DRAFT FINAL REPORT

PLACENTAL AROMATASE ASSAY VALIDATION STUDY: CONDUCT MULTIPLE CHEMICAL STUDIES WITH CENTRALLY PREPARED MICROSOMES

VOLUME II (Appendices B, C and D)

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APPENDIX B

BATTELLE REPORT

DRAFT REPORT

AROMATASE ASSAY VALIDATION:

CONDUCT STUDIES WITH CENTRALLY PREPARED MICROSOMES

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DRAFT REPORT

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COMPLIANCE STATEMENT

This study, Battelle Study Number G608316, was conducted in compliance with the United States (U.S.) Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the standard operating procedures (SOPs) of Battelle Memorial Institute; and the protocol as approved by the Sponsor.

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Study Director

Date

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit and reports were submitted to the study director and management as follows:

Phase Inspected	Date Inspected	Date of Report to Study Director/Management
Protocol review	1/24/2005	1/24/2005
Protocol amendment review	2/4/2005	2/4/2005
Protocol amendment review	2/10/2005	2/10/2005
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Standard preparation	3/1/2005	3/1/2005
Sample homogenization	3/1/2005	3/1/2005
Sample preparation	3/1/2005	3/1/2005
Test system preparation	3/1/2005	3/1/2005
Protein analysis	3/1/2005	3/1/2005
Spectrophotometer absorbance readings	3/1/2005	3/1/2005
Audit study file	8/5/2005	8/5/2005
Audit draft report	1/18/2006	1/18/2006

Quality Assurance Unit Date

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1.0 EXECUTIVE SUMMARY

Four reference chemicals: aminoglutethimide, chrysin, ketoconazole and econazole were tested for their ability to affect CYP19 aromatase activity in human placental microsomes using the classical ^3H_2O method.

Briefly, each inhibitor was incubated with human placental microsomes in the presence of ^{3}H-androstenedione (substrate for aromatase) at pH 7.4 and 37 ± 1°C, for 15 minutes. Eight concentrations of each inhibitor were tested in aromatase assay to construct a dose-response curve from which IC_{50} was calculated. Three (or more) independent replicates of the assay were conducted for each reference chemical. Four types of control samples were included for each replicate (full activity, background activity, positive and negative controls). The average IC_{50} (weighted geometric average) for aminoglutethimide was 4.13 μM (for all five replicates) or 2.99 μM (for three replicates), for chrysin 5.27 μM (all four replicates), for econazole 2.11 nM, and for ketoconazole IC_{50} was 6.47 μM.

From all tested, potential aromatase inhibitors econazole was the most potent. The data obtained with chrysin point out the limitation of the two-parameter model for fitting data that do not span the entire 0 to 100% of control range. The significant differences for all control types (full, positive and negative) between the beginning and the end portion were observed. The end portion was significantly lower than the beginning portion, which may imply a reduction in aromatase activity between the beginning and the end of replicate.

2.0 INTRODUCTION

2.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the United States (U.S.) Environmental Protection Agency (EPA) to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach e.g., a combination of *in-vitro* and *in-vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in-vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Advisory Committee (EDMVAC) will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening

Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed: (1) searching the literature databases; (2) contacting individuals to obtain information on unpublished research; and (3) evaluating the literature and personal communications.

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450arom and consists of ten exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in-vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in-vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in-vitro* subcellular assay using human placental microsomes is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in-vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system.

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays.

2.2 Task Description and Objectives

Three (or more) independent replicates of the aromatase assay were conducted (on separate days) for each reference chemical. Human placental microsomes were prepared by lead laboratory (RTI International). Each reference chemical was tested at eight concentrations and there were three repetitions for each concentration of a given replicate. Four types of control samples were included for each replicate. These included:

- Full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of reference chemical solution] and microsomes.
- Background activity controls (all components that are in the full aromatase activity controls, except NADPH).
- Positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration of 5×10^{-8} M).
- Negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a single concentration of 1×10^{-6} M).

IC_{50}s for each replicate were calculated using Prism software as specified in the protocol.

The objective of presented study was to demonstrate the responsiveness of the aromatase assay to four inhibitors (reference chemicals). An additional aim of the study was to use the optimized assay to obtain intra-laboratory assay variability estimates. The study protocol and Quality Assurance Project Plan (QAPP) can be found in Appendices A and B, respectively.

3.0 MATERIALS AND METHODS

3.1 Preparation of Substrate Solution

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot No. 024K0809) was obtained from Sigma, St. Louis, MO, by the Sponsor's Chemical Repository (CR) and was then distributed to the participating laboratories. It had a reported purity of 100%. The radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN, Lot No. 3538496), was obtained from PerkinElmer Life Sciences, Inc., Boston, MA, and had a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. Radiochemical purity was assessed by high performance liquid chromatography (HPLC) by the lead laboratory (see the Results section).

Preparing the substrate solution involved mixing of non-radiolabeled and radiolabeled [^{3}H]ASDN in order to achieve 100 nM final concentration of ASDN in the assay. The amount of tritium added to each incubation was about 0.1 μCi. This substrate solution should have a concentration of 2 μM with a radioactivity of about 1 μCi/mL.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. A 1:100 dilution of the radiolabeled stock solution in buffer and a 1 mg/mL solution of ASDN in ethanol were prepared. Subsequently, the 1 mg/mL ASDN in ethanol solution was diluted in buffer to a final concentration of 1 μg/mL. Four-and-one half (4.5) mL of the 1 μg/mL solution of ASDN, 800 μL of the [^{3}H]ASDN buffer dilution and 2.7 mL buffer, enough to make 8 mL, were

combined. The weight of each component added to the substrate solution was recorded. After mixing the solution, five aliquots of ca. 20 µL were weighed out and combined with scintillation cocktail for radiochemical content analysis.

3.2 Reference Chemicals and Control Substances

The Sponsor's CR was responsible for chemistry activities required to perform this study. Their responsibilities included chemical procurement, solubility, formulation stability assessment, formulation preparation, formulation analysis, and shipment of stock formulation to the participating laboratories (see the Results section).

3.2.1 Reference Chemicals

The reference chemical formulations and vehicles were received from Sponsor's CR. To each solution of the reference chemical, a specific code was assigned to ensure that replicates were conducted blind for reference chemical identity. Table 1 summarizes all information about used reference chemicals.

Table 1 - Reference Chemicals

Test Substance	Mfr. Purity (%)	CAS No.	Molecular Formula	Stock Solution Code	Stock Solution Concentration (M)	Vehicle	Stock Solution Storage Condition (°C)
Aminoglutethimide	> 99	125-84-8	$C_{13}H_{16}N_2O_2$	1/A	0.1	DMSO	2-8
Chrysin	98.20	480-40-0	$C_{15}H_{10}O_4$	2/B	0.01	DMSO	2-8
Econazole (nitrate)	98	24169-02-6	$C_{18}H_{15}Cl_3N_2O \bullet HNO_3$	1/C	0.1	DMSO	2-8
Ketoconazole	>99	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	2/D	0.01	DMSO	2-8

Dilutions of each reference chemical solution were prepared fresh each day for use in the assay. In each case, the same lot number of vehicle used to prepare the stock solution was used in the preparations of the dilutions. Suppliers and lot numbers for reference chemical vehicles are presented in Table 11 (Section 3.4). The dilution scheme described in Table 2 was used in the preparations of dilutions for the first replicates of aminoglutethimide and econazole (1/A and 1/C, respectively). The scheme described in Table 3 was used for the first replicate of chrysin (2/B) and ketoconazole (2/D).

Table 2 - Dilution Scheme for the First Replicate of Aminoglutethimide (1/A) and Econazole (1/C)

Dilution ID	Vehicle Volume (µL)	Solution Volume (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	0	1000	1/A or 1/C	0.1	0.001
2	9000	1000	Diln. 1	0.01	0.0001
3	9000	1000	Diln. 2	0.001	0.00001
4	9000	1000	Diln. 3	0.0001	0.000001
5	9000	1000	Diln. 4	0.00001	0.0000001
6	9000	1000	Diln. 5	0.000001	0.00000001
7	9000	1000	Diln. 6	0.0000001	0.000000001
8	9000	1000	Diln. 7	0.00000001	0.0000000001

Table 3 - Dilution Scheme for the First Replicate of Chrysin (2/B) and Ketoconazole (2/D)

Dilution ID	Vehicle Volume (µL)	Solution Volume (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	0	1000	2/B or 2/D	0.01	0.0001
2	900	100	2/B or 2/D	0.001	0.00001
3	900	100	Diln. 2	0.0001	0.000001
4	750	250	Diln. 3	0.000025	0.00000025
5	900	100	Diln. 3	0.00001	0.0000001
6	900	100	Diln. 5	0.000001	0.00000001
7	900	100	Diln. 6	0.0000001	0.000000001
8	900	100	Diln. 7	0.00000001	0.0000000001

The results of first replicate for each reference chemical were reviewed and adjustments were made to the tested concentrations in subsequent replicates of the assay in order to obtain data that better described the dose-dependent curve. These new dilution schemes are described in Tables 4, 5, 6 and 7 for 1/A, 2/B, 1/C and 2/D solutions, respectively.

Table 4 - Dilution Scheme for Replicates 2, 3, 4 and 5 of Aminoglutethimide (1/A)

Dilution ID	Vehicle Volume (µL)	Solution Volume (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	0	1000	1/A	0.1	0.001
2	900	100	1/A	0.01	0.0001
3	900	100	Diln. 2	0.001	0.00001
4	500	500	Diln. 3	0.0005	0.000005
5	500	500	Diln. 4	0.00025	0.0000025
6	900	100	Diln. 3	0.0001	0.000001
7	900	100	Diln. 6	0.00001	0.0000001
8	900	100	Diln. 7	0.000001	0.00000001

Table 5 - Dilution Scheme for Replicates 2, 3 and 4 of Chrysin (2/B)

Dilution ID	Vehicle Volume (µL)	Solution Volume (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	0	1000	2/B	0.01	0.0001
2	900	100	2/B	0.001	0.00001
3	750	250	Diln. 2	0.00025	0.0000025
4	900	100	Diln. 2	0.0001	0.000001
5	500	500	Diln. 4	0.00005	0.0000005
6	500	500	Diln. 5	0.000025	0.00000025
7	900	100	Diln. 4	0.00001	0.0000001
8	900	100	Diln. 7	0.000001	0.00000001

Table 6 - Dilution Scheme for Replicates 2 and 3 of Econazole (1/C)

Dilution ID	Vehicle Volume (µL)	Solution Volume (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	49950	50	1/C	0.0001	0.000001
2	900	100	Diln. 1	0.00001	0.0000001
3	900	100	Diln. 2	0.000001	0.00000001
4	500	500	Diln. 3	0.0000005	0.000000005
5	500	500	Diln. 4	0.00000025	0.0000000025
6	900	100	Diln. 3	0.0000001	0.000000001
7	750	250	Diln. 6	0.000000025	0.00000000025
8	900	100	Diln. 6	0.00000001	0.0000000001

Table 7 - Dilution Scheme for Replicates 2 and 3 of Ketoconazole (2/D)

Dilution ID	Vehicle Volume (µL)	Solution Volume (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	0	1000	2/D	0.01	0.0001
2	750	250	2/D	0.0025	0.000025
3	900	100	2/D	0.001	0.00001
4	500	500	Diln. 3	0.0005	0.000005
5	500	500	Diln. 4	0.00025	0.0000025
6	900	100	Diln. 3	0.0001	0.000001
7	750	250	Diln. 6	0.000025	0.00000025
8	900	100	Diln. 6	0.00001	0.0000001

3.2.2 Control Substances

The known aromatase inhibitor, 4-hydroxyandrostenedione (4-OH ASDN), was used as a positive control and the known aromatase non-inhibitor, lindane, was used as a negative control. Stock solution of control chemicals (Table 8) were supplied by the Sponsor's CR and were stored refrigerated. Dilutions were made fresh each day of use in the same vehicle and lot number that was used to prepare the stock solutions (Table 11, Section 3.4). Tables 9 and 10 present the dilution scheme for 4-OH ASDN and lindane, respectively.

Table 8 - Control Substances

Test Substance	Mfr. Purity	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Stock Solution ID	Vehicle	Storage Conditions (°C)
4-hydroxyandrostenedione	99%	566-48-3	$C_{19}H_{26}O_3$	302.41	2-ASDN-1	95% ethanol	2-8
Lindane	99.6%	58-89-9	$C_6H_6Cl_6$	290.83	1-LIN-1	DMSO	2-8

Table 9 - Dilution Scheme for 4-OH ASDN (Positive Control)

Dilution ID	Volume of EtOH (µL)	Volume of Solution (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	9900	100	4-OH ASDN 0.01 M	0.0001	NA
2	1900	100	Diln. 1	0.000005	0.00000005

Table 10 - Dilution Scheme for Lindane (Negative Control)

Dilution ID	Volume of DMSO (µL)	Volume of Solution (µL)	Solution Used	Solution Concentration (M)	Final Concentration In Assay (M)
1	9900	100	Lindane 0.1 M	0.001	NA
2	900	100	Diln. 1	0.0001	0.000001

3.3 Microsomes

Human placental microsomes were provided by RTI International, Lot Sample ID No. 11343-7 and were stored at approximately -70°C until the time of assay. Several vials of the stock microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer, divided into approximately 150 µL aliquots, refrozen in liquid nitrogen and stored at approximately -70°C. On the day of use, microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized by brief vortexing and kept on ice until used. The time between thawing of the microsomes and their use in the assay was limited to less than 2 hours and, in most cases, was about 45 to 50 minutes.

The protein concentration in stock microsomes was approximately 14.0 mg/mL. Microsomes were diluted in assay buffer in two serial dilutions. The first dilution (1:50) was achieved by gently mixing 0.1 mL of microsomes stock suspension with 4.9 mL of buffer (total volume 5 mL). The second dilution (1:10) was obtained by gently mixing 4.5 mL of first microsomes dilution with 40.5 mL of buffer. The first dilution was kept on ice until the protein concentration was measured. In the second dilution, the target protein concentration was ca. 0.025 mg/mL to achieve a final protein concentration in the incubation mixture ca. 0.0125 mg/mL. The second dilution was also kept on ice until it was placed in the water bath just prior to its addition to the incubation mixture to start the reaction.

3.4 Other Assay Components

In addition to substrate, reference chemicals or control substances, vehicle, and microsomes, the aromatase assay contains β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), propylene glycol and phosphate buffer. Suppliers and lot numbers for other aromatase assay components are presented in Table 11.

Table 11 - Suppliers and Lot Numbers for Aromatase Assay Components

Chemical	Supplier	Lot Number
NADPH	Sigma	103K7046
Propylene glycol	Spectrum Chemical	SQ0397
Sodium phosphate dibasic	Sigma	083K0120
Sodium phosphate monobasic	Sigma	054K0144
Ethanol, 95%	Battelle CR	04B10UB
Vehicle (DMSO) for Reference Chemicals 1/A, 2/D and Lindane	Battelle CR	2969A24437
Vehicle (DMSO) for Reference Chemicals 2/B and 1/C	Battelle CR	2969Y30428

3.4.1 β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form (β-NADPH)

β-NADPH is the required co-factor for aromatase. The final concentration in the assay was 0.3 mM. Typically, a 6 mM stock solution was prepared fresh each day by dissolving ca. 20 mg of NADPH in 4 mL of assay buffer, and kept on ice until use.

3.4.2 Assay Buffer

The assay buffer was 0.1 M sodium phosphate buffer, pH 7.4. One liter of 0.1 M solution of sodium phosphate monobasic (NaH_2PO_4) in deionized water and one liter of 0.1 M solution of sodium phosphate dibasic (Na_2HPO_4) in deionized water were prepared. The solutions were combined in the approximate ratio 80:20 (dibasic:monobasic sodium phosphate) to achieve a pH of 7.4. The assay buffer was stored for up to one month in the refrigerator (2 to 8°C).

3.5 Protein Determination

The protein concentration in the microsomes was determined each day the microsomes were used with a DC Protein Assay kit from BioRad (Hercules, CA). The 6-point standard curve was prepared using bovine serum albumin (BSA) reconstituted in Milli-Q water. The standard curve range was from 0.14 to 1.0 mg protein/mL. Briefly, to a 25 µL aliquot of microsomes solution (1:50 dilution in assay buffer) or 25 µL aliquot of each standard, 125 µL of BioRad DC Protein Kit Reagent A was added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard and microsomes solution and gently mixed. The samples were incubated at room temperature for at least 15 minutes. Each sample (standard and microsomes) was transferred to disposable polystyrene cuvettes and the absorbance at 750 nm was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by interpolation, reading the protein concentration on the standard curve that corresponded to its absorbance.

For the protein concentrations, measurements associated with assays that were conducted on February 23, 2005 and later, protein quality control (QC) samples were included in the assay. The QC samples were selected from a set of pre-diluted protein standards (Pierce, Product No. 23208, Lot No. GA93315); protein standards of 0.125, 0.500, and 1.000 mg/mL nominal concentrations were selected as the low, mid and high concentration QC samples. All QC samples were run in duplicate using the same method as described above.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

Three (or more) independent replicates were conducted (on separate days) for each reference chemical with all replicates for a given reference chemical conducted by the same technician. Each reference chemical was tested at eight concentrations and there were three (triplicate) repetitions for each concentration of a given replicate. A single replicate experiment of an example reference chemical is described in Table 12.

Four types of control samples were included with each replicate. These included:

- Full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of reference chemical solutions] and microsomes.
- Background activity controls (all components that are in the full aromatase activity control, except NADPH).
- Positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at the concentration of 5×10^{-8} M.
- Negative controls (all components that are in full aromatase activity controls, except vehicle, and with the addition of lindane at the concentration of 1×10^{-6} M.

Four test tubes of each type of control were included with each replicate and were treated the same as the other samples. The controls sets were split so that two tubes (of each control type) were run at the beginning and two at the end of each replicate set.

The assays were performed in 13 x 100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol, [^{3}H]ASDN, NADPH, and assay buffer were combined in the test tubes with or without inhibitor (as described below) to the total volume of 1.0 mL. The volume of the reference chemical or control substance solutions or vehicle used was 20 µL (1% of total assay volume). The final concentrations for the assay major components are presented in Table 13. The tubes and the microsomal suspension were placed at 37 ± 1°C in the water bath for approximately 5 minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume was 2 mL, and the tubes were incubated for 15 minutes. The incubations were stopped by addition of methylene chloride (2 mL); the tubes were vortex-mixed for approximately 5 seconds and placed on ice. The tubes were then vortex-mixed an additional 20 to 25 seconds, then centrifuged using a Beckman GS-6 centrifuge with a GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. After centrifugation, the methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time discarding the methylene chloride layer. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution. Analysis of the samples was performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represented amount of formed ^3H_2O. The aromatization of one mole of [^{3}H]ASDN resulted in the production of one mole of estrone (non-radiolabeled) and one mole of ^3H_2O.

Table 12 - Reference Chemical Study Design

Sample Type	Repetitions (Test Tubes)	Description	Test Chemical Concentration (M)
Full Activity Control	4	Complete assay[a] with reference vehicle control	N/A
Background Activity Control	4	Complete assay with reference vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay with positive control chemical (4-OH ASDN) added	5×10^{-8}
Negative Control	4	Complete assay with negative control chemical (lindane) added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}

a. The complete assay contains buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH.

Table 13 - Aromatase Assay Conditions using Human Placental Microsomes

Assay Component	Component Volume Added to Assay	Final Concentration In Assay
Microsomal protein	1.0 mL	0.0125 mg/mL
NADPH or assay buffer	100 µL	0.3 mM
[^{3}H]ASDN	100 µL	100 nM
Propylene glycol	100 µL	5% (v/v)
Reference Chemical, Control Substance or Vehicle	20 µL	Varies[a]
Assay buffer	680 µL	~ 0.094 M

a. See Table 12 for details.

3.7 Data Analysis

3.7.1 Aromatase Activity and Percent of Control Calculations

Data were entered into an Excel spreadsheet for calculation of aromatase activity and percent of control. The master spreadsheet used was titled Aromatase_Master_Version1.2.xls.

For each repeat tube (full enzyme activity control, background activity control, positive and negative controls and each reference chemical concentration), the Excel spreadsheet included total observed (uncorrected) disintegration per minute (dpm) per tube and total aromatase activity per tube. The dpm and aromatase activity values were corrected for the background dpm's, as measured by the average of the background activity control tubes. The aromatase activity was calculated by normalizing the corrected dpm by the specific activity of the [^{3}H]ASDN, the mg of protein of the microsomes, and the incubation time.

For each tube, percent of control was determined by dividing the background corrected aromatase activity for that tube by the average background corrected aromatase activity for the four full enzyme activity control tubes and multiplying by 100. Nominally, one may expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the

low inhibitions concentrations. However, due to experimental variation, individual observed percent of control values sometimes extended below 0% or above 100%.

The spreadsheet calculated dpm/mL for each aliquot of extracted aqueous incubation mixture and average dpm/mL and total dpm for each aqueous portion (after extraction). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution radiochemical content (dpm/mL) yielded the total dpm present in the assay tube at initiation. The total dpm remaining in the aqueous portion after extraction divided by the total dpm present in the assay tube at initiation times 100 yielded the percent of the substrate that was converted to product. The total dpm remaining in the aqueous portion after extraction was corrected for background by subtracting the average dpm present in the aqueous portion of the background activity control tubes (for that day/assay). This corrected dpm was then converted to nmol product formed by dividing by the substrate specific activity (dpm/nmol). The activity of the enzyme was expressed in nmol (mg protein)$^{-1}$min^{-1} and was calculated by dividing the amount of estrogen formed (nmol) by the amount of microsomal protein used (in mg) times the incubation time (in min). Average activity in the full activity control samples for a given study was calculated. Percent of activity remaining in the presence of various inhibitor concentrations was calculated by dividing the aromatase activity at a given inhibitor concentration by the average positive full activity control and multiplying by 100.

3.7.2 Statistical Analyses

3.7.2.1 Concentration Response Fits for the Reference Chemicals

For the reference chemicals, an independent concentration response curve fit was carried out for each replicate. Concentration response trend curves were fitted to the percent of control activity values within each of the repeat tubes at each reference chemical concentration. Concentration was expressed on the log scale. In agreement with past convention, logarithms were common logarithms (i.e., base 10). Let X denote the logarithm of the concentration of reference chemical (e.g. if concentration = 10^{-5} then X = -5). Let:

Y ≡ percent of control activity in the inhibitor tube.

X ≡ logarithm (base 10) of the concentration.

DAVG ≡ average dpms across the repeat tubes with the same reference chemical concentration.

β ≡ slope of the concentration response curve (β will be negative).

□≡ $\log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%).

The following concentration response curve was fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\square - X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 (zero) and variance proportional to DAVG (based on the Poisson distribution theory for radiation counts). The variance was approximated by Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits were carried out using Prism software (Version 4.0). Observed individual percent activity values above 100% were set to 99.5%. Observed individual percent activity values below 0% were set to 0.5%.

Concentration response models were fitted for each replicate test within each reference chemical. For each replicate the estimated $\log_{10}IC_{50}$ (μ) and its associated standard error, the IC_{50} and its associated geometric standard error, the slope (β) and its associated standard error, and the "Status" of each response curve was reported (see Appendix G for full statistical analysis).

3.7.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each replicate, the individual percent of control values were plotted versus logarithm of the reference chemical concentration. The fitted concentration response curve was superimposed on the plot. Individual plots were prepared for each replicate.

Additional plots were prepared to compare the percent of control activity values across replicates. For each replicate, the average percent of control values were plotted versus logarithm of reference chemical concentration on the same plot. Plotting symbols distinguished among replicates. The fitted concentration response curves for each replicate were superimposed on the plots. On a separate plot, the average percent of control values for each replicate was plotted versus logarithm of reference chemical concentration. The average concentration response curve across replicates was superimposed on the same plot.

Graphs were prepared for the parameter estimates from the response curve model fits to visually assess the estimates and their variations.

3.7.2.3 Graphical and Analysis of Variance Comparisons of Full Enzyme Activity, Background Activity, and Positive and Negative Control Percent of Control Across Reference Chemicals and Replicates

The means of the full enzyme activity control values within each replicate and chemical were calculated as the reference value for 100% of control for the replicate and chemical. The percent of control for each repetition within each replicate and chemical was calculated as the ratio of the repetition value divided by the corresponding 100% of control reference value. Graphs of the percent of control values indicating the repetition portion by replicate and chemical for background activity, full enzyme activity controls, negative and positive controls were prepared. Graphs displaying the differences of the means of the beginning repetitions and the means of the end repetitions within each replicate and chemical were prepared. A mixed effects model was fit to the percent of control values for each control. The fixed effects were assigned as the chemical type, portion, and portion by chemical interaction. The random effects were assigned as replicated within reference chemical and portion by replicate interaction within chemical.

3.7.2.4 Statistical Software

Concentration response curves were fitted to the data using the non-linear regression analysis features in the Prism statistical analysis package, Version 4.0. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons were carried out using the SAS statistical analysis system, Version 9.

4.0 RESULTS

4.1 Radiochemical Purity

The radiochemical purity for the substrate androstenedione was 97% as reported by RTI International (Appendix C).

4.2 Stock Formulation Analysis

The formulation stability and formulation analysis results for all four reference chemicals and the positive and negative control chemicals from the Battelle CR are included in the reports presented in Appendix D. Some of the formulation analysis data are summarized in Table 14.

Table 14 - Formulation Analysis Data

Chemical/ Substance Code	Chemical ID	Stock Solution ID	Manufacturer's Purity (%)	Stock Solution Preparation Date	Known Stability (days)	Date of Last Use on WA 4-16, Task 5
1/A	Aminoglutethimide	1-AG-1	> 99	1/24/2005	59	3/16/2005
2/B	Chrysin	1-CHRY-1	98.20	1/25/2005	100	3/15/2005
1/C	Econazole	1-ECON-1	98	2/7/2005	56	2/24/2005
2/D	Ketoconazole	1-KET-1	> 99	2/4/2005	60	3/2/2005
N/A	Lindane	1-LIN-1	99.6	1/24/2005	168	3/16/2005
N/A	4-OH ASDN	2-ASDN-1	99	1/25/2005	173	3/16/2005

4.3 Protein Analysis

Protein concentration measurements were done according to the procedure provided in Section 3.5 of this report. To measure the protein concentration, 1:50 microsomes dilution in assay buffer were processed. The results for measuring protein concentration in human placental microsomes for each day of use are presented in Table 16. The average measured protein content of the human placental microsomes stock solution was 11.24 mg/mL (12.79% CV), with the range of 8.841 to 14.23 mg/mL.

In order to better characterize the protein assay, QC samples were included on some runs (see Section 3.5). The QC results are presented in Table 15. The QC sample results indicate that determination of low protein concentrations is very unreliable (approximately -30% difference from known value). This may explain wide day-to day differences found in determinations of protein concentration in placental microsomes. The average concentration of the protein dilution (1:50) that was assayed was about 0.22 mg/mL (11.24/50). This concentration falls near the low end of the reliable range of protein standard curve.

Table 15 - Protein Concentration in QC Samples

Nominal Concentration (mg/mL)	Assay Date	Measured Concentration (mg/mL)	Mean	SD	SEM	% CV	% Difference From Known
0.125	2/23/2005	0.101	0.084	0.025	0.009	29.0	-32.50
	2/24/2005	0.086					
	2/28/2005	0.096					
	3/1/2005	0.099					
	3/2/2005	0.113					
	3/14/2005	0.062					
	3/15/2005	0.037					
	3/16/2005	0.081					
0.500	2/23/2005	0.515	0.502	0.009	0.003	1.7	0.33
	2/24/2005	0.500					
	2/28/2005	0.492					
	3/1/2005	0.499					
	3/2/2005	0.511					
	3/14/2005	0.505					
	3/15/2005	0.490					
	3/16/2005	0.501					
1.000	2/23/2005	0.925	0.952	0.034	0.012	3.6	-4.79
	2/24/2005	0.958					
	2/28/2005	0.930					
	3/1/2005	0.970					
	3/2/2005	0.967					
	3/14/2005	1.016					
	3/15/2005	0.906					
	3/16/2005	0.945					

4.4 Aromatase Activity

Aromatase activity was measured in the presence of eight concentrations of each of four reference chemicals. Each assay set also included four types of controls (see Section 3.6). Three (or more) replicates were run of each reference chemical. Information regarding assay dates, technicians, protein concentration, substrate specific activity and tested reference chemical concentration ranges is presented in Table 16. Radioactivity remaining in each assay tube after extraction of the unreacted substrate represents formed estrone. The aromatase activity is calculated by normalizing the radioactivity present in each tube by the amount of microsomal protein and the reaction time and has the units nmol/mg protein/min.

Table 16 - Aromatase Assay Summary

Reference Chemical Code	Reference Chemical ID	Replicate	Assay Date	Technician	Protein Stock Conc. (measured) (mg/mL)	Substrate Specific Activity (µCi/µg ASDN)	Reference Chemical Concentration Range (M)
1/A	Aminoglutethimide	1	2/2/2005	TD	8.841	0.842	1.00E-03 to 1.00E-10
1/A	Aminoglutethimide	2	2/4/2005	TD	13.384	1.025	1.00E-03 to 1.00E-08
1/A	Aminoglutethimide	3	2/7/2005	TD	9.801	1.044	1.00E-03 to 1.00E-08
1/A	Aminoglutethimide	4	3/14/2005	TD	12.004	1.012	1.00E-03 to 1.00E-08
1/A	Aminoglutethimide	5	3/16/2005	TD	10.192	0.996	1.00E-03 to 1.00E-08
2/B	Chrysin	1	2/15/2005	TD	11.241	0.967	1.00E-04 to 1.00E-10
2/B	Chrysin	2	2/16/2005	TD	11.273	0.921	1.00E-04 to 1.00E-08
2/B	Chrysin	3	2/17/2005	TD	9.813	0.939	1.00E-04 to 1.00E-08
2/B	Chrysin	4	3/15/2005	TD	10.537	0.946	1.00E-04 to 1.00E-08
1/C	Econazole	1	2/22/2005	TD	11.964	1.036	1.00E-03 to 1.00E-10
1/C	Econazole	2	2/23/2005	TD	11.050	1.033	1.00E-06 to 1.00E-10
1/C	Econazole	3	2/24/2005	TD	10.941	0.883	1.00E-06 to 1.00E-10
2/D	Ketoconazole	1	2/28/2005	TD	14.225	1.000	1.00E-04 to 1.00E-10
2/D	Ketoconazole	2	3/1/2005	TD	10.584	0.978	1.00E-04 to 1.00E-07
2/D	Ketoconazole	3	3/2/2005	TD	12.767	1.074	1.00E-04 to 1.00E-07

4.4.1 Control Results

Each replicate set for each reference chemical included four types of controls, each run in quadruplicate. The control types were full aromatase activity, background activity, positive and negative controls. The positive control tubes contained the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN), at a concentration of 5×10^{-8} M and negative control tubes contained the known aromatase non-inhibitor, lindane, at a concentration of 1×10^{-6} M. The control tubes were divided so that two of each type were run at the beginning of the set and two were run at the end of the set. In general, there was about a 10 minute interval between the beginning and the end of each replicate set. The aromatase activity in the full aromatase activity controls represented 100% activity and since all aromatase activities were corrected for background, the background activity controls necessarily were set to 0%. The mean activities for each type of control (except background) for the beginning and end groups and the overall means, standard deviation (SD), standard error of the mean (SEM), and coefficient of variation (CV) across replicates and across chemicals are presented in Table 17. Figures 1 through 4 contain graphical representations of the beginning and end percent control values for each type of control for each reference chemical and replicate. The overall control values for background activity are consistent (0.0000) across the replicates and chemicals. The overall control values for full enzyme activity control and negative control are 0.0490 ± 0.0057 nmol/mg/min (11.70% CV) and 0.0486 ± 0.0064 nmol/mg/min (13.17% CV), respectively. The percent control values of the positive control are consistent, with an overall value of approximately 56%.

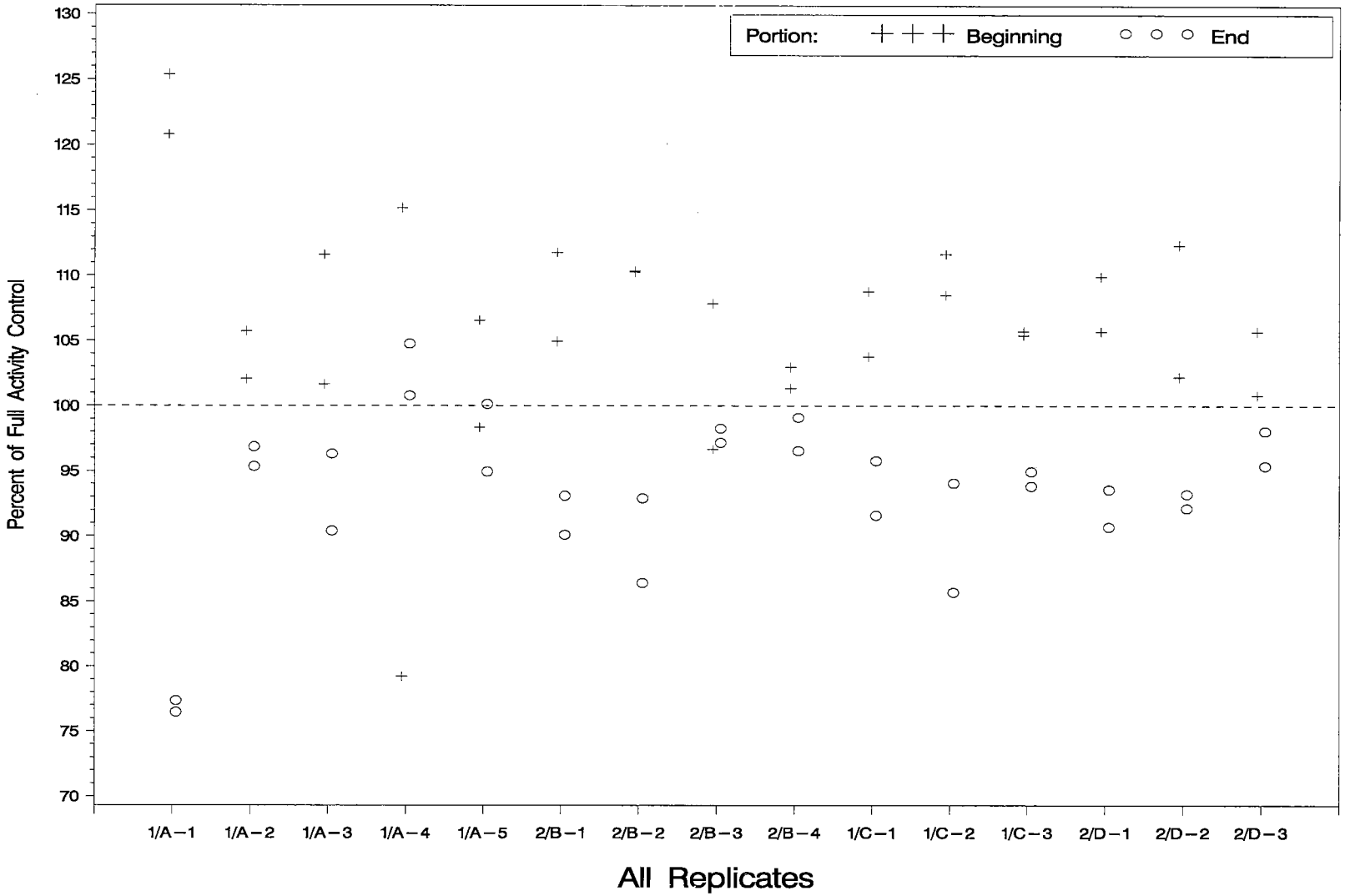


Figure 1 - Full Enzyme Activity Controls. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%.

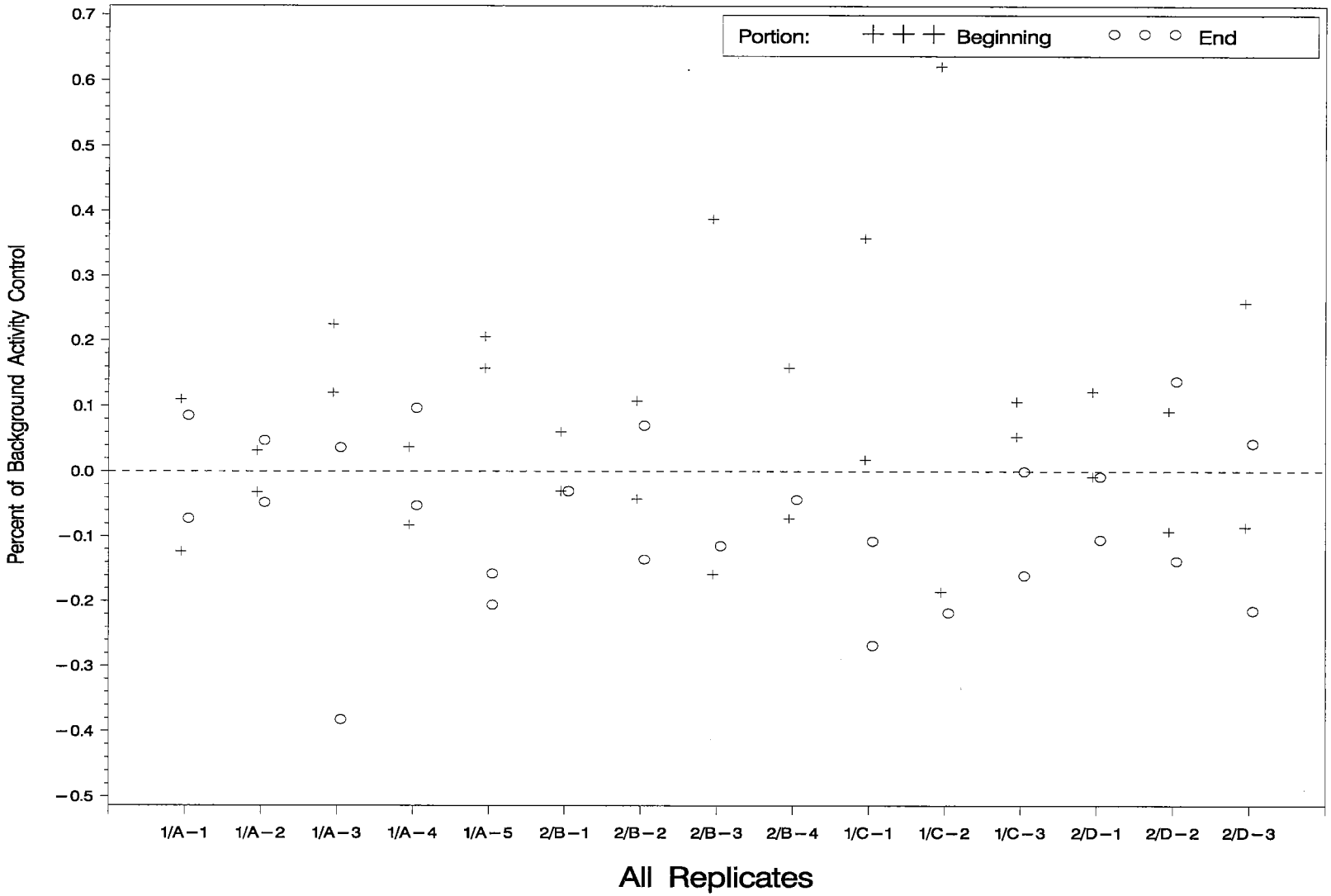


Figure 2 - Background Activity Controls. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%.

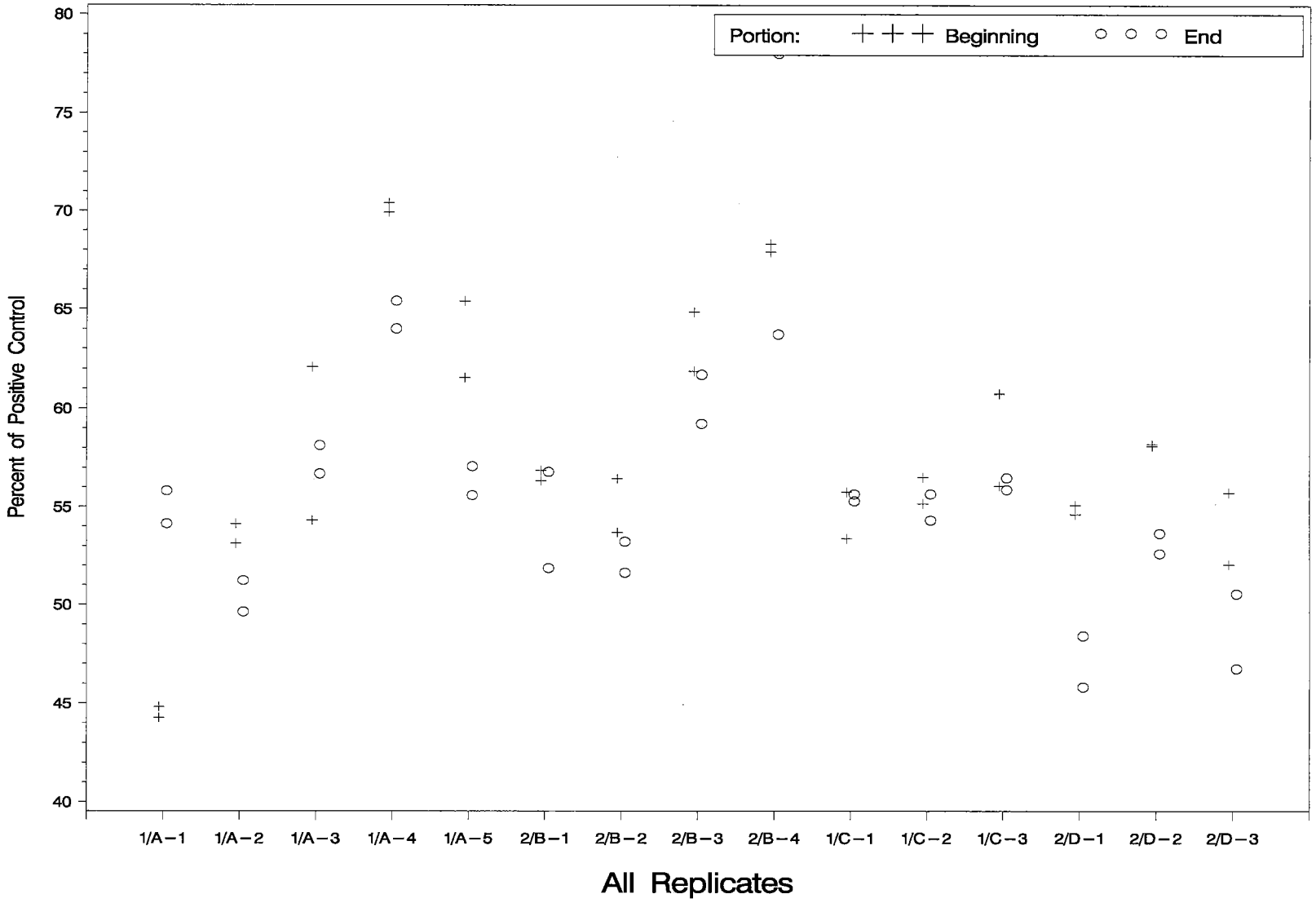


Figure 3 – Positive Control Data. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%.

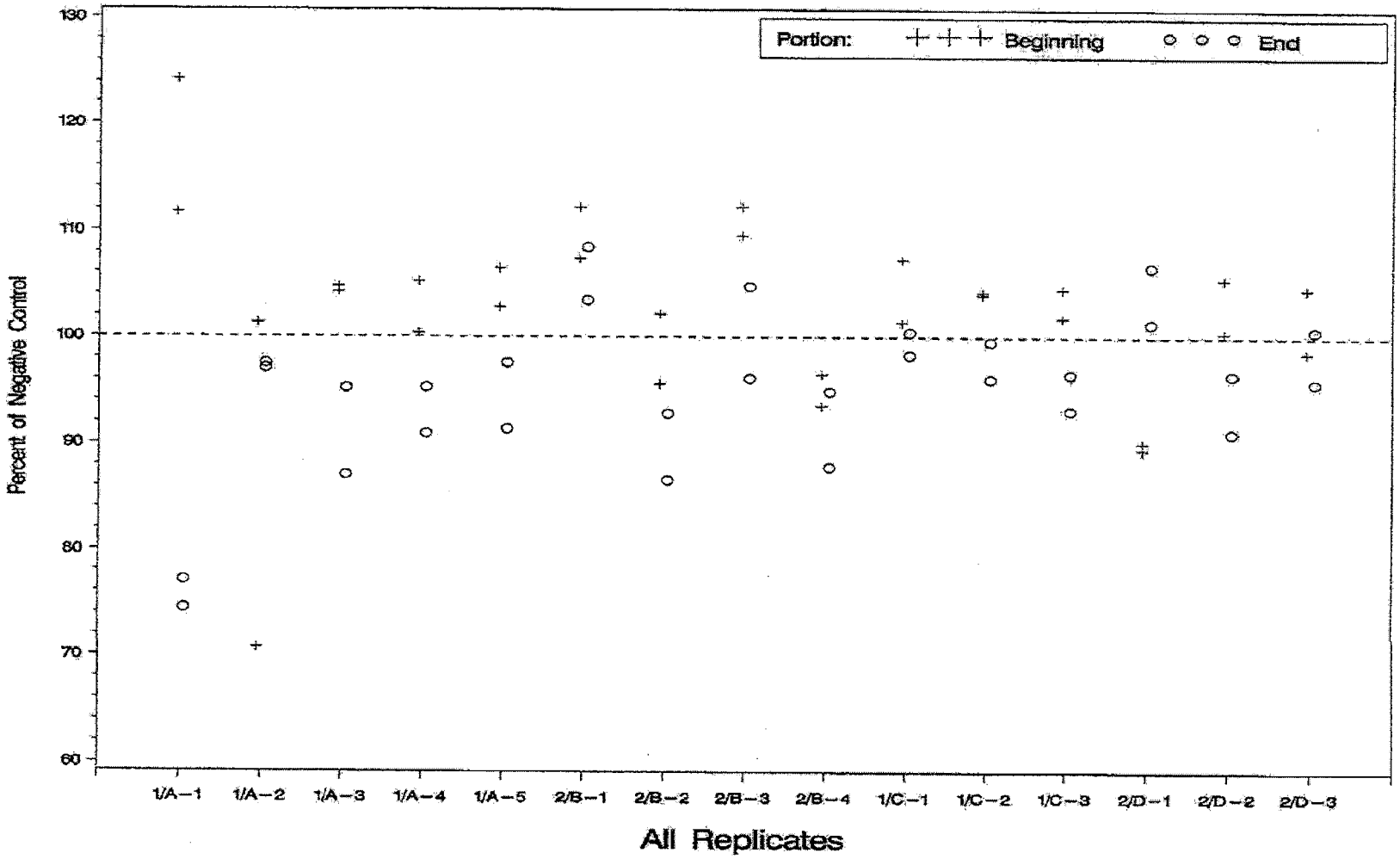


Figure 4 – Negative Controls. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%.

Table 17 - Aromatase Activity in Controls

Reference Chemical	Replicate	Mean Beginning Control (nmol/mg/min)	Mean End Control (nmol/mg/min)	Overall Mean (nmol/mg/min)	SD	SEM	% CV	Overall (by chemicals) Mean (nmol/mg/min)	SD	SEM	% CV
Full Aromatase Activity Controls											
Aminoglutethimide	1	0.0914	0.0571	0.0742	0.0198	0.0099	26.68	0.0476	0.0177	0.0056	37.17
	2	0.0451	0.0417	0.0434	0.0021	0.0011	4.84				
	3	0.0522	0.0457	0.0489	0.0044	0.0022	9.00				
	4	0.0281	0.0298	0.0289	0.0044	0.0022	15.22				
	5	0.0437	0.0416	0.0427	0.0021	0.0011	4.92				
Chrysin	1	0.0693	0.0586	0.0639	0.0065	0.0033	10.17	0.0517	0.0113	0.0040	21.84
	2	0.0596	0.0484	0.0540	0.0066	0.0033	12.22				
	3	0.0533	0.0509	0.0521	0.0028	0.0014	5.37				
	4	0.0374	0.0358	0.0366	0.0010	0.0005	2.73				
Econazole	1	0.0502	0.0443	0.0472	0.0037	0.0019	7.84	0.0550	0.0078	0.0032	14.21
	2	0.0626	0.0511	0.0569	0.0069	0.0035	12.13				
	3	0.0644	0.0576	0.0610	0.0039	0.0020	6.39				
Ketoconazole	1	0.0490	0.0419	0.0455	0.0042	0.0021	9.23	0.0417	0.0057	0.0023	13.68
	2	0.0473	0.0408	0.0440	0.0041	0.0021	9.32				
	3	0.0368	0.0344	0.0356	0.0016	0.0008	4.49				
Overall								0.0490	0.0057	0.0029	11.70
Positive Controls											
Aminoglutethimide	1	0.0331	0.0408	0.0369	0.0045	0.0023	12.20	0.0266	0.0066	0.0021	24.82
	2	0.0233	0.0219	0.0226	0.0009	0.0005	3.98				
	3	0.0285	0.0281	0.0283	0.0016	0.0008	5.65				
	4	0.0203	0.0187	0.0195	0.0009	0.0005	4.62				
	5	0.0271	0.0240	0.0256	0.0019	0.0010	7.42				
Chrysin	1	0.0362	0.0347	0.0355	0.0015	0.0008	4.23	0.0305	0.0041	0.0014	13.30
	2	0.0297	0.0283	0.0290	0.0011	0.0006	3.79				
	3	0.0330	0.0315	0.0323	0.0012	0.0006	3.72				
	4	0.0249	0.0259	0.0254	0.0022	0.0011	8.66				
Econazole	1	0.0258	0.0262	0.0260	0.0005	0.0003	1.92	0.0308	0.0041	0.0017	13.19
	2	0.0318	0.0313	0.0315	0.0005	0.0003	1.59				
	3	0.0356	0.0343	0.0349	0.0014	0.0008	4.30				
Ketoconazole	1	0.0249	0.0214	0.0232	0.0021	0.0011	9.05	0.0220	0.0033	0.0013	14.91
	2	0.0256	0.0234	0.0245	0.0013	0.0007	5.31				
	3	0.0192	0.0173	0.0182	0.0013	0.0007	7.14				
Overall								0.0275	0.0041	0.0021	15.11

Table 17 - Aromatase Activity in Controls (Continued)

Reference Chemical	Replicate	Mean Beginning Control (nmol/mg/min)	Mean End Control (nmol/mg/min)	Overall Mean (nmol/mg/min)	SD	SEM	% CV	Overall (by chemicals) Mean (nmol/mg/min)	SD	SEM	% CV
Negative Controls											
Aminoglutethimide	1	0.0876	0.0562	0.0719	0.0185	0.0093	25.73	0.0461	0.0171	0.0054	37.02
	2	0.0373	0.0422	0.0398	0.0061	0.0031	15.33				
	3	0.0511	0.0446	0.0479	0.0041	0.0021	8.56				
	4	0.0297	0.0269	0.0283	0.0018	0.0009	6.36				
	5	0.0447	0.0403	0.0425	0.0028	0.0014	6.59				
Chrysin	1	0.0702	0.0677	0.0689	0.0023	0.0012	3.34	0.0523	0.0135	0.0048	25.74
	2	0.0534	0.0485	0.0509	0.0035	0.0018	6.88				
	3	0.0578	0.0523	0.0551	0.0037	0.0019	6.72				
	4	0.0348	0.0334	0.0341	0.0014	0.0007	4.11				
Econazole	1	0.0493	0.0469	0.0481	0.0018	0.0009	3.74	0.0553	0.0061	0.0025	11.03
	2	0.0592	0.0556	0.0574	0.0022	0.0011	3.83				
	3	0.0629	0.0578	0.0604	0.0031	0.0016	5.13				
Ketoconazole	1	0.0408	0.0473	0.0440	0.0038	0.0019	8.64	0.0410	0.0049	0.0020	11.94
	2	0.0453	0.0413	0.0433	0.0027	0.0014	6.24				
	3	0.0361	0.0349	0.0355	0.0013	0.0007	3.66				
Overall								0.0486	0.0064	0.0032	13.17

There was a consistent and statistically significant difference in activity for all controls between the beginning and end control tubes. The difference in beginning and end control activities is presented graphically in the statistical report in Appendix G.

4.4.2 Percent of Control

The aromatase activity found in each assay tube was normalized to percent of control by dividing the average full enzyme activity for the replicate. The percent of control values for each reference chemical replicate and tube along with the mean, SD, SEM, and % CV of the percent of control across tubes within a replicate are presented in Table 18.

Table 18 - Percent of Control Aromatase Activity Detected in Assays with Reference Chemicals

Reference Chemical	Replicate	Log [Reference Chemical]	Percent of Control						
			Tube 1	Tube 2	Tube 3	Mean	SD	SEM	% CV
Aminoglutethimide	1	-3.00	0.22	0.56	0.23	0.34	0.19	0.11	57.47
		-4.00	3.10	3.36	3.14	3.20	0.14	0.08	4.38
		-5.00	25.61	24.72	23.92	24.75	0.85	0.49	3.42
		-6.00	75.98	78.06	79.71	77.92	1.87	1.08	2.40
		-7.00	108.28	102.43	92.77	101.16	7.83	4.52	7.74
		-8.00	103.24	105.49	103.49	104.07	1.23	0.71	1.18
		-9.00	102.86	103.13	96.44	101.48	2.63	1.52	2.59
		-10.00	80.75**	77.04**	75.64**	77.81	2.64	1.52	3.39
	2	-3.00	0.21	0.11	0.25	0.19	0.07	0.04	37.95
		-4.00	2.39	2.33	2.38	2.37	0.03	0.02	1.36
		-5.00	19.31	19.09	18.31	18.90	0.53	0.30	2.78
		-5.30	31.67	31.42	31.24	31.44	0.22	0.12	0.69
		-5.60	46.96	46.43	46.02	46.61	0.31	0.18	0.66
		-6.00	66.32	67.33	66.22	66.99	0.58	0.34	0.87
		-7.00	89.46	90.51	92.17	90.71	1.37	0.79	1.51
		-8.00	94.74	94.53	96.48	95.25	1.07	0.62	1.12
	3	-3.00	0.29	0.62	0.33	0.41	0.18	0.10	43.57
		-4.00	3.83	3.85	4.60	4.09	0.44	0.25	10.72
		-5.00	25.89	26.89	26.99	26.59	0.61	0.35	2.29
		-5.30	42.69	41.08	42.46	42.08	0.87	0.50	2.07
		-5.60	60.94	57.26	59.10	59.10	1.84	1.06	3.11
		-6.00	81.14	81.43	78.67	80.41	1.52	0.88	1.89
		-7.00	49.35**	94.89	89.58	77.94	24.90	14.38	31.95
		-8.00	101.21	102.62	103.52	102.45	1.16	0.67	1.14
	4	-3.00	0.66	0.22	0.54	0.47	0.23	0.13	48.05
		-4.00	6.75	7.14	7.61	7.17	0.43	0.25	6.01
		-5.00	40.48	42.80	44.21	42.50	1.88	1.09	4.43
		-5.30	56.55	55.06	59.36	56.99	2.18	1.26	3.83
		-5.60	75.61	76.33	73.49	75.14	1.48	0.85	1.96
		-6.00	80.38	84.05	82.38	82.27	1.84	1.06	2.23
		-7.00	93.15	96.64	96.10	95.30	1.88	1.08	1.97
		-8.00	92.79	101.32	91.81	95.31	5.23	3.02	5.49
	5	-3.00	0.50	0.52	0.59	0.54	0.05	0.03	8.81

Table 18 - Percent of Control Aromatase Activity Detected in Assays with Reference Chemicals (Continued)

Reference Chemical	Replicate	Log [Reference Chemical]	Percent of Control						
			Tube 1	Tube 2	Tube 3	Mean	SD	SEM	% CV
Aminoglutethimide	5	-4.00	8.29	7.93	7.59	7.94	0.35	0.20	4.41
		-5.00	40.24	38.60	39.83	39.56	0.85	0.49	2.16
		-5.30	54.53	57.14	56.71	56.13	1.40	0.81	2.49
		-5.60	74.47	72.17	67.19	71.28	3.72	2.15	5.22
		-6.00	90.74	88.20	85.51	88.15	2.62	1.51	2.97
		-7.00	100.21	91.27	96.19	95.89	4.48	2.59	4.67
		-8.00	99.26	105.85	99.24	101.45	3.81	2.20	3.76
Chrysin	1	-4.00	24.83	24.70	29.04	26.19	2.47	1.43	9.43
		-5.00	14.69	16.48	15.62	15.60	0.90	0.52	5.74
		-6.00	64.44	60.88	54.95	60.09	4.79	2.77	7.98
		-6.60	78.95	80.92	92.95	84.27	7.58	4.38	8.99
		-7.00	92.61	94.25	90.06	92.31	2.11	1.22	2.29
		-8.00	95.45	96.44	96.53	96.14	0.60	0.35	0.62
		-9.00	94.25	95.90	95.14	95.10	0.83	0.48	0.87
		-10.00	95.10	94.10	97.71	95.64	1.86	1.08	1.95
	2	-4.00	22.70	27.38	19.03	23.04	4.19	2.42	18.17
		-5.00	26.46	26.14	28.16	26.92	1.09	0.63	4.03
		-5.60	55.26	56.23	60.14	57.21	2.58	1.49	4.52
		-6.00	74.75	75.61	76.02	75.46	0.65	0.37	0.86
		-6.30	82.56	86.64	78.23	82.48	4.21	2.43	5.10
		-6.60	89.69	93.13	90.70	91.17	1.77	1.02	1.94
		-7.00	94.59	88.08	88.62	90.43	3.61	2.09	4.00
		-8.00	102.54	104.47	100.71	102.57	1.88	1.09	1.83
	3	-4.00	31.60	32.54	26.84	30.33	3.06	1.76	10.08
		-5.00	31.67	31.14	31.75	31.52	0.33	0.19	1.05
		-5.60	68.14	64.12	60.02	64.09	4.06	2.34	6.33
		-6.00	80.03	82.82	82.73	81.86	1.59	0.92	1.94
		-6.30	89.50	94.62	91.73	91.95	2.57	1.48	2.79
		-6.60	92.82	91.49	95.49	93.27	2.04	1.18	2.18
		-7.00	99.12	99.22	97.80	98.71	0.79	0.46	0.80
		-8.00	102.04	101.19	98.79	100.67	1.69	0.97	1.67
	4	-4.00	35.63	40.09	37.01	37.58	2.28	1.32	6.08
		-5.00	46.07	43.74	42.04	43.95	2.02	1.17	4.60
		-5.60	73.60	73.14	73.48	73.41	0.24	0.14	0.33
		-6.00	89.77	87.35	83.38	86.83	3.23	1.86	3.72
		-6.30	87.93	91.20	91.69	90.27	2.04	1.18	2.26
		-6.60	83.61	90.92	95.35	89.96	5.93	3.42	6.59
		-7.00	95.23	88.04	93.36	92.21	3.73	2.15	4.05
		-8.00	95.17	95.92	99.23	96.77	2.16	1.25	2.23
Econazole	1	-3.00	-0.27	-0.23	-0.23	-0.24	0.02	0.01	-9.49
		-4.00	-0.45	-0.27	-0.25	-0.32	0.11	0.06	-34.07
		-5.00	-0.02	-0.36	-0.25	-0.21	0.17	0.11	-82.62
		-6.00	-0.14	0.09	0.18	0.04	0.18	0.11	-84.14
		-7.00	1.75	2.00	2.22	1.99	0.24	0.14	11.82
		-8.00	18.38	17.85	17.97	18.07	0.28	0.16	1.54
		-9.00	70.80	71.62	71.07	71.16	0.42	0.24	0.59
		-10.00	93.30	96.43	98.13	95.95	2.45	1.41	2.55

Table 18. Percent of Control Aromatase Activity Detected in Assays with Reference Chemicals (Continued)

Reference Chemical	Replicate	Log [Reference Chemical]	Percent of Control						
			Tube 1	Tube 2	Tube 3	Mean	SD	SEM	% CV
Econazole	2*	-6.00	0.09	-0.11	0.09	0.02	0.12	0.07	494.87
		-6.00	0.59	0.38	0.23	0.40	0.18	0.10	45.21
		-7.00	1.64	1.69	1.61	1.65	0.04	0.02	2.45
		-7.30	3.63	3.22	3.03	3.29	0.31	0.18	9.31
		-7.60	6.55	6.37	6.37	6.43	0.10	0.06	1.62
		-8.00	15.94	12.59	15.87	14.80	1.91	1.11	12.93
		-8.60	44.32	45.90	45.71	45.31	0.86	0.50	1.90
		-9.00	72.74	68.89	69.30	70.31	2.11	1.22	3.01
	3	-6.00	0.41	0.05	0.37	0.28	0.20	0.11	71.32
		-7.00	1.74	1.90	2.13	1.92	0.20	0.11	10.19
		-8.00	15.09	15.59	15.10	15.26	0.29	0.17	1.87
		-8.30	26.08	25.71	25.51	25.77	0.29	0.17	1.12
		-8.60	44.53	43.11	43.36	43.67	0.76	0.44	1.74
		-9.00	65.92	68.00	69.51	67.81	1.80	1.04	2.66
		-9.60	92.82	88.49	86.98	89.43	3.03	1.75	3.39
		-10.00	90.58	89.63	94.28	91.50	2.46	1.42	2.69
Ketoconazole	1	-4.00	6.02	6.43	6.43	6.29	0.24	0.14	3.76
		-5.00	46.01	46.01	42.10	44.71	2.26	1.30	5.05
		-6.00	87.78	85.88	86.48	86.71	0.97	0.56	1.12
		-6.60	101.45	98.91	99.15	99.84	1.40	0.81	1.40
		-7.00	96.80	98.46	99.19	98.15	1.22	0.71	1.25
		-8.00	94.36	94.31	93.58	94.08	0.44	0.25	0.46
		-9.00	94.31	94.29	91.11	93.24	1.84	1.06	1.98
		-10.00	93.14	94.06	91.89	93.03	1.09	0.63	1.17
	2	-4.00	5.38	5.63	5.91	5.64	0.27	0.15	4.70
		-4.60	22.99	19.94	23.11	22.01	1.80	1.04	8.16
		-5.00	42.81	40.29	40.52	41.21	1.39	0.80	3.38
		-5.30	57.69	55.99	56.43	56.70	0.88	0.51	1.56
		-5.60	72.68	69.90	70.75	71.11	1.42	0.82	2.00
		-6.00	84.41	84.09	66.87	78.46	10.04	5.79	12.79
		-6.60	86.18	88.02	89.63	87.94	1.73	1.00	1.96
		-7.00	91.08	89.81	89.03	89.97	1.03	0.60	1.15
	3	-4.00	6.08	6.21	6.29	6.19	0.11	0.06	1.71
		-4.60	21.63	22.00	21.91	21.85	0.19	0.11	0.88
		-5.00	42.02	39.85	40.19	40.69	1.17	0.67	2.87
		-5.30	57.59	55.66	25.22**	46.16	18.16	10.48	39.34
		-5.60	72.24	68.07	66.76	69.02	2.86	1.65	4.15
		-6.00	76.90	73.53	73.62	74.68	1.92	1.11	2.57
		-6.60	93.27	85.43	88.48	89.06	3.95	2.28	4.44
		-7.00	92.13	90.29	92.18	91.53	1.08	0.62	1.18

Shaded data greater than 100% were reset to 99.5% prior to the calculation of IC_{50}. Shaded data less than zero were reset to 0.5% prior to calculation of IC_{50}.

* Technical error was made during dilution preparation and econazole dilution 1×10^{-6} was used two times, subsequent inhibitor dilutions were as presented in Table 18.

** Data not taken into IC_{50} calculation (technical error occurred during course of the assay).

The % CV at all tested concentrations (except the lowest) of the tested reference chemicals were below 15% with exception of the first replicate for econazole. The higher, 31.95% CV was noted for replicate 3 for aminoglutethimide at the 1 x 10^{-7} M concentration and replicate 3 for ketoconazole at the 5 x 10^{-6} M concentration (39.34% CV).

The mean percent of control activity for each replicate and the overall mean, SD, SEM and % CV across replicates are presented in Table 19. The high % CV values were observed for replicates with aminoglutethimide and chrysin.

Table 19 - Mean Percent of Control per Replicate

Reference Chemical	Log [Ref. Chemical]	Mean Percent of Control					Overall			
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean	SD	SEM	%CV
Aminoglutethimide	-3.00	0.34	0.19	0.41	0.47	0.54	0.39	0.13	0.06	34.35
	-4.00	3.20	2.37	4.09	7.17	7.94	4.95	2.47	1.10	49.78
	-5.00	24.75	18.90	26.59	42.50	39.56	30.46	10.11	4.52	33.20
	-5.30	NA	31.44	42.08	56.99	56.13	46.66	12.23	6.12	26.22
	-5.60	NA	46.61	59.10	75.14	71.28	63.03	12.91	6.45	20.48
	-6.00	77.92	66.99	80.41	82.27	88.15	79.15	7.77	3.48	9.82
	-7.00	101.16	90.71	77.94	95.30	95.89	92.20	8.79	3.93	9.53
	-8.00	104.07	95.25	102.45	95.31	101.45	99.71	4.15	1.85	4.16
	-9.00	101.48	NA	NA	NA	NA	NC	NC	NC	NC
	-10.00	77.81	NA	NA	NA	NA	NC	NC	NC	NC
Chrysin	-4.00	26.19	23.04	30.33	37.58		29.29	6.28	3.14	21.46
	-5.00	15.60	26.92	31.52	43.95		29.50	11.73	5.86	39.76
	-5.60	NA	57.21	64.09	73.41		64.90	8.13	4.69	12.53
	-6.00	60.09	75.46	81.86	86.83		76.06	11.62	5.81	15.28
	-6.30	NA	82.48	91.95	90.27	a	88.23	5.05	2.92	5.73
	-6.60	84.27	91.17	93.27	89.96		89.67	3.85	1.92	4.29
	-7.00	92.31	90.43	98.71	92.21		93.42	3.63	1.82	3.89
	-8.00	96.14	102.57	100.67	96.77		99.04	3.09	1.55	3.12
	-9.00	95.10	NA	NA	NA		NC	NC	NC	NC
	-10.00	95.64	NA	NA	NA		NC	NC	NC	NC
Econazole	-3.00	-0.24	NA	NA			NC	NC	NC	NC
	-4.00	-0.32	NA	NA			NC	NC	NC	NC
	-5.00	-0.21	NA	NA			NC	NC	NC	NC
	-6.00	0.04	0.21	0.28			0.18	0.12	0.07	69.86
	-7.00	1.99	1.65	1.92			1.85	0.18	0.10	9.69
	-7.30	NA	3.29	NA			NC	NC	NC	NC
	-7.60	NA	6.43	NA	b	a	NC	NC	NC	NC
	-8.00	18.07	14.80	15.26			16.04	1.77	1.02	11.03
	-8.30	NA	NA	25.77			NC	NC	NC	NC
	-8.60	NA	45.31	43.67			44.49	NC	NC	NC
	-9.00	71.16	70.31	67.81			69.76	1.74	1.01	2.50
	-9.60	NA	NA	89.43			NC	NC	NC	NC
	-10.00	95.95	NA	91.50			93.73	NC	NC	NC

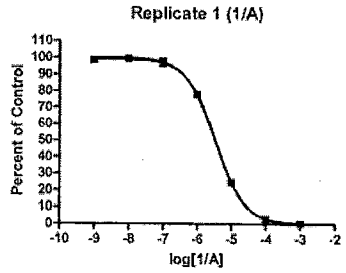
Table 19. Mean Percent of Control per Replicate (Continued)

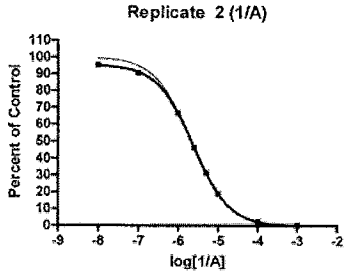
Reference Chemical	Log [Ref. Chemical]	Mean Percent of Control					Overall			
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Mean	SD	SEM	%CV
Ketoconazole	-4.00	6.29	5.64	6.19	b	a	6.04	0.35	0.20	5.79
	-4.60	NA	22.01	21.85			21.93	NC	NC	NC
	-5.00	44.71	41.21	40.69			42.20	2.19	1.26	5.18
	-5.30	NA	56.70	46.16			51.43	NC	NC	NC
	-5.60	NA	71.11	69.02			70.07	NC	NC	NC
	-6.00	86.71	78.46	74.68			79.95	6.15	3.55	7.69
	-6.60	99.84	87.94	89.06			92.28	6.57	3.79	7.12
	-7.00	98.15	89.97	91.53			93.22	4.34	2.51	4.66
	-8.00	94.08	NA	NA			NC	NC	NC	NC
	-9.00	93.24	NA	NA			NC	NC	NC	NC
	-10.00	93.03	NA	NA			NC	NC	NC	NC

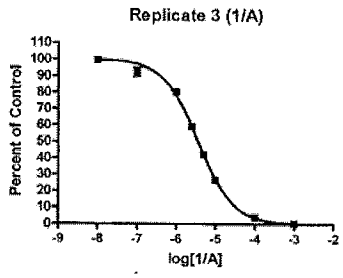
NA = Not applicable- this test concentration was not assayed in this replicate.
NC = Not calculated- only one or two data points - no mean, SD, SEM or % CV calculated.
a There was no replicate 5 for chrysin, econazole and ketoconazole.
b There was no replicate 4 for econazole and ketoconazole.

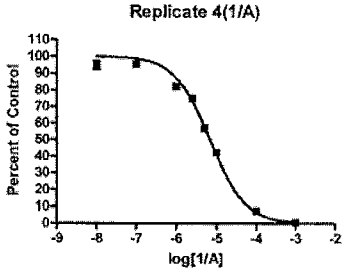
4.4.3 IC_{50} Values

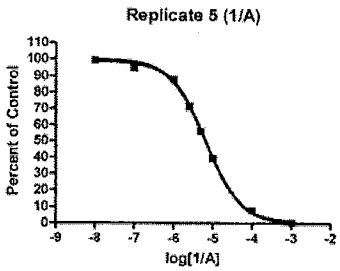
For each reference chemical and replicate the percent of control aromatase activity values were fitted to the equation presented in Section 3.7.2.1 using Prism 4.0 and the IC_{50} and slope were calculated. The concentration response curves for aminoglutethimide, chrysin, econazole, and ketoconazole are presented in Figures 5 through 8, respectively.









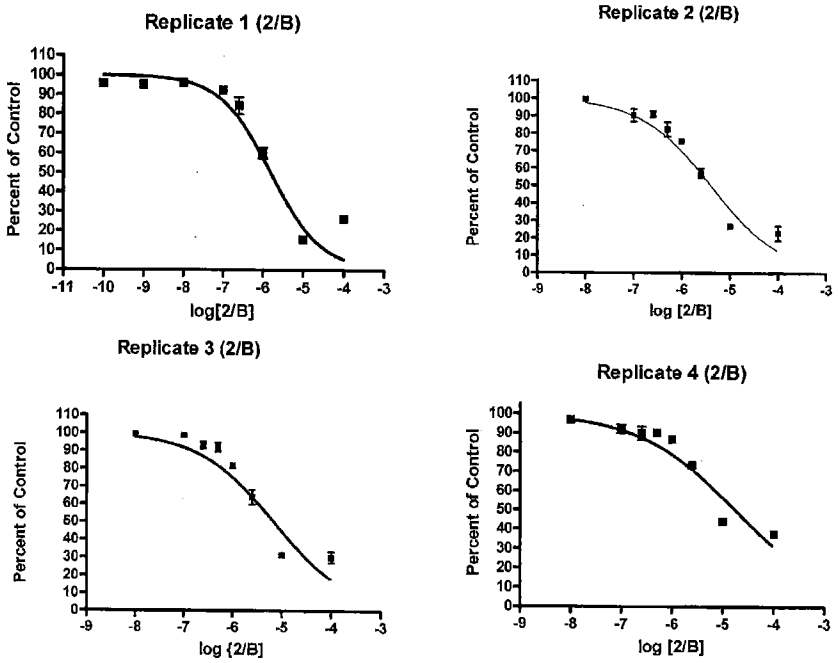


	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Sigmoidal dose-response (variable slope)					
Best-fit values					
BOTTOM	0.0	0.0	0.0	0.0	0.0
TOP	100	100	100	100	100
LOGEC50	-5.470	-5.670	-5.436	-5.161	-5.181
HILLSLOPE	-1.018	-0.9532	-0.9816	-0.9950	-0.9592
EC50	3.385E-06	2.137E-06	3.660E-06	6.906E-06	6.596E-06
Std. Error					
LOGEC50	0.01351	0.01218	0.01089	0.01968	0.01405
HILLSLOPE	0.01797	0.01882	0.01953	0.03027	0.02130
95% Confidence Intervals					
LOGEC50	-5.499 to -5.442	-5.695 to -5.645	-5.459 to -5.414	-5.202 to -5.120	-5.210 to -5.152
HILLSLOPE	-1.056 to -0.9807	-0.9923 to -0.9142	-1.022 to -0.9410	-1.058 to -0.9318	-1.003 to -0.9151
EC50	3.172E-06 to 3.613E-06	2.016E-06 to 2.265E-06	3.474E-06 to 3.856E-06	6.281E-06 to 7.592E-06	6.168E-06 to 7.054E-06
Goodness of Fit					
Degrees of Freedom	19	22	21	22	22
R^2 (unweighted)	0.9986	0.9961	0.9968	0.9904	0.9961
Weighted Sum of Squares (1/Y)	0.8064	1.654	1.469	4.701	2.338
Absolute Sum of Squares	52.30	115.2	93.99	282.0	122.4
Sy.x	1.659	2.288	2.116	3.580	2.359
Constraints					
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0	TOP = 100.0	TOP = 100.0	TOP = 100.0
Data					
Number of X values	7	8	8	8	8
Number of Y replicates	3	3	3	3	3
Total number of values	21	24	23	24	24
Number of missing values	0	0	1	0	0

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Sigmoidal dose-response (variable slope)					
Best-fit values					
BOTTOM	0	0	0	0	0
TOP	100	100	100	100	100
LOGEC50	-5.470	-5.670	-5.436	-5.159	-5.181

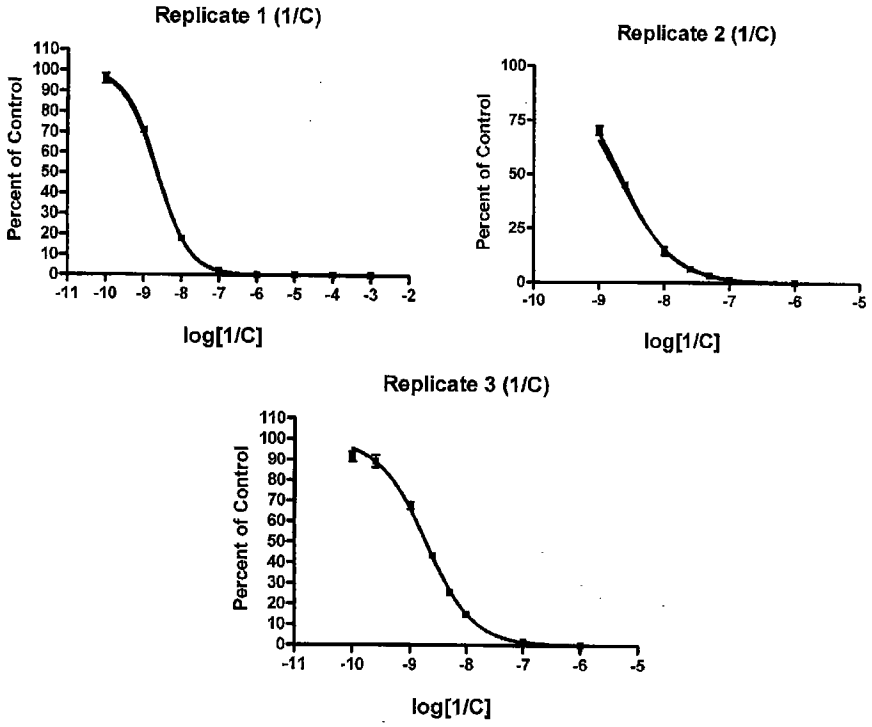
HILLSLOPE	-1.018	-0.9532	-0.9816	-0.9965	-0.9592
EC50	3.385E-06	2.137E-06	3.660E-06	6.941E-06	6.596E-06
Std. Error					
LOGEC50	0.01351	0.01218	0.01089	0.01968	0.01405
HILLSLOPE	0.01797	0.01882	0.01953	0.03027	0.02130
95% Confidence Intervals					
LOGEC50	-5.499 to -5.442	-5.695 to -5.645	-5.459 to -5.414	-5.199 to -5.118	-5.210 to -5.153
HILLSLOPE	-1.056 to -0.9807	-0.9923 to -0.9142	-1.022 to -0.9410	-1.059 to -0.9337	-1.003 to -0.9151
EC50	3.172E-06 to 3.613E-06	2.016E-06 to 2.265E-06	3.474E-06 to 3.856E-06	6.318E-06 to 7.624E-06	6.168E-06 to 7.054E-06
Goodness of Fit					
Degrees of Freedom	19	22	21	22	22
R^2 (unweighted)	0.9986	0.9961	0.9968	0.9905	0.9961
Weighted Sum of Squares (1/Y)	0.8064	1.654	1.469	4.711	2.338
Absolute Sum of Squares	52.30	115.2	93.99	278.8	122.4
Sy.x	1.659	2.288	2.116	3.560	2.359
Constraints					
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0	TOP = 100.0	TOP = 100.0	TOP = 100.0
Data					
Number of X values	7	8	8	8	8
Number of Y replicates	3	3	3	3	3
Total number of values	21	24	23	24	24
Number of missing values	0	0	1	0	0

Figure 5 - Fitted Concentration Response Curves for Aminoglutethimide



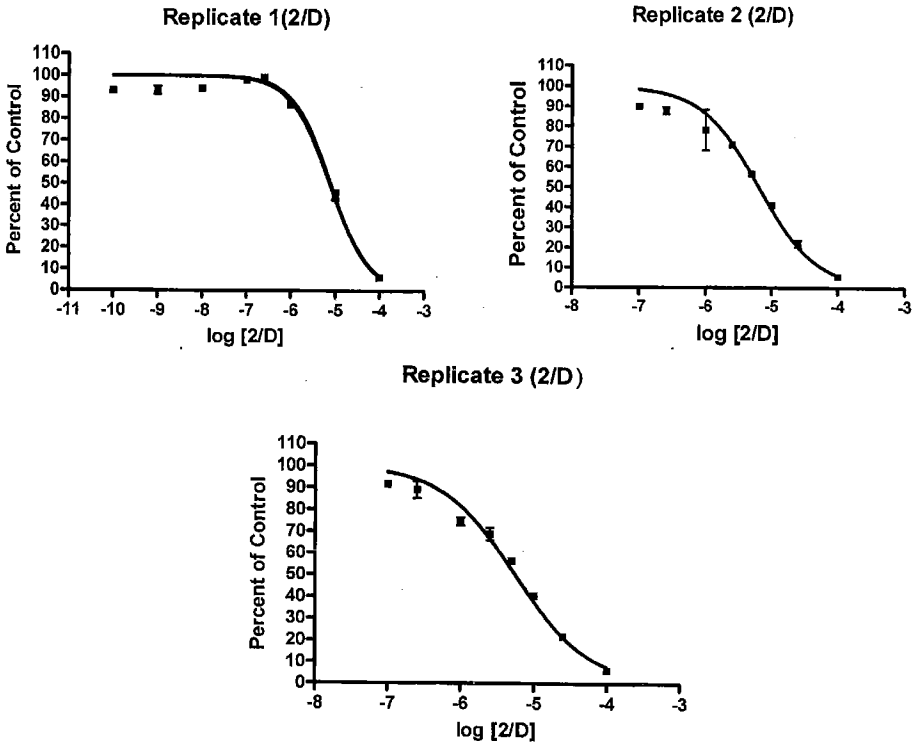
	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Sigmoidal dose-response (variable slope)				
Best-fit values				
BOTTOM	0.0	0.0	0.0	0.0
TOP	100	100	100	100
LOGEC50	-5.807	-5.393	-5.160	-4.752
HILLSLOPE	-0.6870	-0.5970	-0.5723	-0.4597
EC50	1.558E-06	4.042E-06	6.921E-06	1.772E-05
Std. Error				
LOGEC50	0.1134	0.07050	0.09077	0.08748
HILLSLOPE	0.1015	0.05858	0.07143	0.04547
95% Confidence Intervals				
LOGEC50	-6.043 to -5.572	-5.540 to -5.247	-5.348 to -4.972	-4.933 to -4.570
HILLSLOPE	-0.8976 to -0.4765	-0.7185 to -0.4755	-0.7205 to -0.4242	-0.5540 to -0.3654
EC50	9.064E-07 to 2.678E-06	2.887E-06 to 5.660E-06	4.487E-06 to 1.068E-005	1.167E-06 to 2.691E-05
Goodness of Fit				
Degrees of Freedom	22	22	22	22
R^2 (unweighted)	0.9158	0.9426	0.8981	0.9023
Weighted Sum of Squares (1/Y)	63.00	30.02	40.93	20.09
Absolute Sum of Squares	1947	1062	1786	1106
Sy.x	9.409	6.947	9.009	7.090
Constraints				
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0	TOP = 100.0	TOP = 100.0
Data				
Number of X values	8	8	8	8
Number of Y replicates	3	3	3	3
Total number of values	24	24	24	24
Number of missing values	0	0	0	0

Figure 6 - Fitted Concentration Response Curves for Chrysin



	Replicate 1	Replicate 2	Replicate 3
Sigmoidal dose-response (variable slope)			
Best-fit values			
BOTTOM	0.0	0.0	0.0
TOP	100.0	100.0	100.0
LOGEC50	-8.628	-8.678	-8.718
HILLSLOPE	-1.043	-1.083	-1.035
EC50	2.355E-09	2.099E-09	1.913E-09
Std. Error			
LOGEC50	0.03035	0.01378	0.01230
HILLSLOPE	0.04083	0.02014	0.02272
95% Confidence Intervals			
LOGEC50	-8.691 to -8.565	-8.706 to -8.649	-8.744 to -8.693
HILLSLOPE	-1.128 to -0.9588	-1.125 to -1.041	-1.082 to -0.9882
EC50	2.038E-09 to 2.723E-09	1.966E-09 to 2.242E-09	1.804E-09 to 2.028E-09
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9995	0.9982	0.9965
Weighted Sum of Squares (1/Y)	4.895	1.956	1.976
Absolute Sum of Squares	15.73	25.05	100.8
Sy.x	0.8456	1.067	2.140
Constraints			
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0	TOP = 100.0
Data			
Number of X values	8	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	0	0	0

Figure 7 - Fitted Concentration Response Curves for Econazole



	Replicate 1	Replicate 2	Replicate 3
Sigmoidal dose-response (variable slope)			
Best-fit values			
BOTTOM	0.0	0.0	0.0
TOP	100.0	100.0	100.0
LOGEC50	-5.106	-5.216	-5.246
HILLSLOPE	-1.043	-0.9201	-0.8685
EC50	7.830E-06	6.081E-06	5.675E-06
Std. Error			
LOGEC50	0.02991	0.02884	0.02597
HILLSLOPE	0.04869	0.04367	0.03461
95% Confidence Intervals			
LOGEC50	-5.168 to -5.044	-5.276 to -5.156	-5.300 to -5.192
HILLSLOPE	-1.144 to -0.9420	-1.011 to -0.8295	-0.9404 to -0.7965
EC50	6.788E-06 to 9.033E-06	5.298E-06 to 6.978E-06	5.011E-06 to 6.426E-06
Goodness of Fit			
Degrees of Freedom	22	22	21
R^2 (unweighted)	0.9810	0.9662	0.9780
Weighted Sum of Squares (1/Y)	5.043	10.70	6.985
Absolute Sum of Squares	449.0	699.2	448.0
Sy.x	4.518	5.638	4.619
Constraints			
BOTTOM	BOTTOM = 0.0	BOTTOM = 0.0	BOTTOM = 0.0
TOP	TOP = 100.0	TOP = 100.0	TOP = 100.0
Data			
Number of X values	8	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	23
Number of missing values	0	0	1

Figure 8 - Fitted Concentration Response Curves for Ketoconazole

For aminoglutethimide, econazole, and ketoconazole, the percent of control activity values for the tested concentrations essentially span the range 0 to 100%. The model fits the data well for each of these reference chemicals. In the case of chrysin, however, where the percent of control data for the tested concentrations ranges from approximately 23 to 100%, the model does not fit the data as well. The poor fit arises due to the constraint inherent in the equation for the top and bottom plateaus to be 100 and 0%, respectively. In the current case, with the model constrained so that the top and bottom plateaus are 100 and 0%, Prism estimates the $\log_{10}IC_{50}$ value as the log concentration corresponding to 50% remaining activity, even when data do not fully span the 0 to 100% range.

Figure 9 shows the concentration response curve fits of each replicate per reference chemical superimposed on one plot. There are noticeable differences between curves for the same reference chemical across replicates. The highest variations were observed between five replicates of aminoglutethimide and four replicates. When only three first replicates of aminoglutethimide are superimposed on one plot higher similarity (better fit) between curves is observed. The curve fit is poor for chrysin since the data do not span the entire 0 to 100% range.

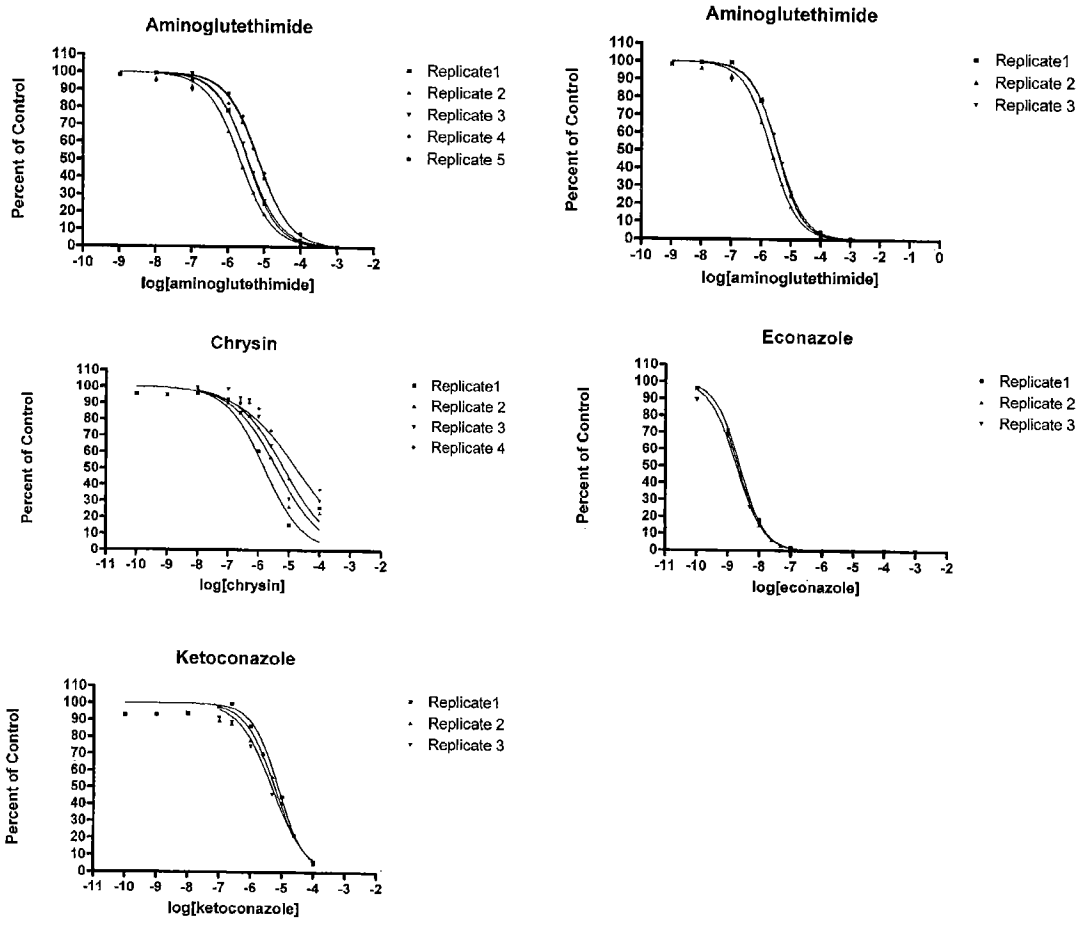


Figure 9 - Concentration Response Curve Fits (Overlay of Individual Replicate Results)

Figure 10 presents the results of curve fitting across the replicates for each reference chemical. The mean percent of control data for repetition within a replicate (as presented in Table 19) were fitted to the model.

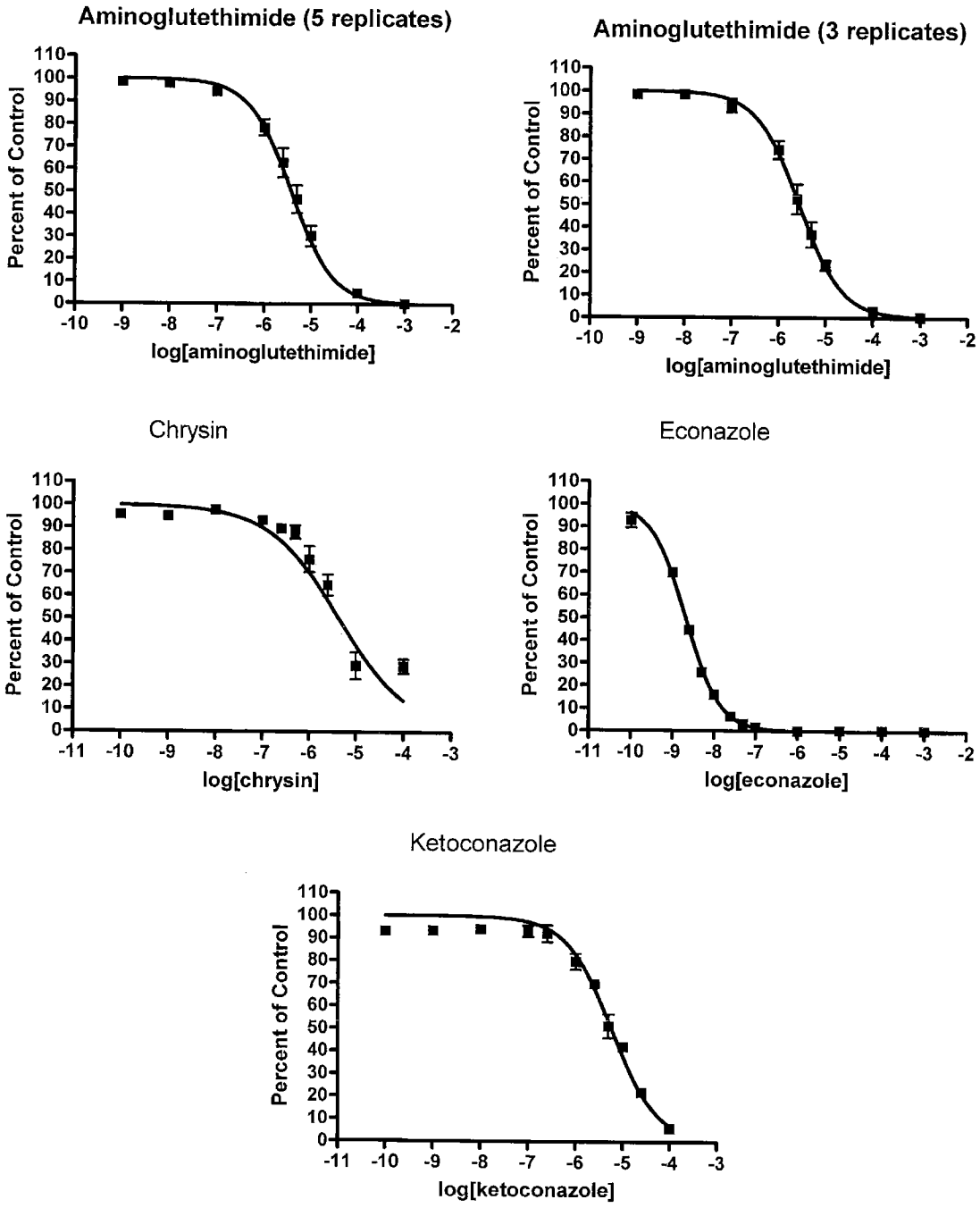


Figure 10 - Concentration Response Curve Fits (Across Replicates)

The IC_{50} and slope data are summarized in Table 20 by chemical and replicate. The status of each response is also indicated in the table. The concentration response curves for aminoglutethimide, ketoconazole, and econazole are characterized as "Complete (C)" since the percent of control data essentially spanned the 0 to 100% range. The curves for chrysin are characterized as "Incomplete (II)" since the percent of control data ranged from ca. 15 to 100%. The standard errors of the parameter estimates of $log_{10}IC_{50}$ are consistent across the replicates with the highest for chrysin. For the response curves estimates of the slope, the standard errors of the slope estimates for all reference chemicals are consistent within the chemicals, with the highest for chrysin.

Table 20 - Summary of $Log_{10}IC_{50}$ and Slope and their Associated Standard Errors (SE)

Reference Chemical	Chemical Code	Replicate	$Log_{10}IC_{50}$	SE $Log_{10}IC_{50}$	Slope	SE Slope	Status	IC_{50}	Mean* IC_{50}
Aminoglutethimide	1/A	1	-5.470	0.01351	-1.018	0.01797	C	3.39 µM	4.13[a] µM
		2	-5.670	0.01218	-0.9532	0.01882	C	2.14 µM	2.99[b] µM
		3	-5.436	0.01089	-0.9816	0.01953	C	3.66 µM	
		4	-5.161	0.01985	-0.9950	0.03047	C	6.91 µM	
		5	-5.181	0.01405	-0.9592	0.02130	C	6.60 µM	
Chrysin	2/B	1	-5.807	0.1134	-0.6870	0.1015	II	1.56 µM	5.27 µM
		2	-5.393	0.07050	-0.5970	0.05858	II	4.04 µM	
		3	-5.160	0.09077	-0.5724	0.07144	II	6.92 µM	
		4	-4.752	0.08748	-0.4597	0.04547	II	17.72 µM	
Econazole	1/C	1	-8.628	0.03035	-1.043	0.04083	C	2.36 nM	2.11 nM
		2	-8.678	0.01378	-1.083	0.02014	C[c]	2.10 nM	
		3	-8.718	0.01230	-1.035	0.02272	C	1.91 nM	
Ketoconazole	2/D	1	-5.106	0.02991	-1.043	0.04869	C	7.83 µM	6.47 µM
		2	-5.216	0.02884	-0.9201	0.04367	C	6.08 µM	
		3	-5.246	0.02597	-0.8685	0.03461	C	5.68 µM	

* Weighted geometric mean.
a. Calculated for 1 to 5 replicates.
b. Calculated for 1 to 3 replicates
c. The curve was not tested at the lowest concentration (10^{-10} M) due to a technical error. If this concentration would have been tested, then the curve would have been characterized to 100 percent and, it was for this reason, that the curve was classified as being "Complete".

The calculated weighted geometric mean IC_{50} value calculated for all five replicates of aminoglutethimide is 4.13 µM and when calculated for first three replicates is 2.99 µM. The IC_{50} weighted geometric mean values calculated for chrysin, econazole and ketoconazole are 5.27 µM, 2.11 nM and 6.47 µM, respectively.

The weighted geometric mean IC_{50} values are slightly different from values reported in the statistical report (Appendix G). During statistical analysis, all experimental data were included into analysis (Appendix G, page 3); as for results presented in the main part of this report, some data were not taken into calculations as specified in Table 18.

5.0 DISCUSSION

The presented study (Task 5) involved testing the response of human placental microsomes aromatase to the presence of four reference chemicals. Eight different concentrations of each reference chemical, ranging from 1×10^{-10} to 1×10^{-3} M were applied to create the dose response curves. Data from the first replicate of each reference

chemical were reviewed and the target concentrations were adjusted to better define the concentration response curve. Five independent replicates for aromatase assays were performed with aminoglutethimide, four with chrysin, and three with econazole and ketoconazole. The additional replicates (two for aminoglutethimide and one for chrysin) were added to the study (as requested by Sponsor) because the results from the second replicate for aminoglutethimide and the first for chrysin appeared aberrant. The overall IC_{50} value for all four reference chemicals are presented in Table 20. Replicate to replicate variation in IC_{50} was high for aminoglutethimide and chrysin and was much lower for econazole and ketoconazole.

The two-parameter model used for concentration-response curve fitting leads to unsatisfactory curve fits where data do not span the entire 0 to 100% range (chrysin). However, the applied model is calculating the IC_{50} properly as the concentration corresponding to 50% inhibition.

The microsomes used in presented study were prepared at RTI. At the time of preparation, the protein content of the microsomal suspension was determined to be 14 mg/mL.

For the presented study, the protein content of the microsomal preparation was measured each time the aromatase assay was run (total 15 runs). The average determined protein concentration was 11.24 mg/mL (12.79% CV). In order to better characterize the protein assay, QC samples were included in some runs (starting from February 23, 2005). The data show acceptable correlation (< 5% difference from known value) of calculated and known amounts for QC samples at 0.500 and 1.000 mg/mL. However, at a nominal concentration of 0.125 mg/mL the difference between measured and known concentration was greater than 30%. The microsomal protein dilutions that were actually measured in the protein assay had a concentration of about 0.22 mg/mL (11.24/ 50).

This concentration falls between the low end of the reliable range of the protein standard curve and the low end of the standard curve range (0.14 to 1.0 mg/mL) and therefore may be subject to a large variance from the true value. Furthermore, the microsomal dilution that was used for protein measurement was not the same dilution that was used in the aromatase assay. The dilution used in the aromatase assay was ten-fold more dilute than that used for protein determination.

Because the concentration of protein used in the aromatase assay is very low (0.0125 mg/mL) and because the aromatase activity is calculated by normalizing the amount of product by the protein content, relatively small variations in protein content can have a large effect on the measured aromatase activity.

Four types of controls were used for the aromatase assay: a full activity control which served as the 100% activity control, a background activity control which was used to correct for non-enzymatic product formation and other coincidental radiochemical content in the assay mixture, a positive control which used a known aromatase inhibitor and a negative control which used a known aromatase non-inhibitor.

All calculated aromatase activities were corrected for the radioactivity in the background activity control, so the average background control activity for a replicate necessarily was 0 (zero). The average full aromatase activity across all 15 runs of the aromatase assay was 0.0490 ± 0.0057 nmol/mg/min (11.70% CV). The positive control activity across 15 runs averaged 0.0275 ± 0.0041 nmol/mg/min (15.11% CV), approximately 56% of full aromatase activity and the negative control activity averaged 0.0486 ± 0.0064 nmol/mg/min (13.17% CV), approximately 99.2% of full aromatase activity.

For almost all controls, a decrease in aromatase activity was noted between the controls run at the beginning of each replicate and those run at the end of the replicate. It may suggest an issue with decreasing aromatase activity over time.

The full statistical analysis report is presented in Appendix G. There are some small differences in data obtained from Prism output and data presented in the statistical report obtained by applying the SAS statistical analysis system.

6.0 CONCLUSION

The responsiveness (in a concentration dependent manner) of the human placental microsomes aromatase assay to aminoglutethimide, chrysin, econazole, and ketoconazole was confirmed.

It was determined that econazole was the most potent aromatase activity inhibitor (IC_{50} = 2.11 nM) from the group of four studied reference chemicals.

The importance of microsomal protein accuracy and precision determination was established.

The dependency between the amount of protein used in the aromatase assay and determined aromatase activity was observed. The high variability in calculated aromatase activity may be explained by inaccurate protein concentration determination.

The applied model for fitting the concentration dependent curve worked well for aminoglutethimide, econazole, and ketoconazole, but needs some improvements for compounds like chrysin.

APPENDIX A

Protocol (with Amendments and Deviations)

Aromatase Assay Validation: Conduct Studies with Centrally Prepared Microsomes (with Amendments) A-1

BATTELLE STUDY PROTOCOL

AROMATASE ASSAY VALIDATION: CONDUCT STUDIES WITH CENTRALLY PREPARED MICROSOMES

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EPA Contract: 68-W-01-023
EPA Work Assignment: WA 4-16, Task 5

Battelle
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Experiment Start Date: January 31, 2005
Experiment End Date: March 15, 2005

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AROMATASE ASSAY VALIDATION: CONDUCT STUDIES WITH CENTRALLY PREPARED MICROSOMES

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AROMATASE ASSAY VALIDATION: CONDUCT STUDIES WITH CENTRALLY PREPARED MICROSOMES

1.0 OBJECTIVES

The objective of this protocol is to describe procedures for conduct of the aromatase assay using human placental microsomes. In this task, studies will be conducted with four potential aromatase inhibitors (reference chemicals) to demonstrate the responsiveness of the assay. Microsomes will be prepared by the lead laboratory (RTI International).

Justification for test system: The test system for this study is human placental microsomes. This test system was selected because it provides a biological source of the aromatase enzyme and, since the assay is being evaluated for its potential to serve as a screening assay, the use of human tissue enhances its predictive potential.

This *in vitro* test method involves combining microsomes, substrate, appropriate cofactors and test substances in a common reaction vessel. The effect of the test substances on microsomal enzyme activity is evaluated by measuring the amount of the product formation.

There is no applicable route of administration in the sense of a dose administration route for this *in vitro* test.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and human placental microsomes will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

Procedure for identification of the test system: Each test tube used in the conduct of the aromatase assay will be uniquely identified by applying a label or writing directly on the test tube.

2.1 Substrate

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1 β-^{3}H]-androstenedione, [^{3}H]ASDN) will be provided to the laboratories by Battelle's Chemical Repository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN (of each lot that is used) will be assessed by the lead laboratory (RTI). The radiochemical purity will be greater then approximately 95%, if less then 95%, then the Sponsor will be notified.

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2.1.2 Preparation of Substrate Solution for use in Aromatase Assay

A solution containing a mixture of non-radiolabeled and radiolabeled [^{3}H]ASDN will be prepared to achieve 100 nM final concentration of ASDN in the assay and the amount of tritium added to each incubation about 0.1 µCi. This substrate solution should have a concentration of 2 µM with a radiochemical content of about 1 µCi/mL.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Prepare a 1:100 dilution of the radiolabeled stock in buffer. Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL. Combine 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for 80 tubes). Record the weight of each component added to the substrate solution. After mixing the solution well, weigh aliquots (ca 20 µL) and combine with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yields a final [^{3}H]ASDN concentration of 100 nM with 0.1 µCi/tube.

2.2 Reference Chemicals

The reference chemicals for this task are aminoglutethimide, chrysin, econazole and ketoconazole (Table 1).

Table 1. Reference Chemicals for Task 5

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Basis of Selection
Aminoglutethimide	125-84-8	$C_{13}H_{16}N_2O_2$	232.3	Non-steroidal aromatase inhibitor
Chrysin	480-40-0	$C_{15}H_{10}O_4$	254.2	Potent flavonoid
Econazole (nitrate)	24169-02-6	$C_{18}H_{15}Cl_3N_2O$-HNO_3	444.7	Potent imidazole anti-fungal
Ketoconazole	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	531.43	Weak imidazole anti-fungal

2.2.1 Reference Chemical Formulation and Analysis

Reference chemical stock solution will be prepared and analyzed by the CR and distributed to the laboratories. Reference chemicals will be formulated in buffer, absolute ethanol and dimethyl sulfoxide (DMSO). The total volume of reference chemical formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilution of the stock solution will be prepared in the same solvent as the stock solution on the day of use such that the target concentration of reference chemical can be achieved by the addition of the 20 µL of the dilution to a 2 mL assay volume. Information on the storage conditions for reference chemical stock solutions will be provided by the CR.

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2.3 Control Substances

The known aromatase inhibitor, 4-hydroxyandrostenedione (4-OH ASDN), will be used as the positive control substance. A known aromatase non-inhibitor, lindane, will be used as the negative control substance. Table 2 contains identity and property information for these substances.

Table 2. Control Substances

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Target Concentration in Assay (M)	Basis for Selection
4-OH ASDN	566-48-3	$C_{19}H_{26}O_3$	302.4	5×10^{-8}	Known aromatase inhibitor
Lindane	58-89-9	$C_6H_6Cl_6$	290.8	1×10^{-6}	Affects StAR and cholesterol metabolism; no aromatase activity

2.3.1 Control Substance Formulation and Analysis

Control substances stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. Control Substances will be formulated in buffer, absolute ethanol or DMSO. The total volume of control substance formulation used in each assay should be no more than 1 % of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dilutions will be prepared such that the target concentration of control substance (Table 2) can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Information on storage conditions for control substance stock solution will be provided by the CR.

2.4 Microsomes

Human placental microsomes will be supplied to each laboratory by the lead laboratory. These samples should be treated as potentially infectious and appropriate precautions must be employed. The microsomes must be stored at approximately -70°C. The approximate protein content of the microsomes will be 14 mg/mL.

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

On the day of use, microsomes will be thawed quickly in a 37 ± 1°C water bath and then immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) prior to use. The microsomes will be diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome

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samples will be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. The microsomes should not be left on ice for longer than approximately 2 hours before proceeding with the assay or the microsomal enzyme activity may be decreased.

Excess undiluted stock microsomes may be flash frozen in liquid nitrogen and returned to ca. -70 °C storage for future use. It is strongly recommended that stock microsomes to be refrozen be divided into aliquots appropriate for use prior to refreezing in order to minimize the number of freeze/thaw cycles.

Diluted microsomes must be used only on the day of preparation. Under no conditions should diluted microsomes be refrozen for later use in the assy.

2.5 Other Assay Components

2.5.1 Buffer

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic and sodium phosphate dibasic will be used in the preparation of the buffer. Solutions of each reagent at 0.1 M will be prepared in deionized water and then the solutions will be combined to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (ca. 2-8 °C).

2.5.2 Propylene Glycol

Propylene glycol will be added to the assay directly as described below.

2.5.3 NADPH

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form), is the required co-factor for aromatase. The final concentration in the assay will be 0.3 mM. Typically, a 6 mM stock solution will be prepared in assay buffer and then 100 µL of the stock will be added to the 2 mL assay volume. NADPH solution must be prepared fresh each day and kept on ice until use.

3.0 PROTEIN ASSAY

The protein concentration in the microsomes will be determined each day of microsome use in the aromatase assay by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). A 6-point standard curve will be prepared; target range will be from 0.14 to 1.0 mg protein/mL. The protein standards will be made from bovine serum albumin (BSA). To a 25 µL aliquot of microsomes solution (1:50 dilution of microsomes may be required) or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or microsomes solution and the samples will be gently mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow color development. (The absorbances are stable for about 1 hour.) Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (750 nm)

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will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined by interpolation, reading the concentration of protein on the standard curve that corresponds to its absorbance.

4.0 AROMATASE ASSAY METHOD

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1.0 mL). The final concentrations for the assay components are presented in Table 3. The tubes and the microsomal suspension will be placed at 37 ± 1°C in the water bath for approximately five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes will be then vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6 centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution.

Table 3. Optimized Aromatase Assay Conditions

Assay Factor (units)	Human Placental
Microsomal Protein (mg/mL)[a]	0.0125
NADPH (mM)[a]	0.3
[^{3}H]ASDN (nM)[a]	100
Incubation Time (min)	15

[a] Final concentrations

Analysis of the samples will be performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represents amount of formed ^3H_2O.

5.0 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO REFERENCE CHEMICALS

Each replicate will test the response of aromatase activity to the presence of eight concentrations of reference chemical. The reference chemicals must be coded prior to distribution to the assaying technicians in order that the replicates are conducted blind for reference chemical identity. This

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task will be conducted in three independent replicates. All three replicates for a given reference chemical must be conducted by the same technician. However, the same technician is not required to perform the three replicates for all four reference chemicals. Multiple reference chemicals may be conducted by a single technician in a given day. Each replicate for a given reference chemical must be conducted entirely independently of the other replicates for that reference chemical. Thus, it is recommended that if multiple replicates are conducted on a given day by a single technician, those replicates should use different reference chemicals. Each reference chemical will be tested at eight concentrations and there will be three (triplicate) repetitions for each concentration of a give replicate. A single replicate study of a given chemical is described in Table 4.

Four types of control samples will be included for each replicate. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of the test substance solutions] and microsomes)
- background activity controls (all components that are in full aromatase activity controls, except NADPH)
- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration)
- negative controls (all components that are in the full aromatase controls, except vehicle, and with the addition of lindane at a single concentration).

Four test tubes of each type control are included with each replicate and are treated the same as the other samples. The controls sets will be split so that two tubes (of each control type) are run at the beginning and two at the end of each replicate set.

The assay will be conducted as described in Section 4.0 with the following modification. Reference chemical solution (or vehicle) will be added to the mixture of propylene glycol, substrate, NADPH and buffer in a volume not to exceed 20 µL prior to preincubation of that mixture. The volume of buffer used will be adjusted so the total incubation volume remains at 2 mL.

After completion of the first replicate, the data will be reviewed and, if necessary, the concentration of reference chemical used in the second and third replicates can be adjusted. The decision whether to adjust test concentration will rest with the Study Director. The decisions should be based on the results from the first replicate with the following guidelines in mind:

- If insolubility is observed at the high concentration (10^{-3} M), then set the highest concentration for the second and third replicates at the highest concentration that appeared to be soluble (limited to 10^{-4} or 10^{-5} M). Do not use a concentration lower than 10^{-5} M for the highest concentration tested.
- If the highest concentration to be tested is lowered to 10^{-4} or 10^{-5} M, then add midlog concentration(s) near estimated IC_{50} based on the replicate one results in order to keep eight concentrations in the test set.
- The lowest concentration to be tested is 10^{-10} M.

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Table 4. Reference Chemical Study Design

Sample type	Repetitions (test tubes)	Description	Test Chemical concentration (M)
Full Activity Control	4	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control	4	Complete assay with reference vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay[a] with positive control chemical (4-OH ASDN)added	5×10^{-8}
Negative Control	4	Complete assay[a] with negative control chemical (lindane) added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}

[a]The Complete Assay contains buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH

6.0 DATA ANALYSIS

6.1 Aromatse Activity and Percent of Control Calculations

Relevant data will be entered into the latest version of the spreadsheet Aromatase_Master_Versionx.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. The version of the spreadsheet used will be included in the reports. A working document detailing the use of this spreadsheet appears in Appendix A.

6.2 Statistical Analyses

6.2.1 Concentration Response Fits for the Test Substance

For the reference chemicals multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three.
For each replicate two repeat tubes of the full enzyme activity controls (FEAC), the

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background activity controls (BAC) and the positive and negative controls will be run prior to the repetition of the graded concentrations of the reference chemical and two repeat tubes of each control will be run following the repetition of the reference chemical. Three repetitions will be prepared for each concentration of the reference chemical.

For each repeat tube (FEAC, BAC, positive and negative controls and each reference chemical concentration) the Excel database spreadsheet will include total observed (uncorrected) disintegrations per minutes (dpms) per tube and total aromatase activity per tube. The dpm and aromatase activity values will be corrected for background dpms, as measured by average of BAC control tubes. The aromatase activity will be calculated as the corrected dpm, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the microsomes, and the incubation time. The average (corrected) dpms and aromatase activity across the four BAC control repeat tubes must necessarily be equal to 0 within each replicate.

For each tube percent of control will be determined by dividing the background corrected aromatase activity for that tube by average background corrected aromatase for activity for the four FEAC tubes and multiplying by 100. Nominally one might expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations. However due to experimental variation individual observed percent control values will sometimes extend below 0% or above 100%.

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each reference chemical concentration. Concentration will be expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of reference chemical (e.g. if concentration = 10^{-5} then X = -5). Let

$Y \equiv$ percent of control activity in the inhibitor tube
$X \equiv$ logarithm (base 10) of the concentration
DAVG $\equiv$ average dpms across the repeat tubes with the same reference chemical concentration
$\beta \equiv$ slope of the concentration response curve (β will be negative)
$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%).

The following concentration response curve will be fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu-X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance is approximated by Y.

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits will be carried out using Prism software (Version 3 or higher). Observed individual percent activity values above 100% will be set to 99.5%.

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Observed individual percent activity values below 0% will be set to 0.5%. Model fits will be carried out using Prism software (Version 3 or higher).

Concentration response models will be fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate the extent of aromatase inhibition will be summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for a reference chemical will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β and the replicate-to-replicate component of variation will be calculated based on a one-way random effects analysis of variance model fit. For each reference chemical and replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve will be indicated as:

- "C" Complete. i.e. ranging from essentially 0 percent to 100 percent of control.
- "II" Incomplete. But can interpolate to $\log_{10}IC_{50}$.
- "IX" Incomplete. But must extrapolate to $\log_{10}IC_{50}$.

Replicates for which a concentration response curve cannot be fitted (and so an IC_{50} cannot be estimated) will be referred to as "noninhibitors".

6.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each replicate the individual percent of control values will be plotted versus logarithm of the reference chemical concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each replicate.

Additional plots will be prepared to compare the percent of control activity values across replicates. For each replicate the average percent of control values will be plotted versus logarithm of reference chemical concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curves for each replicate will be superimposed on the plots. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of reference chemical concentration. The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}). Let X and Y ($0< Y <100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}].$$

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on one-way random effects analysis of variance, treating the replicates as random effects. For each of β and μ, plots will be prepared that display the parameters within each replicate with

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associated 95% confidence intervals based on the within replicate standard error and average across replicates with associated 95% confidence interval incorporating replicate-to replicate variation.

6.2.3 Graphical and Analysis of Variance Comparisons of FEAC, BAC, and Positive and Negative Control Percent of Control Across Reference Chemicals and Replicates

Within each replicate of each reference chemical quadruplicate repetitions will be made of the FEAC control, BAC control, and negative and positive control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are consistent throughout the replicate test, the control tubes at the beginning should be equivalent to those at the end.

To assess whether this is the case the control responses will be adjusted for background dpms, divided by the average of the (background adjusted) FEAC control values, and expressed as percent of control. The average of the four BAC controls within a replicate must necessarily be 0 percent and the average of the four FEAC controls within a replicate must necessarily be 100 percent. The FEAC controls percent of control, the BAC controls percent of control, and the negative and positive controls percent of control values will be plotted across reference chemical and replicate within reference chemical, with plotting symbol distinguishing between beginning and end, and with reference line 0% (BAC control) or 100% (FEAC control) respectively. These plots will display the extent of consistency across reference chemicals and replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Additional plots will be prepared displaying the difference of the average of the first two percent of control values (i.e., those based on the "beginning" tubes) and the average of the last two percent of control values (i.e., those based on the "end" tubes) across reference chemicals and replicates within reference chemicals. Each plot will have a reference line of 0.

Three-factor mixed effects analysis of variance models will be fitted, separately for the FEAC control, the BAC control, and the positive and negative control tubes. The fixed effect factors in the analysis of variance will be:

- reference chemical
- portion (beginning or end)
- portion by reference chemical interaction.

The random effects will be:

- replicate nested within reference chemical
- portion by replicate within reference chemical interaction.

The residual error variation corresponds to repetition within reference chemical, replicate, and portion. The response will be percent of control. Since for the BAC and FEAC controls the average of the repetitions within a reference chemical and replicate are constrained to be 0 and 100 respectively, by the way in which "percent of control" is defined, the variation associated with the reference chemical effect and the replication within reference chemical effect are both necessarily constrained to be 0.

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If the daily replicates are in control the portion main effect and the portion by replicate within reference chemical interaction should be nonsignificant. If the portion by replicate within reference chemical interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate within reference chemical to the portion effect averaged across replicates within reference chemical, adjusting for simultaneity by Bonferroni's method.

6.2.4 Statistical Software

Concentration response curves will be fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using PRISM, the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS), as convenient.

6.2.4 Interlaboratory Statistical Analysis

The lead laboratory and each of the participating laboratories will carry out "intra-laboratory" statistical analyses based on their test data, according to this common statistical analysis plan, developed by the Data Coordination Center (Battelle). The Data Coordination Center will carry out the "inter-laboratory" statistical analysis. It will combine summary values developed in each of the intra-laboratory analyses to assess relationships among the laboratory results, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

7.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the archives for the life of the contract.

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study. The study will be conducted in compliance with the Federal Register, 40 CFR Part 160. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practices Standards.

9.0 REPORTS

Interim data summaries, draft and final reports will be submitted as described in Section 9.5 of the QAPP.

The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and test

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chemical repetition), percent of control activity, IC_{50} ,slope and graphs of activity versus log chemical concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the intra-laboratory statistical analyses described in Section 6 of this document.

10.0 STUDY RECORDS TO BE MAINTAINED

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments
- List of any Protocol Deviations
- List of Standard Operating Procedures
- Quality Assurance Project Plan (QAPP) and any Amendments
- List of any QAPP Deviations

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APPENDIX A

Notes for use of the spreadsheet: Aromatase_Master_Version1.1.xls

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Substrate Specific Activity Worksheet

This worksheet calculates:
1. the radiochemical content (dpm/mL) of the substrate solution.
2. the new specific activity of the [^{3}H]ASDN in the substrate solution.

The first item is based on the results of LSC analysis of weighed aliquots of the substrate solution.

The second item is calculated by:
1. determining the mass of ASDN (both radiolabeled and nonradiolabeled)/g of solution. This calculation uses both the measured mass of nonradiolabeled ASDN used in the solution preparation and also the specific activity of the stock [^{3}H] ASDN.

2. the radiochemical content (mCi/g) of the solution is then divided by the mass of ASDN/g solution to arrive at the new specific activity for [^{3}H]ASDN in the substrate solution.

Data to be input include:
- substrate solution aliquot weights (g) and dpm results,
- weight (mg) of ASDN used in original stock and volume (mL) of the original stock,
- all dilution factors for the dilution of ASDN stock to the solution that was finally used in substrate preparation,
- weight (g) of ASDN dilution used to prepare substrate solution and total weight (g) of substrate solution, and
- specific activity of the stock [^{3}H]ASDN (μCi/mmol).

Protein Worksheet

This worksheet calculates protein content based on absorbance data of standards and unknown samples obtained when samples are analyzed using a commercially available kit.

Data to be input include the concentration of protein standard stock solution (mg/10 mL), protein stock ID, Sample IDs, absorbance data (in triplicate) for standards and unknowns and appropriate dilution factors.

Absorbance values are corrected for blank absorbance. A calibration curve is prepared by linear regression of the standards data (corrected absorbance vs. mg protein measured). The concentration of protein in the unknowns is calculated based on the standard curve.

Microsomes and Chemical Dilutions Worksheet

This worksheet calculates the concentration of protein in the final microsomes dilution. It also serves as the data input center for the test chemical concentrations used in the assay.

Data input include volumes used in the preparation of microsomes dilutions. Also entered is the protein concentration of the stock microsomes. Normally, this value will be determined using the protein worksheet described above.

Test chemical concentrations are entered in molar units of the final concentrations used in the assay.

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Activity Calculation Worksheet

The primary aim of this worksheet is to calculate aromatase activity for each sample in a set based on measured dpm, protein concentration and incubation time.

The function of each section is described below:

Section 1 (Columns A-B)
This section contains fields for sample identification.

Section 2 (Columns C-I)
This section calculates the total dpm that remain in the incubation mixture after extraction (this is a measure of the ^3H_2O formed in the reaction).
Data input includes:
1. aliquot volume.
2. dpm measured for each aliquot of each sample.

Output:
The worksheet calculates the dpm/mL for each repetition and the total dpm contained in the sample (based on the aliquots and total sample volumes).

Section 3: (Columns J-L)
This section calculates the percent turnover of the substrate to product.

Data input: Volume of substrate solution used in each assay tube.

Linked Data: Column K links to radiochemical content value for the substrate that is calculated in the substrate specific activity worksheet.

Output: Percent conversion to product.

Section 4 (Columns M-N)
This section calculates the nmol ^3H_2O formed.

Data input: None.

Linked Data: Column N links to specific activity value for the substrate that is calculated in the substrate specific activity worksheet.

Calculations: Column M corrects the total dpm in each tube for background dpm determined in negative control tubes.

Column N converts dpm data to nmol using the substrate specific activity.

Data output: nmol ^3H_2O formed.

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Section 5 (Columns O-R)

This section calculates aromatase activity in each tube.

Data input: Volume of diluted microsomes used in assay tube and incubation time.

Output: Aromatase activity (nmol/mg protein/min).

Results Summary Worksheet

This worksheet summarizes the results.

Section 1 (Columns A-D, Rows 3-15)

This section summarizes control data.

Data input: None.

Output: Average and SD for control samples for beginning, end and overall portions.

Section 2 (Columns A-F, Rows 18-42)

This section summarizes activity values according to inhibitor level and replicate.

Data input: None.

Output: Log [test inhibitor].

Section 3 (Columns H-L, Rows 18-28)

This section calculates percent of control values for each test chemical concentration and replicate and organizes the data in a format suitable for importation into Prism Software.

Data input: None.

Output: Percent of control values with data arranged in a format suitable for importation into Prism Software.

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PROTOCOL AMENDMENT NUMBER 1

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Conduct Studies with Centrally Prepared Microsomes (WA 4-16, Task 5)**

PART TO BE CHANGED: Section 5.0, page 9, third bullet mark after *at a single concentration,* i.e. 5 x 10^{-8} M, will be added.
Section 5.0, page 9, fourth bullet mark after *at a single concentration,* i.e. 1x 10^{-6} M, will be added.
Section 6.0. Through entire section abbreviations FEAC and BAC will be replaced with full enzyme activity and background activity, respectively.

Section 6.2.1, page 12. The "Status" of each response curve will be indicated as:
"C" Complete. i.e. ranging from essentially 0 to 100 percent of control.
"II" Incomplete. But can interpolate to $\log_{10}IC_{50}$.
"IX" Incomplete. But must extrapolate to $\log_{10}IC_{50}$.

CHANGE TO: The "Status" of each response curve will be indicated as:
"Complete" curve obtained for any particular chemical (100-0%).
"Incomplete" curve but data points to at lest 50% inhibition.
"No inhibition" (no points below 80%). (See Attachment A).

REASON FOR CHANGE: Revision done on the request by Sponsor.

EFFECTIVE DATE: February 3, 2005

APPROVED BY:

Bozena D. Lusiak
Bozena D. Lusiak, Study Director

02-15-05
Date

Jerry D. Johnson
Sponsor Representative

2-15-05
Date

G608316

ATTACHMENT A

Table 4. Reference Chemical Study Design

Sample type	Repetitions (test tubes)	Description	Test Chemical concentration (M)
Full Activity Control	4	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control	4	Complete assay with reference vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay[a] with positive control chemical (4-OH ASDN)added	5×10^{-8}
Negative Control	4	Complete assay[a] with negative control chemical (lindane) added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}

[a]The Complete Assay contains buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH

6.0 DATA ANALYSIS

6.1 Aromatse Activity and Percent of Control Calculations

Relevant data will be entered into the latest version of the spreadsheet Aromatase_Master_Versionx.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. The version of the spreadsheet used will be included in the reports. A working document detailing the use of this spreadsheet appears in Appendix A.

6.2 Statistical Analyses

6.2.1 Concentration Response Fits for the Test Substance

For the reference chemicals multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three.
For each replicate two repeat tubes of the full enzyme activity controls, the background

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activity controls and the positive and negative controls will be run prior to the repetition of the graded concentrations of the reference chemical and two repeat tubes of each control will be run following the repetition of the reference chemical. Three repetitions will be prepared for each concentration of the reference chemical.

For each repeat tube (full enzyme activity, background activity, positive and negative controls and each reference chemical concentration) the Excel database spreadsheet will include total observed (uncorrected) disintegrations per minutes (dpms) per tube and total aromatase activity per tube. The dpm and aromatase activity values will be corrected for background dpms, as measured by average of background activity control tubes. The aromatase activity will be calculated as the corrected dpm, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the microsomes, and the incubation time. The average (corrected) dpms and aromatase activity across the four background activity control repeat tubes must necessarily be equal to 0 within each replicate.

For each tube percent of control will be determined by dividing the background corrected aromatase activity for that tube by average background corrected aromatase for activity for the four full enzyme activity tubes and multiplying by 100. Nominally one might expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations. However due to experimental variation individual observed percent control values will sometimes extend below 0% or above 100%.

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each reference chemical concentration. Concentration will be expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of reference chemical (e.g. if concentration = 10^{-5} then X = -5). Let

Y ≡ percent of control activity in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average dpms across the repeat tubes with the same reference chemical concentration
β ≡ slope of the concentration response curve (β will be negative)
$\mu \equiv \log_{10} IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%).

The following concentration response curve will be fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance is approximated by Y.

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits will be carried out using Prism software (Version 3 or higher). Observed individual percent activity values above 100% will be set to 99.5%.

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Observed individual percent activity values below 0% will be set to 0.5%. Model fits will be carried out using Prism software (Version 3 or higher).

Concentration response models will be fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate the extent of aromatase inhibition will be summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for a reference chemical will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β and the replicate-to-replicate component of variation will be calculated based on a one-way random effects analysis of variance model fit. For each reference chemical and replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve will be indicated as:

- "Complete" curve obtained for any particular chemical (100-0%).
- "Incomplete" curve but data points to at least 50% inhibition.
- "No inhibition" (no points below 80%).

Replicates for which a concentration response curve cannot be fitted (and so an IC_{50} cannot be estimated) will be referred to as "noninhibitors".

6.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each replicate the individual percent of control values will be plotted versus logarithm of the reference chemical concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each replicate.

Additional plots will be prepared to compare the percent of control activity values across replicates. For each replicate the average percent of control values will be plotted versus logarithm of reference chemical concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curves for each replicate will be superimposed on the plots. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of reference chemical concentration. The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}). Let X and Y ($0< Y <100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is

$$Y_{avg} = 100/[1 + 10^{\beta_{avg}(\mu_{avg} - X)}].$$

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on one-way random effects analysis of variance, treating the replicates as random effects. For each of β and μ, plots will be prepared that display the parameters within each replicate with

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associated 95% confidence intervals based on the within replicate standard error and average across replicates with associated 95% confidence interval incorporating replicate-to replicate variation.

6.2.3 Graphical and Analysis of Variance Comparisons of Full Enzyme Activity, Background Activity, and Positive and Negative Control Percent of Control Across Reference Chemicals and Replicates

Within each replicate of each reference chemical quadruplicate repetitions will be made of the full enzyme activity control, background activity control, and negative and positive control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are consistent throughout the replicate test, the control tubes at the beginning should be equivalent to those at the end.

To assess whether this is the case the control responses will be adjusted for background dpms, divided by the average of the (background adjusted) full enzyme activity control values, and expressed as percent of control. The average of the four background activity controls within a replicate must necessarily be 0 percent and the average of the four full enzyme activity controls within a replicate must necessarily be 100 percent. The full enzyme activity controls percent of control, the background activity controls percent of control, and the negative and positive controls percent of control values will be plotted across reference chemical and replicate within reference chemical, with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity control) or 100% (full enzyme activity control) respectively. These plots will display the extent of consistency across reference chemicals and replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Additional plots will be prepared displaying the difference of the average of the first two percent of control values (i.e., those based on the "beginning" tubes) and the average of the last two percent of control values (i.e., those based on the "end" tubes) across reference chemicals and replicates within reference chemicals. Each plot will have a reference line of 0.

Three-factor mixed effects analysis of variance models will be fitted, separately for the full enzyme activity control, the background activity control, and the positive and negative control tubes. The fixed effect factors in the analysis of variance will be:

- reference chemical
- portion (beginning or end)
- portion by reference chemical interaction.

The random effects will be:

- replicate nested within reference chemical
- portion by replicate within reference chemical interaction.

The residual error variation corresponds to repetition within reference chemical, replicate, and portion. The response will be percent of control. Since for the background activity and full enzyme activity controls the average of the repetitions within a reference chemical and replicate are constrained to be 0 and 100 respectively, by the way in which

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"percent of control" is defined, the variation associated with the reference chemical effect and the replication within reference chemical effect are both necessarily constrained to be 0.

If the daily replicates are in control the portion main effect and the portion by replicate within reference chemical interaction should be nonsignificant. If the portion by replicate within reference chemical interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate within reference chemical to the portion effect averaged across replicates within reference chemical, adjusting for simultaneity by Bonferroni's method.

6.2.4 Statistical Software

Concentration response curves will be fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using PRISM, the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS), as convenient.

6.2.4 Interlaboratory Statistical Analysis

The lead laboratory and each of the participating laboratories will carry out "intra-laboratory" statistical analyses based on their test data, according to this common statistical analysis plan, developed by the Data Coordination Center (Battelle). The Data Coordination Center will carry out the "inter-laboratory" statistical analysis. It will combine summary values developed in each of the intra-laboratory analyses to assess relationships among the laboratory results, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

7.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the archives for the life of the contract.

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study. The study will be conducted in compliance with the Federal Register, 40 CFR Part 160. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practices Standards.

9.0 REPORTS

Interim data summaries, draft and final reports will be submitted as described in Section 9.5 of the QAPP.

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The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and test chemical repetition), percent of control activity, IC_{50} ,slope and graphs of activity versus log chemical concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the intra-laboratory statistical analyses described in Section 6 of this document.

10.0 STUDY RECORDS TO BE MAINTAINED

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments
- List of any Protocol Deviations
- List of Standard Operating Procedures
- Quality Assurance Project Plan (QAPP) and any Amendments
- List of any QAPP Deviations

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PROTOCOL AMENDMENT NUMBER 2

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Conduct Studies with Centrally Prepared Microsomes (WA 4-16, Task 5)**

PART TO BE CHANGED: Section 2.4., fourth paragraph, page 7.
Excess undiluted stock microsomes may be flash frozen in liquid nitrogen and returned to ca. -70 °C storage for future use. It is strongly recommended that stock microsomes to be refrozen be divided into aliquots appropriate for use prior to refreezing in order to minimize the number of freeze/thaw cycles.

CHANGE TO: Section 2.4, third paragraph, page 6 (*it is moved to precede the current third paragraph).*
Human placental microsomes (7 tubes of ca. 500 µL each) will be thawed quickly in a 37 ± 1°C water bath and then immediately transfer to an ice bath. The microsomes will be pooled and rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes). The pooled sample will be aliquoted into portions appropriate for use in a single experiment (ca. 150 µL) and the samples will be flash frozen and stored at ca. -70 °C for future use. Each tube will provide enough protein for a single experiment and any excess of thawed microsomal solution will be discarded.

REASON FOR CHANGE: Revision done on the request by Sponsor. This change will standardize the number of freeze/thaw cycles experienced by the microsomes used in this task to 2. The first freeze was at microsomes preparation, the first thaw and second freeze will occur during the repacking described above and the second thaw will occur at assay.

PART TO BE CHANGED: Section 2.4, third paragraph, page 7, last sentence.
The microsomes should not be left on ice for longer than approximately 2 hours before proceeding with the assay or the microsomal enzyme activity may be decreased.

CHANGE TO: Microsomes are not be left on ice for longer than approximately 2 hours before proceeding with the assay. Appropriate documentation of time from thaw to use must be maintained.

REASON FOR CHANGE: Revision done on the request by Sponsor.

G608316

EFFECTIVE DATE: February 2, 2005

APPROVED BY:

Bozena D. Lusiak, Study Director — 02-10-05 Date

Sponsor Representative — 2-10-05 Date

PROTOCOL AMENDMENT NUMBER 3

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Conduct Studies with Centrally Prepared Microsomes (WA 4-16, Task 5)**

PART TO BE CHANGED: Section 2.4., fourth paragraph, second sentence, page 7. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) prior to use.

CHANGE TO: Section 2.4, fourth paragraph, second sentence, page 7. The microsomes aliquot will be vortexed about 5 seconds in the microcentrifuge tube in which they are stored and then an aliquot directly from that tube will be removed for dilution for use in the assay.

REASON FOR CHANGE: Revision done on the request by Sponsor.

EFFECTIVE DATE: February 11, 2005

APPROVED BY:

Bozena D. Lusiak, Study Director — 02-16-05 Date

Sponsor Representative — 2-17-05 Date

PROTOCOL AMENDMENT 4

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Conduct Studies with Centrally Prepared Microsomes (WA 4-16, Task 5)**

PART TO BE CHANGED: The following sentence is being added in to Section 3.0 entitled Protein Assay, page 7 after third sentence in this section.

Three Quality Control (QC) standards (0.125, 0.5 and 1.0 mg protein/mL), obtained from Pierce (Rockford, IL) will be run in duplicate with each assay.

REASON FOR CHANGE: The above sentence is being added as per the Sponsor request.

EFFECTIVE DATE: February 23, 2005

APPROVED BY:

Bozena D. Lusiak

Bozena D. Lusiak, Study Director

2-2-06

Date

Sponsor Representative

2-2-06

Date

DEVIATION REPORT

G608316

AROMATASE ASSAY VALIDATION: CONDUCT STUDIES WITH CENTRALLY PREPARED MICROSOMES

Type of Deviation:	Protocol
Date of Deviation:	From 2-15-2005 through the Study
Nature of Deviation:	The protocol stated that the first replicate concentrations were to be 1×10^{-3} to 1×10^{-10} M.
Cause of Deviation:	The concentration of stock solutions of chrysin and ketoconazole (send by the Sponsor) was 0.01 M. Using above stock solutions directly (without dilution) to the aromatase assay resulted in the final inhibitor concentration of 1×10^{-4} M.
Impact of Deviation on the Study:	None.
Corrective Action:	None

Approved By: Bozena D. Sunjak Date: 07-07-06

Original: Study File
Copies : H. Flory

DEVIATION REPORT

G608316

AROMATASE ASSAY VALIDATION: CONDUCT STUDIES WITH CENTRALLY PREPARED MICROSOMES

Type of Deviation:	Protocol
Date of Deviation:	02/24/05 and 02/28/05
Nature of Deviation:	The protocol guidelines for using inhibitor concentrations after first replicate stated not to use a concentration lower than $1x\ 10^{-5}$ M.
Cause of Deviation:	After examining the results obtained from the first replicate for econazole the right range for inhibitor concentration was chosen as $1x\ 10^{-6}$ to $1x\ 10^{-10}$ M. Econazole exhibited a very potent inhibition property, almost 100% of inhibition was noticed when $1\ x10^{-3}$ to $1x\ 10^{-6}$ M concentrations were used. Choosing inhibitor concentrations between $1x10^{-6}$ to $1x10^{-10}$M allowed constructing better concentration response curve for second and third replicate.
Impact of Deviation on the Study:	None.
Corrective Action:	None

Approved By: B D Lunich Date: 07-07-06

Original: Study File
Copies : H. Flory

APPENDIX B

Quality Assurance Project Plan (QAPP) B-1

1.0 TITLE AND APPROVAL

Quality Assurance Project Plan (QAPP)
For Work Assignment 4-16
Placental Aromatase Validation Study

Task 5 -Conduct Multiple Chemical Studies
with Centrally Prepared Microsomes

for

EPA CONTRACT NUMBER 68-W-01-023

January 24, 2005

SIGNATURE PAGE

Quality Assurance Project Plan for WA 4-16, Task 5
Placental Aromatase Validation Study
EPA CONTRACT NUMBER 68-W-01-023

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Appendix A: Template Protocol for Task 5

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4.0 PROJECT ORGANIZATION

The U.S. Environmental Protection Agency (EPA) is implementing the Endocrine Disruptor Screening Program (EDSP). To support this program, the EPA has contracted with Battelle to provide comprehensive toxicological and ecotoxicological testing services, including chemical, analytical, statistical, and quality assurance (QA)/quality control (QC) support, to assist EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and tests for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize and validate methods, prepare appropriate guidance documents for peer review of the methods, and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs. The validation studies will be conducted under the EDSP Quality Management Plan (QMP), study protocols, applicable Quality Assurance Project Plans (QAPPs), relevant program and facility Standard Operating Procedures (SOPs), guidance documents, and Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice Standards (GLPs).

One of the assays recommended for validation and consideration for inclusion in the screening program is the aromatase assay. A Detailed Review Paper (DRP) was prepared for the U.S. EPA in 2002 to review the scientific basis of the aromatase assay and examine assays reported in the literature used to measure the effect of chemical substances on aromatase.

Prevalidation studies on the aromatase assay (Work Assignment [WA] 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placental microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10).

The objectives of this work assignment are to use the now optimized assay: (1) to obtain intra- and interlaboratory assay variability estimates by conducting experiments at multiple laboratories, (2) to conduct microsome preparation and analysis experiments at multiple laboratories, and (3) to test up to 10 reference chemicals with different modes of action in order to evaluate assay relevance.

This work assignment is composed of multiple studies that are to be conducted by the lead laboratory (Research Triangle Institute International [RTI], Research Triangle Park, NC) and three participating laboratories (Battelle, Columbus, OH; In Vitro Technologies, Baltimore, MD; WIL Research Laboratories, LLC, Ashland, OH). This QAPP will address the work to be conducted in Task 5 of the work assignment.

A summary of the work assignment organization is shown in Figure 4-1.

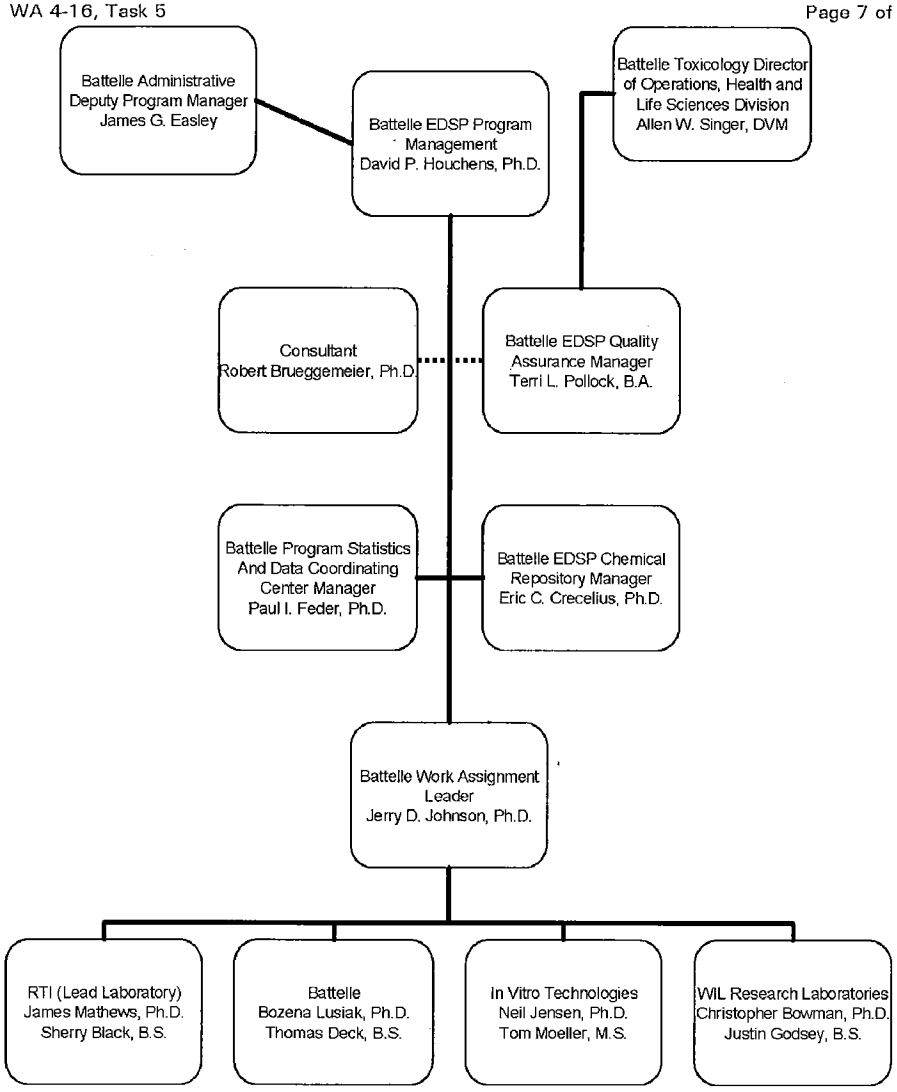

Figure 1. WA 4-16 Project Organization Overview

Portions of this work assignment will be managed at RTI, Battelle, WIL, and In Vitro. At each of these laboratories, there will be a person responsible for preparing the protocol, assigning appropriate staff to complete specified tasks within the protocol, and monitoring the progress of both technical and fiscal milestones as outlined in the technical work plan. A study director from each laboratory will report on the progress of the work assignment to Drs. David Houchens and Jerry D. Johnson at Battelle through a series of planned conference calls and through the use of written monthly reports.

General scientific direction and supervision of the work performed under this work assignment will be provided by Dr. Jerry D. Johnson, Battelle and Dr. James Mathews, RTI International. Dr. Johnson will serve as the Work Assignment Leader (WAL) for the participating laboratories and Dr. Mathews for the lead laboratory (RTI).

Each laboratory will have a study director in charge of overseeing the daily operation and conduct of the study. The individual laboratory teams will execute the necessary tasks required in the study protocols and ensure the data are collected and handled appropriately. All of these tasks are clearly defined in the study protocol.

The Quality Assurance Unit (QAU) representative for each laboratory will administer the QAPP for the EDSP facility QA team members. The specific responsibilities include:

- Interact with the Study Director to ensure that QA and QC procedures are understood by WA personnel.
- Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the program WAs with respect to the EDSP QMP, the WA QAPPs and/or GLP protocol, and applicable program and facility SOPs.
- Prepare and track reports of deficiencies and submit them to both line and program management.
- Consult with the Study Director and, as necessary, the EDSP Battelle QA Manager and Program Manager on actions required to correct deficiencies noted during the conduct of the WA.
- Ensure that all data produced as part of the EDSP WAs are maintained in a secure, environmentally-protected archive.
- Ensure, during the conduct of TSAs, that all staff participating on the EDSP are adequately trained.

- Maintain complete facility-specific QA records related to the program.
- Submit copies of resolved audits to the EDSP Battelle QA Manager.
- Submit a QA Statement to the EDSP Battelle QA Manager and Program Manager with each written deliverable that describes the audit and review activities completed and any outstanding issues that could affect data quality or interpretation of the results discussed in the report.
- Maintain effective communication with the EDSP QA Manager.
- Act as the facility's EDSP SOP Custodian for all SOPs received from the SOP Administrator.

As EDSP manager, Dr. David Houchens will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He also will serve as the principal interface with the EPA's project officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an administrative deputy manager, Mr. James Easley. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control. He has played a similar role on ten other large, multi-year, level-of-effort task-order contracts for EPA. Thus, he will be able to assure that all purchases are compliant with government regulations and that EPA is provided timely, accurate accounting of these substantial costs in our monthly progress reports.

Ms. Terri Pollock, the EDSP QA manager at Battelle, will direct a team of QA specialists to monitor the technical activities on the chemical repository program, and provide oversight to all associated QA functions. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock reports, for the purposes of this program, to Dr. Allen W. Singer, Director of Operations in the Toxicology Product Line in Battelle's Health and Life Sciences Division. This reporting relationship assures that the QA function is independent of the technical activities on the program.

5.0 PROBLEM DEFINITION/BACKGROUND

5.1 Problem Definition

Prevalidation studies on the placental aromatase assay (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of

the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10).

With the prevalidation studies successfully completed, this work assignment directs Battelle to conduct the interlaboratory studies to determine the performance of several laboratories in conducting the assay and should complete the validation of the placental aromatase assay. A companion work assignment (WA 4-17) has been issued for the conduct of the recombinant aromatase assay.

The work assignment is comprised of 9 tasks of which five tasks involve experimentation. Task 3 is a training task. The work in Tasks 4 through 7, is described in this QAPP. Table 1 summarizes the validation tasks and the laboratory(ies) involved for each experimental task.

Table 1. Validation Study Plan Experiments

Task Number	Description of Experimental Task	Experimental Task Assignment
1	Not applicable (Develop work plan, study plan, and identify/select participating laboratories)	Not an experimental task
2	Not applicable (Develop QAPP and protocols)	Not an experimental task
3	Training Participating Laboratories in the Conduct of the Assay	Lead Laboratory + 3 Participating Laboratories
4	Conduct Positive Control Studies in the Participating Laboratories	3 Participating Laboratories
5	Conduct Multiple Chemical Studies with Centrally Prepared Microsomes (RTI/Participating Laboratories)	Lead Laboratory + 3 Participating Laboratories
6	Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Lead and One Participating Laboratory	Lead Laboratory + 3 Participating Laboratories
7	Conduct Multiple Chemical Studies with Microsomes Prepared in Participating Laboratories (RTI/Participating Laboratories)	Lead Laboratory + 3 Participating Laboratories
8	Prepare Study Reports (RTI/Participating Laboratories)	Not an experimental task
9	Prepare Presentation for EDMVAC*	Not an experimental task

*EDMVAC = Endodrine Disruptor Method Validation Committee

5.2 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an EDSP. In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the EDMVAC will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) evaluating the literature and personal communications.

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450arom and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay using human placental microsomes, is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system, with IC_{50} values for aromatase inhibition ranging from 0.04 mM to greater than 50 mM.

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10). The objective of the current work assignment is to use the now optimized assay to obtain intra- and interlaboratory assay variability estimates to complete the validation of the human placental microsome aromatase assay.

6.0 PROJECT/TASK DESCRIPTION

Only Task 5 is under the control of this QAPP. However, this QAPP also addresses the other three experimental tasks in this work assignment and will be reissued prior to the start of each new task together with a finalized task-specific template protocol included as an attachment. The Task 5 template protocol is attached to the present QAPP. The task numbering scheme for the original work assignment is employed in this document for ease of cross-referencing.

Task 4: Conduct Positive Control Studies in the Participating Laboratories

This task was completed by staff at Battelle, WIL and In Vitro. RTI staff did not conduct any experiments on this task but were involved in the review of the data produced by the other laboratories. RTI provided human placental microsomes to the other laboratories for use in this task. Battelle/RTI provided a boilerplate protocol for this Task to the participating laboratories which they used to prepare their laboratory-specific protocols. These protocols contained all necessary technical detail for the conduct of this Task. Briefly, the Task required that each laboratory conduct three independent replicates of a Positive Control Study. In this Study, 4-OH androstenedione (4-OH ASDN, a known aromatase inhibitor) was tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an IC_{50} may be calculated. Control runs also were included in the assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). Battelle's Chemical Repository (CR) supplied 4-OH ASDN to each laboratory as a stock solution and conducted all necessary pre-assay chemistry activities for 4-OH ASDN.

Each laboratory presented its results in a separate spreadsheet for each of the three replicates and the results were compared both within and between laboratories.

The results of this experiment require technical review and approval prior to proceeding to Task 5.

Task 5: Conduct Studies with Centrally Prepared Microsomes

This Task will be completed by staff at RTI, Battelle, WIL and In Vitro. RTI will provide human placental microsomes to the other laboratories for use in this task. Battelle/RTI will provide a boilerplate protocol for this Task to the participating laboratories which they will use to prepare their laboratory-specific protocols. These protocols will contain all necessary technical detail for the conduct of this Task. Briefly, the Task requires that each laboratory conduct three independent replicate studies on each of four test chemicals. All three replicates for a given chemical will be conducted by the same technician within a laboratory. Control runs are also included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). In additional positive control samples (containing a known aromatase inhibitor) and negative control samples (containing a known aromatase non-inhibitor) will be included in each assay set. Battelle's CR will supply the test and control chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test and control chemicals.

Each laboratory will present their results in a separate spreadsheet for each of the three replicates and the results will be compared both within and between laboratories.

The results of this experiment would require technical review and approval prior to proceeding to Task 7.

Task 6: Prepare Microsomes in Two Participating Laboratories

There are two activities in this Task. The first, to be conducted by Battelle and In Vitro, requires those laboratories to obtain a human placenta, prepare microsomes and then to analyze their microsome preparations for protein content and (uninhibited) aromatase activity. In addition, those laboratories will conduct two independent replicates of the Positive Control Study (as used in Task 4) using their microsomal preparations. RTI/Battelle will supply a template protocol that includes all technical detail required for the conduct of these experiments. Battelle's CR will supply 4-OH ASDN to each laboratory as a stock solution. The laboratories will submit the results of these studies to Battelle and the data will be reviewed by Battelle and RTI prior to submission to EPA. After EPA approves the results, the second portion of the Task can be initiated.

For the second activity in this Task, Battelle and In Vitro will each ship portions of their placental microsomes preparations to the other three participating laboratories. Each laboratory will measure the protein content and (uninhibited) aromatase activity of the microsomal preparations from both laboratories.

Each laboratory will present their results in a separate spreadsheet for each replicate and the results will be compared both within and between laboratories.

Task 7: Conduct Studies with Microsomes Prepared in Participating Laboratories

Battelle and In Vitro will conduct the studies in this task with microsomes prepared in their laboratory in Task 6. RTI and WIL will receive microsomes from Battelle and In Vitro, respectively, for use on this task.

RTI/Battelle will supply a template protocol describing all technical details for this task to the participating laboratories from which they will prepare their laboratory-specific protocols. Each laboratory will conduct three independent replicate studies with each of 10 chemicals. All three replicates for a given chemical will be conducted by the same technician within a laboratory. Control runs also will be included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). In addition, positive control samples (containing a known aromatase inhibitor) and negative control samples (containing a known aromatase non-inhibitor) will be included in each assay set. Battelle's CR will supply the test and control chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test and control chemicals.

7.0 QUALITY OBJECTIVES AND CRITERIA

The endpoints for WA 4-16 include the aromatase activity measured in the control and inhibitor samples, the inter- and intralaboratory variance, and the IC_{50} and slope values for each inhibitor tested.

7.1 Data Quality Indicators

7.1.1 Precision

The activities of replicate tubes should be within the mean activity ± 15%. Each control activity for each assay/laboratory should be within the overall mean ± 15% activity for that control type for that laboratory.

Variance between laboratories and within laboratories will be assessed for an appropriate level of precision as part of this WA. It is anticipated that full aromatase control activity between and within laboratories should be statistically equivalent at the $p > 0.1$ level. Any modifications to this criterion will be discussed with the sponsor and added to the QAPP by amendment.

IC_{50} and slope values calculated for each inhibitor should be statistically equivalent at the $p>0.1$ level both between and within laboratories. If data from an assay are statistical outliers, the assay may be repeated.

7.1.2 Bias

The control samples that are run with each assay will be used to control for bias. If the control samples for any assay do not meet the precision criteria described above, the assay may be rerun. Assays will be conducted blind at the technician level for test chemical identity.

7.1.3 Accuracy

Accuracy of the liquid scintillation spectrometry (LSS) data (from which is derived the aromatase activity) will be assessed by analysis of a sealed standard of known radioactive content. If the radioactivity in the sealed standard is more than 5% different from the known value, the data will not be used. Samples may be recounted on another LSS or on the same LSS after any problems with the instrument are corrected.

8.0 SPECIAL TRAINING/CERTIFICATION

All personnel involved in handling radiolabeled materials will have completed a Radiation Safety Training course. Training documentation will be maintained in the individual training files. Each laboratory will be licensed to receive radiolabeled materials.

All personnel involved in handling human placental microsomes will have appropriate training in the handling and disposition of biohazards. Training documentation will be maintained in the individual training files.

Staff from the participating laboratories have been trained on the performance of the aromatase assay at RTI International as part of Task 3 of this work assignment. Personnel participating in this training conducted the aromatase assay including full aromatase control and background control samples and a series of samples containing varying amounts of a known aromatase inhibitor (4-OH ASDN). The resultant data were evaluated by Battelle and RTI International and then submitted to EPA for review.

9.0 DOCUMENTS AND RECORDS

9.1 Retention of Specimens and Records

Archiving procedures will be specified in the individual protocols.

9.2 Quality Assurance Project Plan

This QAPP will be distributed to project participants initially, and whenever revised. Previous versions will be marked as "obsolete" when newer versions are distributed, or collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month, Year
Page 1 of 1

is used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually and a determination made to either modify the document based on new or modified project requirements, or leave as is.

Controlled copies of the QAPP will be maintained, tracked, and managed by the laboratories' QAU through the use of a master distribution list.

9.3 Data Forms

All data forms will include a title identifying the type of data to be recorded, a unique study code or protocol number, and the initials and date of the data recorder(s) to authenticate the records.

Corrections to data entries will be made by drawing a single line through the error, recording the correct entry, initials, date, and error code that explains the reason for the correction.

9.4 Microsome Storage Conditions

Microsomes must be stored at -70 to -80°C and the freezer temperature records must be maintained.

9.5 Reports

9.5.1 Interim Data Summary, and Draft and Final Reports

An interim data summary from each laboratory will be submitted to the EPA after completion of each task. These data summaries will not be audited by Quality Assurance but will be checked for accuracy by technical staff. This procedure is necessary to provide a rapid turn around of the data so that approval to proceed can be given by EPA.

Each laboratory will prepare an individual report for each task to be based on a template provided by Battelle and will submit these reports to Battelle. The purpose of these reports is to provide a complete description about how the experiments were performed, present the results that were obtained (including tables and graphs), and state the conclusions that were made for each applicable WA task. RTI/Battelle will prepare a report for each task that summarizes all work on the particular task and incorporates the reports from the participating laboratories as Appendices for submission to EPA. After EPA comments have been received on each task report and, if applicable, incorporated into a new version of the draft task report, then it will be issued as a final report.

Each final task report will include:

- Abstract
- Objectives
- Materials and Methods
- Results
- Discussion
- Conclusions

- References
- Summary data with statistical analyses
- Appendices which will include final reports with compliance statements for each participating laboratory
- Protocol, any amendments, or any deviations from the protocol
- QAPP, any amendments, or any deviations from the QAPP.

RTI/Battelle will prepare a final Work Assignment report that summarizes the results of the entire Work Assignment. This report will consist of a statement of the objectives of the work assignment, a summary of the results and a statement of conclusions for the Work Assignment. The individual task reports will be referenced within this final report.

9.5.2 QA Assessment Reports

QA assessment reports are maintained as confidential files in the QAU.

9.5.3 Status Reports

Status/progress reports will be submitted to the EPA Project Officer by Battelle on a monthly basis as stipulated in the contract.

10.0 SAMPLING PROCESS DESIGN (EXPERIMENTAL DESIGN

The details of the experimental design for the task subject to this QAPP will be contained in a GLP compliant protocol. A template protocol for this task is attached as an Appendix to this document.

11.0 SAMPLING METHODS

The entire aqueous portion of the incubation mixtures remaining after extraction with methylene chloride (CH_2Cl_2) will be placed in appropriate containers. The samples will be mixed well prior to the removal of aliquots for liquid scintillation counting (LSC). If there is insufficient time for preparing LSC samples on the day the assay is run, the samples will be refrigerated overnight. Samples remaining after preparation of LSC aliquots should be frozen and stored at about -20°C. These samples may be thawed, mixed and realiquoted, if necessary, due to problems with LSC samples.

Each test and standard chemical will be supplied to the participating laboratories by Battelle as a stock solution at the highest concentration necessary for use in the assay. These

solutions will be well-mixed prior to the preparation of dilutions of these stock solutions by the individual participating laboratories.

12.0 SAMPLE HANDLING AND CUSTODY

12.1 Test and Reference Chemical Solutions

The test and standard chemical stock solutions will be transferred to the Laboratories' Material Handling Facility with a study specific transfer of material form. The samples will be processed according to the SOPs for packing, shipment and documentation of shipment and receipt.

12.2 Sample Collection Documentation

All samples (or sample sets) will be labeled with enough information to allow for unequivocal identification of each sample along with suitable storage conditions in accordance with applicable regulations.

13.0 ANALYTICAL METHODS

Analytical methods are described in the study protocol (Appendix). Failures of analytical systems are addressed in the relevant SOPs.

14.0 QUALITY CONTROL

14.1 Methods

Control samples are run with each assay. These include 1) full aromatase enzyme activity controls (FEAC), 2) background controls (BAC), 3) positive controls and 4) negative controls. Acceptance criteria and corrective actions where acceptance criteria are not met are described in Section 7. Replicates are used as a means to monitor variability of the assay. Replicates will be assessed for variance and those that are outside the acceptable range (mean ± 15%) will be flagged as statistical outliers.

14.2 Data Collection

Data collection documentation will be as described in applicable SOPs or protocols.

Assay data, including weights and/or volumes of chemicals, solvents or other materials used to prepare necessary solutions or samples, will be recorded manually on data sheets. Protein assay absorbance data may also be recorded manually on data sheets. All data sheets include a title identifying the type of data to be recorded, the unique study code or protocol number, and the initials and date of the data recorder(s) to authenticate the records.

Scintillation counter data will be automatically saved to a data file that will automatically be assigned a unique filename. The data must be annotated to identify samples with the sequential vial number. Procedures for converting CPM data to DPM data must be documented.

Relevant data from the data sheets and scintillation counter output (as DPM) will be typed into a validated MS Excel spreadsheet for calculation of 1) substrate specific activity 2) protein content and/or 3) aromatase activity. All transcribed data will be verified (100% QC) before they are reported and this QC check will be documented on the spreadsheet printouts by technician initials and date.

Aromatase activity data will be entered automatically (through linked validated spreadsheets) or manually into Prism data files for calculation of IC_{50}. Data will be entered automatically (through linked validated spreadsheets) or manually into spreadsheets for import into SAS data files for statistical analysis. All manually entered data will undergo a 100% QC check.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The following types of equipment are required for this WA: temperature controlled shaking water bath, pH meter, analytical balances, centrifuges (low and high speed and ultracentrifuges), pipettors, scintillation counters, spectrophotometer, and high performance liquid chromatography (HPLC) equipment (injector, pumps, detectors [radiochemical and ultraviolet {UV}], data collection system). The equipment will be tested, inspected and maintained according to schedules contained in the relevant SOPs.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

Balances used to obtain weight measurements, as well as the check weights that are used to verify a balance's calibration status will be calibrated and maintained according to the schedule specified in relevant SOPs. Balances that do not meet the criteria specified in the SOP will not be used for this work assignment.

Scintillation Counters will be calibrated using procedures described in the relevant SOPs. Calibration of pH meters occurs as specified in relevant SOPs. The water bath, pipettes,

spectrophotometer, and HPLC equipment will be calibrated using the procedures and schedule in applicable SOPs. Any equipment or instrument that does not meet acceptance criteria as described in the relevant SOP will not be used for this work assignment.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items must be inspected for conformance to quality requirements prior to use. All use of the product must be prior to the expiration dates, if applicable. Chemicals will be received and stored in accordance with applicable SOPs.

18.0 NON-DIRECT MEASUREMENTS

No collection of any samples or sample data will be obtained from non-direct measures such as computer data bases or programs.

19.0 DATA MANAGEMENT

19.1 Data Management Overview

Data will be maintained in notebooks and/or files according to applicable facility SOPs. The records will be kept in the appropriate rooms until there is a signed final report at which time they will be inventoried and placed in the facility archives according to applicable facility SOPs, unless the sponsor requests that they be transferred to another archive location.

19.2 Data Transfer

Information will be sent to the Data Coordination Center in electronic format as specified in SOP EDSP.D-003-01. Specifically all raw data, all tables, graphs summarizing results of statistical analyses as presented in study reports, statistical analysis data files, statistical analysis programs, and all study documents will be sent to the EDSP Data Coordination Center in electronic format.

20.0 ASSESSMENTS AND RESPONSE ACTIONS

EDSP QA team members will perform assessments on WA activities and operations affecting data quality and the raw data and final report. They will report any findings to the Study Director and management to ensure that the requirements in relevant SOPs, study protocols and

WA QAPP, the QMP, and the FIFRA GLPs are met. The assessments for this study include TSAs and ADQs. Performance Evaluations do not apply to this QAPP.

20.1 Technical Systems Audits

A TSA is a process by which the quality of a study is assessed through evaluating a study activity's conformance with the protocols, applicable facility or program SOPs, QAPP, QMP, and GLPs. The acceptance criteria are that WA activities and operations must meet the requirements of these planning documents and the GLPs or be explained and evaluated in a deviation report. Deviations from the GLPs, QAPP, protocol, or SOPs will be properly documented and assessed by management and the study director as to their impact on the study.

20.2 Type, Scheduling, and Performance of Technical Systems Audits

The following paragraphs provide an example of how the laboratories may perform technical system audits.

Prior to the experimental start, the facility QA Team Member will convey a list of inspections targeted for the study to the study director. Whenever possible, TSAs should be done at the commencement of the WA critical phase to ensure WA integrity based on compliance with the protocol, QAPP, SOPs, and GLPs. Critical phases targeted for TSAs include, but are not limited to:

- Protocol review
- Placental collection and microsome preparation
- Aromatase assay sample preparation and analysis.

During the TSA, EDSP QA team members will record observations to be used later in preparing the audit report. EDSP QA team members will observe the procedure, data recording, and any equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the study protocols and QAPP, applicable SOPs, QMP, and the GLPs. Any findings will be communicated to the technical personnel at the completion of the procedure unless an error could compromise the study (e.g., misdiluting the stock solution). EDSP QA team members immediately notify the Study Director by telephone and/or e-mail of any adverse findings that could impact the conduct of the study. This direct communication will also be documented in the audit report.

20.3 Audits of Data Quality

An ADQ is a process by which the accuracy of data calculations and reporting will be assessed to ensure that the reported results are of high quality and accurately reflect the raw data and accurately describe the materials used in the study. The acceptance criteria for the ADQ are

that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA protocols and QAPP, QMP, and the FIFRA GLPs, or be explained and evaluated in a deviation report, as previously described.

20.4 Scheduling and Performance of Audits of Data Quality

Direct and frequent communication between the WA Leader/Study Director, laboratory supervisor, and the QA Manager will provide for sufficient time to perform an ADQ so that the submission date of the draft final report meets that specified in the study protocol. The scheduling process should also allow for a reasonable amount of time for corrections and subsequent verification of the corrections by QA.

EDSP QA team members will audit the study records at a frequency adequate to ensure that approved protocol requirements are met. The frequency required is specified by the type of data in the QMP, Section 2.4.1. Findings will be reported and corrective actions undertaken as described earlier. EDSP QA team members will review the final report using the audited data and corrected tables. The report text will be reviewed to ensure that every statement is supported by the data and any discussions or conclusions drawn from the study are supported by the data. Findings will then be reported and corrective actions undertaken as described earlier.

20.5 Audit Report Format

The following paragraphs provide an example of how the laboratories may format an audit report.

The audit report consists of a cover page for study information and additional page(s) with the audit findings. All pages have header information containing the study protocol number, audit report date, and audit type. The audit report date is the date on which the EDSP QA team member signs the audit report and sends it to the Study Director and management.

The cover page contains the study protocol title, number, and code; Sponsor; Study Director; audit type; audit date(s); EDSP QA team member; distribution list; the dated signature of the auditor; the date that the Study Director received the audit report; and the dated signatures of the Study Director and management. The distribution list may include additional names for individuals who have findings pertaining to their area of responsibility (e.g., the ARF Manager would address a finding pertaining to the ARF) and is used to ensure that the report is sent to all who need to respond. Subsequent page(s) contain the audit finding(s), any recommended remedial actions, and space for the Study Director to respond to the findings and document remedial actions taken or to be taken.

20.6 Response Actions and Resolution of Issues

The Study Director will respond to the TSA report within a specified number of working days of receipt of the report as required by the laboratory's SOPs. There is no deadline for the Study Director's response to an ADQ report except for the time constraint deriving from the submission date of the final WA report. The Study Director forwards the audit report to management for review. Management adds comments as necessary, signs and dates the report and returns it to the EDSP QA team member. The EDSP QA team member assesses the responses and verifies the corrective actions. If a disagreement between the Study Director and EDSP QA team member arises over a finding, it will be discussed among the other EDSP QA team members. The EDSP QA team member will then present the majority opinion to the Study Director for further consideration. If the disagreement remains, the issue will be reported to the Study Director's management. The action decided on by management will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA objectives of acceptable quality cannot be achieved, the auditor follows the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.7 Independent Assessments

The EDSP Battelle QA Manager (QAM), or designee, may conduct an independent TSA and ADQ during the conduct of this work assignment. Typically one independent audit may be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3).

In addition, the EDSP EPA QAM, or designee, has the option of conducting external TSAs/ADQs.

21.0 REPORTS TO MANAGEMENT

The QA Manager will send periodic reports to the study director and management, which detail significant regulatory, protocol, and SOP issues. Also, the participating laboratories will report to the EDSP Program Manager and WAL.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel for the validation process and by EDSP QA team members for the verification process (see

section 23). The criteria used for validation depend on the type of data. For dose solution sample data, information regarding the condition of the containers and whether or not samples were compromised is recorded in the sample chain-of-custody records. Compromised samples are not analyzed. The criteria for validating data are those found in Section 7 (Data Quality Objectives).

23.0 VERIFICATION AND VALIDATION METHODS

23.1 Chain of Custody for Data

Study data, records, and specimens will be maintained in a secure and designated location, e.g., in the respective laboratory offices until study completion. Chain-of-custody procedures will be implemented according to facility SOPs. Chain-of-custody information, including the date, study record(s) removed or returned, and the name of the person removing or returning the data will be documented. At study completion, the Study Director will follow the procedures specified in the facility SOP for archiving study materials.

23.2 Data Validation

Data validation is a process by which the WA Leader/Study Director and/or other technical personnel evaluate the data for conformance to the stated requirements for methodology and quality. These personnel are responsible for reviewing the data, evaluating any technical deviations or non-conformances, and then determining the degree to which the data meet the quality criteria stated in Section 7.

23.3 Data Verification

Data verification constitutes part of the ADQ process performed by EDSP QA team members and described earlier. Verification ensures that 1) the data are of high quality and were collected according to the planning documents' requirements, and 2) the reported results accurately reflect the raw data. Each data type will be evaluated against its collection and reduction requirements specified in the planning documents. Errors discovered during the data evaluation will be corrected. The reported conclusions drawn from the data are verified by EDSP QA team members during the report audit to confirm that they are true and accurate. The procedure for resolving issues of data verification has been detailed in prior sections of this document.

24.0 RECONCILIATION AND USER REQUIREMENTS

Proposed methods for data analysis, including a test for statistical outliers, are specified in the Study Plan and/or protocols.

25.0 REFERENCES

The following references were used to prepare the QAPP. Not all references are cited in the text.

Battelle (2003). Endocrine Disruptor Screening Program Quality Management Plan, Version 2. May 12, 2003.

Battelle (2004). Technical Work Plan on Microsomal Aromatase Validation Study, EPA Contract Number 68-W-01-023, Work Assignment 4-16. September 8, 2004.

FQPA (1996). Food Quality Protection Act of 1996, U.S. Public Law 104-170, 21 U.S.C. 46a(p), Section 408(p), 110 STAT.1489. August 3, 1996.

APPENDIX A

TEMPLATE PROTOCOL FOR TASK 5

EPA Contract No.:
EPA Work Assignment No.:

TITLE: Template Protocol for WA 4-16 Task 5:
Conduct Studies with Centrally Prepared Microsomes

SPONSOR:

TESTING FACILITY:

PROPOSED EXPERIMENTAL START DATE:
PROPOSED EXPERIMENTAL END DATE:

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			

Approved By:

Laboratory's Study Director Date	Jerry Johnson, Ph.D. DABT Date Work Assignment Leader, Battelle
Laboratory's Manager Date	David P. Houchens, Ph.D. Date EDSP Program Manager, Battelle
Linda J. Phillips, Ph.D. Date EPA Project Officer	Gary Timm, M.S., M.A. Date EPA Work Assignment Manager

Reviewed By:

Laboratory's Quality Assurance Specialist Date	Terri L. Pollock, B.A. Date EDSP Quality Assurance Manager, Battelle
J. Thomas McClintock, Ph.D. Date EPA Quality Assurance Manager	

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1.0 OBJECTIVES

Task 5: Conduct Studies with Centrally Prepared Microsomes

The objective of this protocol is to describe procedures for conduct of the aromatase assay using human placental microsomes. In this task, the participating laboratories will conduct studies with four potential aromatase inhibitors (reference chemicals) to demonstrate the responsiveness of the assay. Microsomes will be prepared by the lead laboratory and supplied to the participating laboratories.

1.1 Justification for Test System

The test system for this study is human placental microsomes. This test system was selected because it provides a biological source of the aromatase enzyme and, since the assay is being evaluated for its potential to serve as a screening assay, the use of human tissue enhances its predictive potential.

1.2 Test Method

This *in vitro* test method involves combining microsomes, substrate, appropriate co-factors and test substances in a common reaction vessel. The effect of the test substances on microsomal enzyme activity is evaluated by measuring the amount of the product of the enzyme-catalyzed substrate oxidation that is formed.

There is no applicable route of administration in the sense of a dose administration route for this *in vitro* test.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and microsomal preparation from the human placenta will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies.

2.1 Substrate

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN) will be provided to the laboratories by

Battelle's Chemical Respository (CR). The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN will be assessed by the lead laboratory and this information will be included in the study reports. The radiochemical purity of the [^{3}H]ASDN will be greater than approximately 95 percent.

2.1.2 Preparation of Substrate Solution for use in Aromatase Assay

Since the specific activity of the stock [^{3}H]ASDN is too high for use directly in the assay, a solution containing a mixture of nonradiolabeled and radiolabeled [^{3}H]ASDN is prepared such that the final concentration of ASDN in the assay is 100 nM and the amount of tritium added to each incubation is about 0.1 µCi. This substrate solution should have a concentration of 2 µM with a radiochemical content of about 1 µCi/mL.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Prepare a 1:100 dilution of the radiolabeled stock in buffer. Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL. Combine 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for 80 tubes). Record the weight of each component added to the substrate solution. After mixing the solution well, weigh aliquots (ca 20 µL) and combine with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yields a final [^{3}H]ASDN concentration of 100 nM with 0.1 µCi/tube.

2.2 Reference Chemicals

The reference chemicals for this task are aminoglutethimide, chrysin, econazole and ketoconazole (Table 1).

Table 1. Reference Chemicals for Task 5

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection
aminoglutethimide	125-84-8	$C_{13}H_{16}N_2O_2$	232.3	Non-steroidal aromatase inhibitor
chrysin	480-40-0	$C_{15}H_{10}O_4$	254.2	Potent flavonoid
econazole (nitrate)	24169-02-6	$C_{18}H_{15}Cl_3N_2O$-HNO_3	444.7	Potent imidazole anti-fungal
ketoconazole	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	531.43	Weak imidazole anti-fungal

2.2.1 Reference Chemical Formulation and Analysis

Reference chemical stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. Reference chemicals will be formulated in buffer, absolute ethanol or dimethylsulfoxide (DMSO). The total volume of reference chemical formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 μL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use such that the target concentration of reference chemical can be achieved by the addition of 20 μL of the dilution to a 2 mL assay volume. Information on storage conditions for reference chemical stock solutions will be provided by the CR.

2.3 Control Substances

The known aromatase inhibitor, 4-hydroxyandrostendione (4-OH ASDN), is used as the positive control substance. A known aromatase non-inhibitor, lindane, will be used as the negative control substance. Table 2 contains identity and property information for these substances.

Table 2. Control Substances

Test Substance	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Target Concentration in Assay (M)	Basis for Selection
4-OH ASDN	566-48-3	$C_{19}H_{25}O_3$	302.4	5E-8	Known aromatase inhibitor
Lindane	58-89-9	$C_6H_6Cl_6$	290.8	1E-6	Affects StAR and cholesterol metabolism; no aromatase activity

2.3.1 Control Substance Formulation and Analysis

Control substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. Control substances will be formulated in buffer, absolute ethanol or DMSO. The total volume of control substance formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 μL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dilutions will be prepared such that the target concentration of control substance (Table 2) can be achieved by the addition of 20 μL of the dilution to a 2 mL assay volume. Information on storage conditions for control substance stock solutions will be provided by the CR.

2.4 Microsomes

Human placental microsomes will be supplied to each laboratory by the lead laboratory. These samples should be treated as potentially infectious and appropriate precautions must be employed. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes is 14 mg/mL.

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. that is used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

On the day of use, microsomes are thawed quickly in a 37°C water bath and then are immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (about 5-10 passes) prior to use. The microsomes are diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 2 h before proceeding with the assay or microsomal enzyme activity may be decreased.

Excess undiluted stock microsomes may be flash frozen in liquid nitrogen and returned to -70 to -80 °C storage for future use. It is strongly recommended that stock microsomes to be refrozen be divided into aliquots appropriate for use prior to refreezing in order to minimize the number of freeze/thaw cycles.

Diluted microsomes must be used only on the day of preparation. Under no conditions should diluted microsomes be refrozen for later use in the assay.

2.5 Other Assay Components

2.5.1 Buffer

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic (JT Baker, cat # 4011-01, 137.99 g/mol) and sodium phosphate dibasic (JT Baker, cat # 4062-01, 141.96 g/mol) are used in the preparation of the buffer. Solutions of each reagent at 0.1 M are prepared in distilled, deionized water and then the solutions are combined to a final pH of 7.4. The assay buffer may be stored for up to one month in the refrigerator (2-8 °C).

2.5.2 Propylene Glycol

Propylene glycol (JT Baker, cat # 9402-01, 76.1 g/mol) is added to the assay directly as described below.

2.5.3 NADPH

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt, Sigma, cat # 1630, 833.4 g/mol) is the required co-factor for CYP19. The final concentration in the assay is 0.3 mM. Typically, a 6 mM stock solution is prepared in assay buffer and then 100 µL of the stock is added to the 2 mL assay volume. NADPH must be prepared fresh each day and is kept on ice.

3.0 PROTEIN ASSAY

The protein concentration of the microsome preparation will be determined on each day of use of the microsomes in the aromatase assay. A 6-point standard curve will be prepared, ranging from 0.13 to 1.5 mg protein/mL. The protein standards will be made from bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 25 µL aliquot of unknown or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or unknown and the samples will be vortex mixed. The samples will be allowed to sit at room temperature for at least 15 min to allow for color development. The absorbances are stable for about 1 h. Each sample (unknown and standards) will be transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined by extrapolation of the absorbance value using the curve developed using the protein standards.

4.0 AROMATASE ASSAY METHOD

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The final concentrations for the assay components are presented in Table 3. The tubes and the microsomal suspension will be placed at 37 ± 1°C in the water bath for five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca.

5 s and placed on ice. The tubes are then vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

Table 3. Optimized Aromatase Assay Conditions

Assay factor (units)	Human Placental
Microsomal Protein (mg/mL)[a]	0.0125
NADPH (mM)[a]	0.3
[^{3}H]ASDN (nM)[a]	100
Incubation Time (min)	15

[a] Final concentrations

Analysis of the samples will be performed using liquid scintillation spectrometry (LSS). Radiolabel found in the aqueous fractions represents ^3H_2O formed.

5.0 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO REFERENCE CHEMICALS

Each replicate will test the response of aromatase activity to the presence of eight concentrations of a reference chemical. The reference chemicals must be coded prior to distribution to the assaying technicians in order that the replicates are conducted blind for reference chemical identity. This task will be conducted in three independent replicates by each laboratory. At each laboratory, all three replicates for a given reference chemical must be conducted by the same technician. However, the same technician is not required to perform the three replicates for all four reference chemicals. Multiple reference chemicals may be conducted by a single technician in a given day. Each replicate for a given reference chemical must be conducted entirely independently of the other replicates for that reference chemical. Thus, it is recommended that if multiple replicates are conducted on a given day by a single technician, those replicates should use different reference chemicals. Each reference chemical will be tested at eight concentrations and there will be three (triplicate) repetitions for each concentration of a given replicate. A single replicate study of a given reference chemical is described in Table 4.

Four types of control samples will be included for each replicate. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)
- background activity controls (all components that are in the full aromatase activity controls, except NADPH)
- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration)
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a single concentration)

Four test tubes of each type of control are included with each replicate and are treated the same as the other samples. The controls sets will be split so that two tubes (of each control type) are run at the beginning and two at the end of each replicate set.

The assay will be conducted as described in Section 4.0 with the following modification. Reference chemical solution (or vehicle) will be added to the mixture of propylene glycol, substrate, NADPH and buffer in a volume not to exceed 20 µL prior to preincubation of that mixture. The volume of buffer used will be adjusted so the total incubation volume remains at 2 mL.

After completion of the first replicate, the data will be reviewed and, if necessary, the concentration of reference chemical used in the second and third replicates can be adjusted. The decision whether to adjust test concentrations rests with each participating laboratory. The decision should be based on the results from the first replicate with the following guidelines in mind:

- If insolubility is observed at the high concentration (10^{-3} M), then set the highest concentration for the second and third replicates at the highest concentration that appeared to be soluble (limited to 10^{-4} or 10^{-5} M). Do not use a concentration lower than 10^{-5} M for the highest concentration tested.
- If the highest concentration to be tested is lowered to 10^{-4} or 10^{-5} M, then add mid-log concentration(s) near the estimated IC_{50} based on the replicate one results in order to keep eight concentrations in the test set.
- The lowest concentration to be tested is 10^{-10} M.

Table 4. Reference Chemical Study Design

Sample type	Repetitions (test tubes)	Description	Test Chemical concentration (M final)
Full Enzyme Activity Control	4	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control	4	Complete assay with reference chemical vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay[a] with positive control chemical (4-OH ASDN) added	5×10^{-8}
Negative Control	4	Complete assay[a] with negative control chemical (lindane) added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}

[a]The Complete Assay contains buffer, propylene glycol, microsomal protein, [^{3}H]ASDN and NADPH

6.0 DATA ANALYSIS

6.1 Aromatase Activity and Percent of Control Calculations

Relevant data are entered into the latest version of the spreadsheet Aromatase_Master_Versionx.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. The version of the spreadsheet used will be included in the reports. A working document detailing the use of this spreadsheet appears in Appendix A.

6.2 Statistical Analyses

6.2.1 Concentration Response Fits for the Test Substance

For the reference chemicals multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three.

For each replicate two repeat tubes of the full enzyme activity controls (FEAC), the background activity controls (BAC) and the positive and negative controls will be run prior to the to repetitions of the graded concentrations of the reference chemical and two repeat tubes of each control will be run following the repetition of the reference chemical. Three repetitions will be prepared for each concentration of the reference chemical.

For each repeat tube (FEAC, BAC, positive, and negative controls and each reference chemical concentration) the Excel database spreadsheet will include total observed (uncorrected) disintegrations per minute (DPMs) per tube and total aromatase activity per tube. The DPM and aromatase activity values are corrected for the background DPMs, as measured by the average of the BAC control tubes. The aromatase activity is calculated as the corrected DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The average (corrected) DPMs and aromatase activity across the four BAC control repeat tubes must necessarily be equal to 0 within each replicate.

For each tube percent of control is determined by dividing the background corrected aromatase activity for that tube by the average background corrected aromatase activity for the four FEAC tubes and multiplying by 100. Nominally one might expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations. However due to experimental variation individual observed percent of control values will sometimes extend below 0% or above 100%.

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each reference chemical concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of reference chemical (e.g. if concentration = 10^{-5} then X = -5). Let

Y ≡ percent of control activity in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average DPMs across the repeat tubes with the same reference chemical concentration
β ≡ slope of the concentration response curve (β will be negative)
$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%)

The following concentration response curve will be fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance is approximated by Y.

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits will be carried out using Prism software (Version 3 or higher). Observed individual percent activity values above 100% will be set to 99.5%. Observed individual percent activity values below 0% will be set to 0.5%. Model fits will be carried out using Prism software (Version 3 or higher).

Concentration response models will be fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate the extent of aromatase inhibition will be summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for a reference chemical will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β and the replicate-to-replicate component of variation will be calculated based on a one-way random effects analysis of variance model fit. For each reference chemical and replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve is indicated as:

- "C" Complete. i.e. ranging from essentially 0 percent to 100 percent of control.
- "II" Incomplete. But can interpolate to $\log_{10}IC_{50}$.
- "IX" Incomplete. But must extrapolate to $\log_{10}IC_{50}$.

Replicates for which a concentration response curve cannot be fitted (and so an IC_{50} cannot be estimated) will be referred to as "noninhibitors".

6.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits

For each replicate the individual percent of control values will be plotted versus logarithm of the reference chemical concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each replicate.

Additional plots will be prepared to compare the percent of control activity values across replicates. For each replicate the average percent of control values will be plotted versus logarithm of reference chemical concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curves for each replicate will be superimposed on the plots. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of reference chemical concentration. The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}). Let X and Y ($0< Y <100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is

$$Y_{avg} = 100/[1 + 10^{\beta_{avg}(\mu_{avg} - X)}].$$

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on one-way random effects analysis of variance, treating the replicates as random effects. For each of β and μ, plots will be prepared that display the parameters within each replicate with associated 95% confidence intervals based on the within replicate standard error and the average across replicates with associated 95% confidence interval incorporating replicate-to-replicate variation.

6.2.3 Graphical and Analysis of Variance Comparisons of FEAC, BAC, and Positive and Negative Control Percent of Control Across Reference Chemicals and Replicates

Within each replicate of each reference chemical quadruplicate repetitions will be made of the FEAC control, BAC control, and negative and positive control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are consistent throughout the replicate test, the control tubes at the beginning should be equivalent to those at the end.

To assess whether this is the case the control responses will be adjusted for background DPMs, divided by the average of the (background adjusted) FEAC control values, and expressed as percent of control. The average of the four BAC controls within a replicate must necessarily be 0 percent and the average of the four FEAC controls within a replicate must necessarily be 100 percent. The FEAC controls percent of control, the BAC controls percent of control, and the

negative and positive controls percent of control values will be plotted across reference chemical and replicate within reference chemical, with plotting symbol distinguishing between beginning and end, and with reference line 0% (BAC control) or 100% (FEAC control) respectively. These plots will display the extent of consistency across reference chemicals and replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Additional plots will be prepared displaying the difference of the average of the first two percent of control values (i.e., those based on the "beginning" tubes) and the average of the last two percent of control values (i.e., those based on the "end" tubes) across reference chemicals and replicates within reference chemicals. Each plot will have a reference line of 0.

Three-factor mixed effects analysis of variance models will be fitted, separately for the FEAC control, the BAC control, and the positive and negative control tubes. The fixed effect factors in the analysis of variance will be

- reference chemical
- portion (beginning or end)
- portion by reference chemical interaction.

The random effects will be

- replicate nested within reference chemical
- portion by replicate within reference chemical interaction.

The residual error variation corresponds to repetition within reference chemical, replicate, and portion. The response will be percent of control. Since for the BAC and FEAC controls the average of the repetitions within a reference chemical and replicate are constrained to be 0 and 100 respectively, by the way in which "percent of control" is defined, the variation associated with the reference chemical effect and the replication within reference chemcial effect are both necessarily constrained to be 0.

If the daily replicates are in control the portion main effect and the portion by replicate within reference chemical interaction should be nonsignificant. If the portion by replicate within reference chemical interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate within reference chemical to the portion effect averaged across replicates within reference chemical, adjusting for simultaneity by Bonferroni's method.

6.2.4 Statistical Software

Concentration response curves will be fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using PRISM, the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS), as convenient.

6.2.5 Interlaboratory Statistical Analysis

The lead laboratory and each of the participating laboratories will carry out "intra-laboratory" statistical analyses based on their test data, according to this common statistical analysis plan, developed by the Data Coordination Center (Battelle). The Data Coordination Center will carry out the "inter-laboratory" statistical analysis. It will combine summary values developed in each of the intra-laboratory analyses to assess relationships among the laboratory results, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

7.0 RETENTION OF RECORDS

All records that remain the responsibility of the testing laboratory will be retained in the archives for the life of the contract.

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study. The study will be conducted in compliance with the Federal Register, 40 CFR Part 160. Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practices Standards.

9.0 REPORTS

Interim data summaries, draft and final reports will be submitted as described in Section 9.5 of the QAPP.

The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and test chemical repetition), percent of control activity, IC_{50}, slope and graphs of activity versus log chemical concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the intra- and inter-laboratory statistical analyses described in Section 6 of this document.

10.0 STUDY RECORDS TO BE MAINTAINED

- All records that document the conduct of the laboratory experiments and results obtained, as well as the equipment and chemicals used
- Protocol and any Amendments

- List of any Protocol Deviations
- List of Standard Operating Procedures
- QAPP and any Amendments
- List of any QAPP Deviations

Appendix A

Notes for use of the spreadsheet: Aromatase_Master_Version1.1.xls

Substrate Specific Activity Worksheet

This worksheet calculates:
1. The radiochemical content (DPM/mL) of the substrate solution
2. The new specific activity of the [^{3}H]ASDN in the substrate solution

The first item is based on the results of LSC analysis of weighed aliquots of the substrate solution

The second item is calculated by:
1. determining the mass of ASDN (both radiolabeled and nonradiolabeled)/g of solution. This calculation uses both the measured mass of nonradiolabeled ASDN used in the solution preparation and also the specific activity of the stock [^{3}H]ASDN.

2. the radiochemical content (mCi/g) of the solution is then divided by the mass of ASDN/g solution to arrive at the new specific activity for [^{3}H]ASDN in the substrate solution.

Data to be input include
- Substrate solution aliquot weights (g) and DPM results
- Weight (mg) of ASDN used in original stock and volume (mL) of the original stock
- All dilution factors for the dilution of ASDN stock to the solution that was finally used in substrate preparation.
- Weight (g) of ASDN dilution used to prepare substrate solution and total weight (g) of substrate solution
- Specific activity of the stock [^{3}H]ASDN (μCi/mmol)

Protein Worksheet

This worksheet calculates protein content based on absorbance data of standards and unknown samples obtained when samples are analyzed using a commercially available kit.

Data to be input include the concentration of protein standard stock solution (mg/10 mL), protein stock ID, Sample IDs, absorbance data (in triplicate) for standards and unknowns and appropriate dilution factors.

Absorbance values are corrected for blank absorbance. A calibration curve is prepared by linear regression of the standards data (corrected absorbance vs. mg protein measured). The concentration of protein in the unknowns is calculated based on the standard curve.

Microsome and Chemical Dilutions Worksheet

This worksheet calculates the concentration of protein in the final microsome dilution. It also serves as the data input center for the test chemical concentrations used in the assay.

Data input include volumes used in the preparation of microsome dilutions. Also entered is the protein concentration of the stock microsomes. Normally, this value will be determined using the protein worksheet described above.

Test chemical concentrations are entered in molar units of the final concentrations used in the assay.

Activity Calculation Worksheet

The primary aim of this worksheet is to calculate aromatase activity for each sample in a set based on measured DPM, protein concentration and incubation time.

The function of each section is described below:

Section 1 (Columns A-B)
This section contains fields for sample identification

Section 2 (Columns C-I)
This section calculates the total DPM that remain in the incubation mixture after extraction (this is a measure of the ^3H_2O formed in the reaction).
Data input:
1. Aliquot volume
2. DPM measured for each aliquot of each sample.

Output:
The worksheet calculates the average DPM/mL for each sample, the average DPM/mL for each sample and the total DPM contained in the sample (based on the aliquots and total sample volumes)

Section 3: (Columns J-L)
This section calculates the percent turnover of the substrate to product.

Data input:. Volume of substrate solution used in each assay tube

Linked Data: Column K links to radiochemical content value for the substrate that is calculated in the substrate specific activity worksheet

Output: Percent conversion to product

Section 4 (Columns M-N)
This section calculates the nmol ^3H_2O formed

Data input: None

Linked Data: Column N links to specific activity value for the substrate that is calculated in the substrate specific activity worksheet

Calculations: Column M corrects the total DPM in each tube for background DPM determined in negative control tubes

Column N Converts DPM data to nmol using the substrate specific activity

Data output: nmol ^3H_2O formed

Section 5 (Columns O-R)

This section calculates aromatase activity in each tube.

Data input: Volume of diluted microsomes used in assay tube and incubation time

Output: Aromatase activity (nmol/mg protein/min)

Results Summary Worksheet

This worksheet summarizes the results.

Section 1 (Columns A-D, Rows 3-15)
This section summarized control data.

Data input: none

Output: average and SD for control samples for beginning, end and overall portions

Section 2 (Columns A-F, Rows 18-42)

This section summarizes activity values according to inhibitor level and replicate

Data input: None

Output: Log[test inhibitor]

Section 3 (Columns H-L, Rows 18-28)

This section calculates percent of control values for each test chemical concentration and replicate and organizes the data in a format suitable for importation into Prism Software.

Data input: None

Output: Percent of control values with data arranged in a format suitable for importation into Prism Software.

APPENDIX C

[^{3}H]ASDN Purity Assessment Report (RTI) C-1

FINAL ANALYSIS REPORT

PLACENTAL AROMATASE VALIDATION STUDY

[^{3}H]ASDN Radiochemical Purity Determination

EPA Contract Number 68-W-01-023
Work Assignment 4-16

Sponsor:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

Performing Laboratory:
Drug Metabolism and Pharmacokinetics
RTI International
Post Office Box 12194
Research Triangle Park, NC 27709

RTI INTERNATIONAL

FINAL REPORT

Title:	PLACENTAL AROMATASE VALIDATION STUDY [^{3}H]ASDN Radiochemical Purity Determination
Author:	Sherry Black
Performing Laboratory:	Drug Metabolism and Pharmacokinetics RTI International Post Office Box 12194 Research Triangle Park, NC 27709
Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
Sponsor's Representatives:	David Houchens, Ph.D. EDSP Program Manager Battelle Jerry D. Johnson, Ph.D. Diplomate, A.B.T. Work Assignment Leader Battelle
Analysis Date:	January 5, 2005
Final Report Date	September 28, 2005

Author:

Sherry Black 9/28/05

Sherry Black Date
Research Chemist

Approved:

9-28-05

James Mathews, Ph. D, DABT Date
Study Director

RTI INTERNATIONAL

Quality Assurance Statement

Study Title:	**[^{3}H] ASDN Radiochemical Purity Determination WA 4-16 and WA 4-17**
Sponsor:	**Battelle Memorial Institute**
Study Code:	**An05-928**
Protocol Number:	**RTI-928-AN**

This study was audited by the Science and Engineering – Health Sciences Quality Assurance Unit and the results of the inspections and audits were reported to the study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Study Director and Management
Data and Report Audit	March 24, 2005	March 25, 2005

K. Collier — 9/28/2005

K. Collier
Quality Assurance Specialist

Date

Approval:

Carrie Ingalls — 09/28/2005

Carrie Ingalls
Quality Assurance Assistant Manager

Date

Introduction

The objective of this work is to determine the radiochemical purity of the [3H]ASDN to be used in the conduct of WA 4-16 and WA 4-17. The criteria for acceptance of the material for this use is 95% radiochemical purity as determined by high performance liquid chromatography (HPLC) and liquid scintillation counting.

Materials and Methods

[^{3}H]Androstenedione ([^{3}H]ASDN) of lot number 3538496 was received from Perkin Elmer Life Science (Boston, MA).

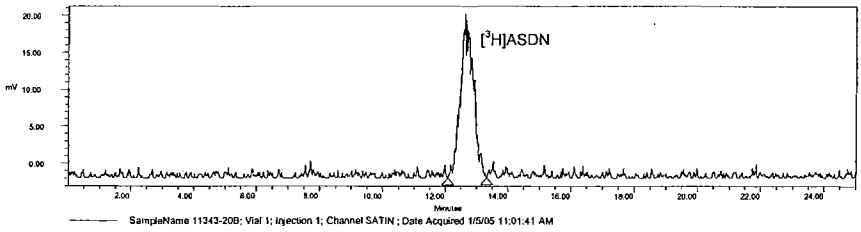
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through radioactivity detector (IN/US, Inc., Tampa, FL) with a 250 µL glass scintillant cell. Data was collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method used a Zorbax Rx-C_{18} column (4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant was monitored by ultraviolet (UV) absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions were collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS)

Results

The HPLC radiochromatogram of the [^{3}H]ASDN, lot number 3538496, is presented in Figure 1. The measured radiochemical purity of the [^{3}H]ASDN was 97%.

Figure 1. HPLC Radiochromatogram of [^{3}H]ASDN



Conclusion

[^{3}H]ASDN, lot number 3538496, is acceptable for use on WA 4-16 and WA 4-17.

APPENDIX D

Chemistry Reports (from Battelle)

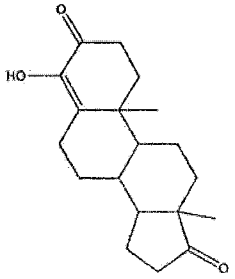
4-Hydroxyandrostenedione (4-OH ASDN) D-1
Aminoglutethimide D-18
Chrysin D-31
Econazole D-46
Ketoconazole D-62
Lindane D-73

Battelle
The Business of Innovation

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3
Lot No.: 063K4069 (Sigma Aldrich)
Receipt Date: 10/22/04
Amount Received: 3.1 g
Appearance: Solid
Vendor Purity: 99% by TLC
Storage Conditions (@ Battelle): Refrigerated (~5°C)

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	302.41 g/mol	$C_{19}H_{26}O_3$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/04	10/26/04
Formulation preparation	12/2/04	12/2/04
Dispensing	12/2/04	12/2/04
Formulation analysis	12/2/04	12/2/04
Audit analytical report	10/20/05	10/20/05
Audit study file	10/20/05	10/20/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 12-14-05 Date

EXECUTIVE SUMMARY

The title compound, 4-hydroxyandrostenedione (4-OH ASDN), was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation Work, Work Assignment 4-16/17.

The solubility of 4-hydroxyandrostenedione was determined to be acceptable in 95% ethanol for preparing formulations.

A formulation analysis method was developed and validated to analyze 4-hydroxyandrostenedione in 95% ethanol at a concentration of 3.02 mg/mL (0.01M). This method was used to analyze samples from both formulation and formulation storage stability studies at 3.02 mg/mL.

The storage stability study indicated that a 3.02 mg/mL formulation, stored in sealed amber glass bottles and protected from light, was stable for 173 days at approximately 5°C.

The stock formulation prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for 4-hydroxyandrostenedione on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in 95% ethanol.
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle of 4-hydroxyandrostenedione, 063K4069, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 22, 2004. The label amount indicated 3.1 grams was sent. The chemical was received and subsequently stored at approximately 5°C.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99% based on thin layer chromatography (TLC).

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of 4-hydroxyandrostenedione (4-OH ASDN) in 95% ethanol, at a concentration of at least 30.2 mg/mL. The 4-OH ASDN (0.30200 ± 0.03020 g) was weighed into a 10-mL volumetric flask, diluted to approximately 80% volume with 95% ethanol, sealed and shaken to mix. The flask was diluted to volume with 95% ethanol, sealed, shaken, sonicated for approximately 50 minutes and stirred. The 4-OH ASDN did not go into solution.

A second solubility study was conducted to determine the solubility of 4-OH ASDN in 95% ethanol, with a solubility of at least 3.02 mg/mL being required for acceptability. The 4-OH ASDN (0.03020 ± 0.00302 g) was weighed into a 10-mL volumetric flask, diluted to approximately 80% volume with 95% ethanol, sealed and shaken to mix. The flask was diluted to volume with 95% ethanol, sealed, shaken and sonicated for approximately 2 minutes. The 4-OH ASDN went into solution. This experiment showed that 95% ethanol was an acceptable solvent for the 3.02 mg/mL formulation (0.01M).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	4-Androsten-4-ol-3,17-dione
Product Number	A5791
Product Brand	SIGMA
CAS Number	566-48-3
Molecular Formula	$C_{19}H_{26}O_3$
Molecular Weight	302.41

TEST	SPECIFICATION	LOT 063K4069 RESULTS
APPEARANCE		WHITE POWDER
SOLUBILITY		CLEAR COLORLESS SOLUTION AT 10 MG/ML OF METHANOL
ELEMENTAL ANALYSIS		75.45% CARBON
PROTON NMR SPECTRUM		CONSISTENT WITH STRUCTURE
PURITY BY THIN LAYER CHROMATOGRAPHY		99%
QC ACCEPTANCE DATE		JUNE 2003

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of 4-hydroxyandrostenedione in 95% ethanol at a target concentration of 3.02 mg/mL (0.01 M) for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic columns and conditions. The selected method was one which produced acceptable retention time for the major peak, apparent resolution of significant impurities and acceptable peak shape. The detection method chosen was gas chromatography with flame ionization detection (GC/FID).

4.2 Method

The GC parameters for 4-hydroxyandrostenedione are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5 MS, 15 m × 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at 2 mL/minute
Oven Temperature	150°C, hold for 1 minute, increase at 15°C/minute to 320°C
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at 30 mL/minute; Air at 380 mL/minute
Detector Temperature	320°C
Injector Temperature	250°C
Injection Volume	1 µL
Injection Mode	Split 1:10
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Fifty (50) milligrams ± 4 mg of benzophenone was added to a 25-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 50 ± 1 mg of 4-OH ASDN each into individual 50-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 1000 µg/mL each.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with methanol, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	IS (mL)	95% Ethanol (mL)	Final Volume (mL)
VS1	500	A	5	1	1	10
VS2	300	B	3	1	1	10
VS3	200	A	2	1	1	10
VS4	100	B	1	1	1	10

4.3.1.4 Blanks

Triplicate blanks without IS were prepared by pipetting 1 mL of 95% ethanol into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 1 mL of 95% ethanol into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

4.3.2 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.3 Calculations

The integration of the 4-OH ASDN and IS peaks by the chromatography data system was evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation weighted 1/x was calculated relating the response ratio of 4-OH ASDN divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standard at each concentration.

4.3.4 Results

Specificity is shown by representative overlaid chromatograms from low and high vehicle/calibration standards, blank with IS, and a blank from the validation data as presented in Figure 2.

The blank and blank with IS exhibited no peaks that would significantly interfere with the 4-OH ASDN or IS peaks.

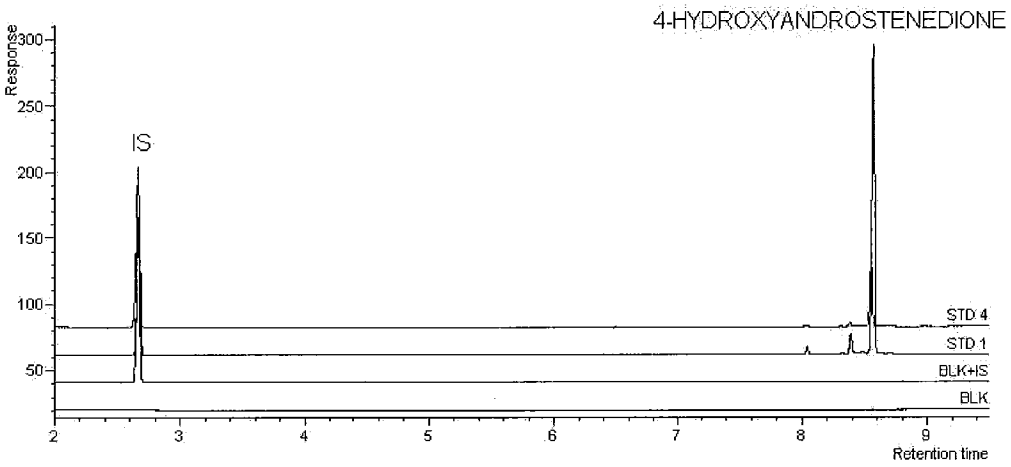


Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

The regression analysis results from the validation standard curve indicate linearity and are shown in Table 3.

Table 3 – Regression Analysis Validation Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0038	-0.0272	0.9975	0.0565

The vehicle/calibration standard validation results are shown in Table 4.

Table 4 –Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	%RE	Avg %RE
	496.8				-1.9	
506.4	494.5	509.6	24.2	4.7	-2.3	0.6
	537.5				6.1	
298.1	289.4	NA	NA	NA	-2.9	NA
202.6	198.8	NA	NA	NA	-1.9	NA
	100.7				1.3	
99.38	99.89	100.4	0.4	0.4	0.5	1.0
	100.5				1.1	

The method validation sensitivity was 1.266 µg/mL, the LOD, which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 13 µg/mL when a formulation is diluted 1 to 10 for analysis. The LOQ was 4.219 µg/mL, defined as ten times the standard deviation of the lowest standard because there was no blank response. This is equivalent to a formulation concentration of 42 µg/mL when a formulation is diluted 1 to 10 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 99.38 µg/mL.

4.3.5 Conclusions

The method met all acceptance criteria for precision, accuracy, linearity, sensitivity and specificity. The method was suitable for the stability study and subsequent formulation analyses for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a concentration of 3.02 mg/mL (0.01 M) in 95% ethanol for 173 days in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A sample was analyzed on the day of preparation (Day 0) and Day 14. A second sample was analyzed on the day of preparation Day 0, Days 27, 54, 83 and 173. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on November 10, 2004 (Day 0) for the storage stability study at a target concentration of 3.02 mg/mL (0.01 M) in 95% ethanol by accurately weighing 75.50 ± 0.75 mg of 4-OH ASDN into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with 95% ethanol. The flask was sealed, sonicated for 10 minutes and allowed to cool to room temperature. The contents of the flask was diluted to volume with 95% ethanol, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four, 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After 14 days of storage, a vial was removed from the refrigerator, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation was prepared on December 2, 2004 (Day 0) at a target concentration of 3.02 mg/mL (0.01 M) in 95% ethanol by accurately weighing 151.00 ± 0.50 mg into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with 95% ethanol, sealed and mixed well. The contents of the flask were diluted to volume with 95% ethanol and mixed well. Approximately 18 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0, 27, 54, 83 and 173 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards, blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report with the exception that the standard stocks were prepared by accurately weighing 25 ± 1 mg of 4-OH ASDN into 25-mL volumetric flasks.

In triplicate, 1 mL of the formulation and 1 mL of IS were pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

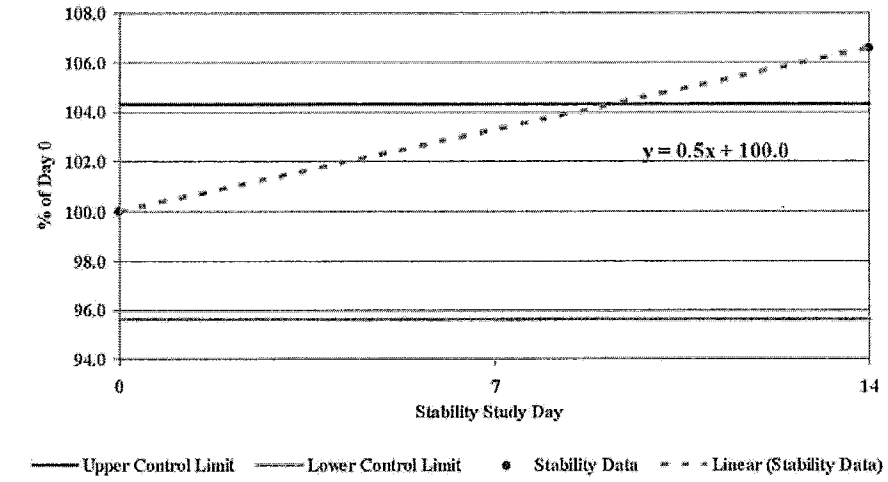
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (3.02 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
11/10/04	11/10/04	0	2.871	2.873	2.928	2.891 ± 0.032	100.0 ± 1.1
11/10/04	11/24/04	14	3.006	3.085	3.149	3.080 ± 0.072	106.5 ± 2.5
12/2/04	12/2/04	0	3.005	3.022	3.005	3.011 ± 0.010	100.0 ± 0.3
12/2/04	12/29/04	27	3.168	3.123	3.117	3.136 ± 0.028	104.2 ± 0.9
12/2/04	1/25/05	54	3.008	3.126	3.110	3.081 ± 0.064	102.3 ± 2.1
12/2/04	2/23/05	83	3.027	3.131	3.217	3.125 ± 0.095	103.8 ± 3.2
12/2/04	5/24/05	173	3.126	3.142	3.129	3.133 ± 0.008	104.1 ± 0.3

For the sample prepared November 10, 2004, the pooled RSD of the analytical method was 1.9%. This means that there would have to be a difference of more than 4.4% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the sample prepared December 2, 2004, the pooled RSD of the analytical method was 1.8%. This means that there would have to be a difference of more than 4.0% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.



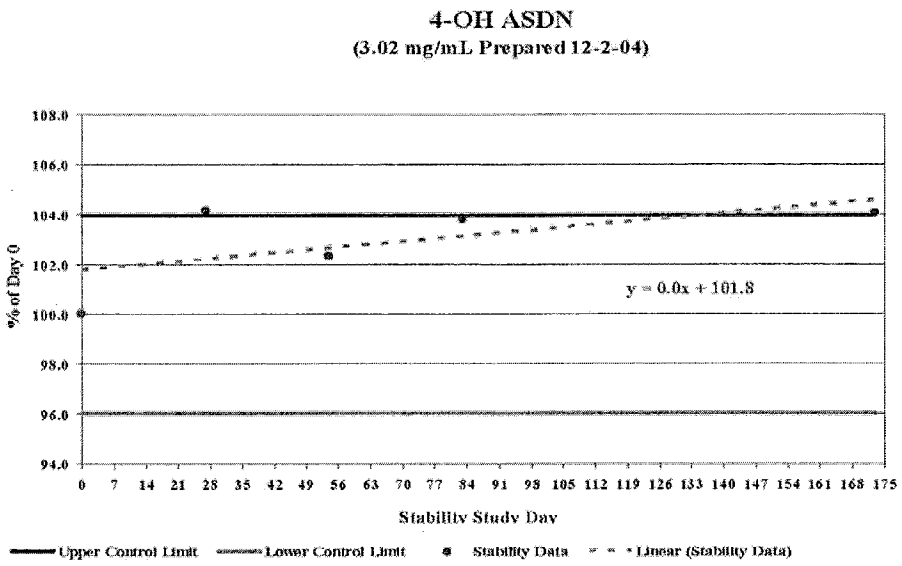


Figure 3 – Control Charts for the Storage Stability Studies

5.5 Discussion and Conclusions

The concentration of the samples stored at approximately 5°C protected from light in amber glass vials for Day 14 was above the upper significance level, but was within 6.5% of the Day 0 value (prepared November 10, 2004). Concentrations for Days 54 and 83 samples were within the upper and lower significance levels and Days 27 and 173 were just above the upper significant level. A linear trend analysis indicated there was no significant trend to changing concentration over time for the samples. These data indicate the formulation was stable when stored protected from light at approximately 5°C for 173 days.

6 FORMULATION PREPARATIONS AND ANALYSES

Formulations were prepared and analyzed on December 2, 2004, January 25, 2005, March 21, 2005, and June 27, 2005, according to SOP No. COMSPEC.II-027, "Standard Operating Procedure (SOP) for the Formulation and Analysis of 4-Hydroxyandrostenedione (4-OH ASDN) in 95% Ethanol." This section describes the method, results, and conclusions.

6.1 Preparation of Formulations

An accurate weight of 151.00 ± 0.50 mg of 4-OH ASDN was added to a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with 95% ethanol, sealed and mixed well. The contents of the flask were diluted to volume with 95% ethanol and mixed well. This produced a target concentration of 3.02 mg/mL (0.01 M) 4-OH ASDN in 95% ethanol.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the method validation, Section 4.3.1 of this report.

6.3 Preparation of Formulation Samples

One (1) mL of the formulation and 1-mL of IS were pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well.

6.4 Analysis

Autoinjector vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the conditions from the method validation (Table 1).

6.5 Calculations

The peaks for 4-hydroxyandrostenedione and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation weighted 1/x was calculated relating the response ratio (4-hydroxyandrostenedione/IS) (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios

were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/ calibration standards and formulation samples when applicable.

6.6 Results

Specificity is shown by the representative overlaid chromatograms of the high and low standards, blank with IS and a blank presented in Figure 4.

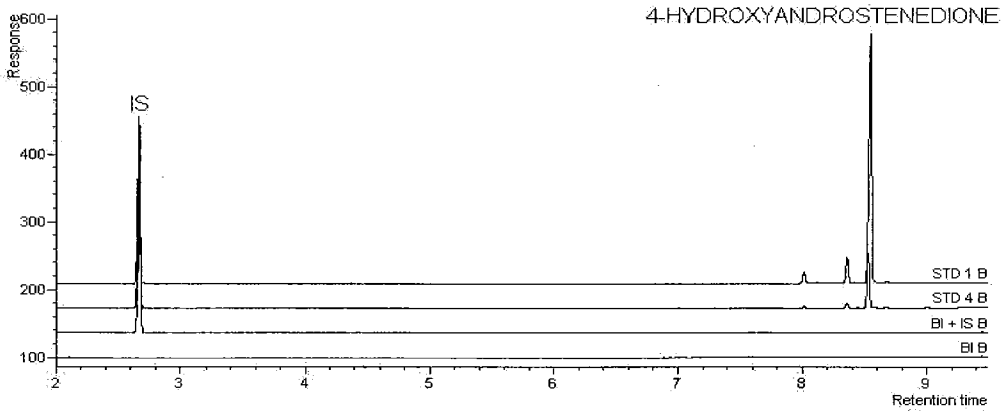


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

The regression analysis results of the vehicle/calibration standard curves indicated linearity and are shown in Table 6.

Table 6 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0038	-0.0140	0.9999	0.0117
0.0035	-0.0037	1.000	0.0061
0.0036	-0.0251	0.9999	0.0100
0.0038	-0.0218	0.9999	0.0104

The results of the formulation analyses are shown in Table 7.

Table 7 – Formulation Analysis Results

Batch	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	% RSD
1-ASDN	3.005	3.022	3.005	3.011	-0.3	0.3
2-ASDN	3.056	3.089	3.049	3.065	1.4	0.7
3-ASDN	3.112	3.053	3.063	3.076	1.9	1.0
4-ASDN	2.943	2.945	2.950	2.946	-2.5	0.1

The formulations met acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

6.7 Conclusions

The average concentration of the stock formulations and their percent RSD were within acceptance criteria. Therefore, the formulations were suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Sandy Runyon, Christina Zielinski, Tudor Fernando, Kevin Carrico, and Darren Brown. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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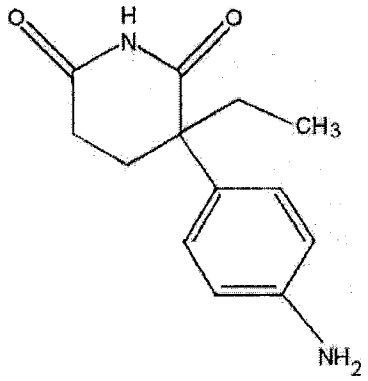
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

AMINOGLUTETHIMIDE

CAS No.: 125-84-8	Lot No.: 043K0939 (Sigma Aldrich)
Receipt Date: 10/27/04	Amount Received: 2.40 g
Appearance: Solid	Vendor Purity: >99% by TLC
Receipt Date: 6/24/05	Lot No.: 06016JS (Sigma Aldrich)
Appearance: Solid	Amount Received: 3.0 g
Storage Conditions (@ Battelle): Room Temperature (~25°C)	Vendor Purity: 99% by TLC

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	232.28 g/mol	$C_{13}H_{16}N_2O_2$

Prepared By:

Denise A. Contos, M.S.

Approved By:

10-18-05

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and Management as follows:

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Test substance receipt*	10/26/04	10/26/04
Formulation preparation*	12/2/04	12/2/04
Dispensing*	12/2/04	12/2/04
Formulation analysis*	12/2/04	12/2/04
Audit study file	7/26/05	7/26/05
Audit analytical report	7/26/05	7/26/05
Audit study file	10/5/05	10/5/05
Audit analytical report	10/5/05	10/5/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Hillary Long 10-14-05
Quality Assurance Unit Date

EXECUTIVE SUMMARY

The title compound, aminoglutethimide (AG), was analyzed in support of the Environment Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

Solubility of aminoglutethimide was determined to be acceptable in dimethylsulfoxide (DMSO) at a concentration of 23.2 mg/mL (0.1 M).

An aminoglutethimide formulation analysis method was validated on the previous EPA WA 3-10 study. This method was used without technical modification for analysis of formulation and stability samples on the current study.

Storage stability was previously determined (EPA WA 3-10 study) as 39 days when stored at approximately 5°C and protected from light at a target formulation concentration of 27.6 mg/mL in DMSO. In the current study, a formulation sample at a target concentration of 23.2 mg/mL in DMSO was stable when stored refrigerated and protected from light for 59 days.

The stock formulation prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for aminoglutethimide on EPA Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO)
- Preparing and analyzing a stock formulation and a formulation stability sample.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle containing 2.40 grams of aminoglutethimide, Lot No. 043K0939, and one 30-mL clear glass bottle containing 3.0 grams of aminoglutethimide, Lot No. 06016JS was received on October 27, 2004 and on June 24, 2005, respectively, from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA. The chemicals were received and subsequently stored at room temperature.

A copy of the manufacturer's Certificates of Analysis for these lots are shown in Figures 1 and 2. The purity of the chemicals were > 99% and 99%, respectively, based on thin layer chromatography.

SIGMA-ALDRICH

Certificate of Analysis

Product Name	DL-Aminoglutethimide
Product Number	A9657
Product Brand	SIGMA
CAS Number	125-84-8
Molecular Formula	$C_{13}H_{16}N_2O_2$
Molecular Weight	232.28

TEST	SPECIFICATION	LOT 043K0939 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML OF ACETIC ACID:METHANOL (1:1)	CONFORMS
IDENTITY	CONSISTENT WITH STRUCTURE BY IR OR NMR	IR SPECTRUM CONFORMS
CARBON	66.0 TO 68.5%	67.2% *
NITROGEN	11.8 TO 12.3%	12.1% *
PURITY BY THIN LAYER CHROMATOGRAPHY	98% MINIMUM	> 99%
		* SUPPLIER'S INFORMATION
SHELF LIFE SOP QC-12-006	7 YEARS	OCTOBER 2007
QC ACCEPTANCE DATE		APRIL 2003
PRODUCT CROSS REFERENCE INFORMATION		REPLACEMENT FOR ALDRICH #259195

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis, Lot No. 043K0939

SIGMA-ALDRICH

3050 Spruce Street
Saint Louis, Missouri 63105 USA
Telephone (800) 521-8956 • (314) 771-5765
Fax (800) 325-5052 • (314) 77-5757
Visit Us At www.sigma-aldrich.com

Certificate of Analysis

11236693MEC
BATTELLE NORTHWEST
1529 W SEQUIM BAY RD
SEQUIM WA 98382

PO NBR: 11236693MEC

PRODUCT NUMBER: 259195-500MG
LOT NUMBER: 06016JS

PRODUCT NAME: AMINOGLUTETHIMIDE, 99%

FORMULA: C13H16N2O2
FORMULA WEIGHT: 232.29

APPEARANCE	WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 3072B OF EDITION I, VOLUME 2 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
ELEMENTAL ANALYSIS	CARBON 66.60% HYDROGEN 6.93% NITROGEN 11.94%
THIN-LAYER CHROMATOGRAPHY	CONSISTENT WITH 99% PURITY
SOLUBILITY	5% HOAC:MEOH 1:1; SLIGHTLY HAZY, FAINT YELLOW SOLUTION.
QUALITY CONTROL ACCEPTANCE DATE	JULY 1998

ALDRICH CHEMICAL COMPANY
RONNIE MARTIN
NOVEMBER 5, 2003

We are Committed to the success of our Customers, Employees and Shareholders through leadership in Life Science, High Technology and Service.

Figure 2 – Certificate of Analysis, Lot No. 06016JS

3 SOLUBILITY STUDY

A solubility study was conducted to determine the solubility of aminoglutethimide in 100% dimethylsulfoxide (DMSO) at a target concentration of 23.2 mg/mL (0.1 M). The solution was prepared by weighing 0.23228 ± 0.02322 g of aminoglutethimide into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and the contents were mixed. The flask was diluted to volume with DMSO, sealed and mixed. The aminoglutethimide easily went into solution with shaking. This experiment showed that DMSO was an acceptable solvent for a 23.2 mg/mL (0.1 M) formulation.

4 FORMULATION PREPARATION AND ANALYSIS

A formulation was prepared and analyzed on January 24, 2005 according to SOP No. COMSPEC.II-007-01, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Aminoglutethimide (AG) in 100% DMSO." In addition, the January 24, 2005 formulation was re-analyzed to determine stability on March 24, 2005, 59 days of storage at approximately 5°C and protection from light. A second formulation was prepared and analyzed on June 30, 2005 according to SOP No. COMSPEC.II-007-01. The following sections describe the method, results, and conclusions.

4.1 Preparation of Formulation

Aminoglutethimide formulations with a target concentration of 23.2 mg/mL (0.1M) in DMSO were prepared on January 24, 2005 (Batch 1-AG) and on June 30, 2005 (Batch 2-AG) by accurately weighing 1160.00 ± 46 mg of aminoglutethimide into a tarred 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and mixed well until the aminoglutethimide dissolved. The content of the flask was diluted to volume with DMSO, sealed, and mixed well.

4.2 Preparation of Standards and Blanks

4.2.1 Internal Standard (IS)

An internal standard (IS) solution was prepared by pipetting 100 µL of octanophenone into a 100-mL volumetric flask. The content of the flask was diluted to volume with acetone, sealed, and mixed well.

4.2.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 35 ± 1 mg and 30 ± 1 mg of aminoglutethimide into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with acetone. This produced stocks A and B with target concentrations of 1.4 and 1.2 mg/mL, respectively.

4.2.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 1. The contents of the flasks were diluted to volume with acetone, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 1 – Preparation of Vehicle/Calibration Standards

Vehicle/ Calibration Std	Target Conc (µg/mL)	Source	Source Volume (mL)	IS Volume (mL)	DMSO Volume (mL)	Final Volume (mL)
VS1	56	A	2	1	0.10	50
VS2	48	B	2	1	0.10	50
VS3	28	A	1	1	0.10	50
VS4	24	B	1	1	0.10	50

4.2.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.10 mL of DMSO into three individual 50-mL volumetric flasks. The contents of the flasks were diluted to volume with acetone, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.10 mL of DMSO into three individual 50-mL volumetric flasks. The contents of the flasks were diluted to volume with acetone, sealed, and mixed well.

4.3 Preparation of Formulation and Formulation Stability Samples

In triplicate, 0.10 mL of the formulation and 1 mL of the IS were pipetted into individual 50-mL volumetric flasks, diluted to volume with acetone, sealed, and mixed well.

4.4 Analysis

A portion of each vehicle/calibration standard, blank, and sample was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the GC parameters for aminoglutethimide shown in Table 2.

Table 2 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-1, 30 m × 0.53 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at 10 mL/minute
Oven Temperature	160°C, hold for 1 minute, increase at 10°C/minute to 300°C
Detector Type	FID
Detector Flow Rates	Hydrogen at 30 mL/minute; Air at 300 mL/minute
Detector Temperature	300°C
Injector Temperature	260°C
Injection Volume	1 µL using a split ratio of 2 and a flow of 20 mL/minute
Run Time	15 minutes

4.5 Calculations

The integration of the aminoglutethimide and the IS peaks by the chromatography data system were evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation, un-weighted, was calculated relating the response ratio, aminoglutethimide/IS, (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each vehicle/calibration standard and formulation sample. The percent relative error (%RE) for each vehicle/calibration standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent relative error for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.6 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank as presented in Figure 3. The blank and blank with IS exhibited no peaks that would significantly interfere with the aminoglutethimide or IS peaks.

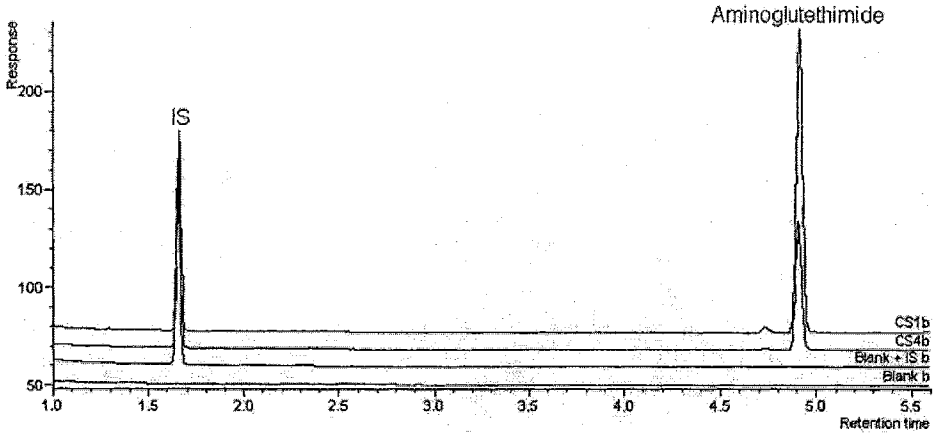

Figure 3 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from 1/24/2005 Formulation Analysis (Shown Top to Bottom)

The regression analysis results from the vehicle/calibration standard curves for the analyses indicated linearity and are shown in Figures 4 and 5.

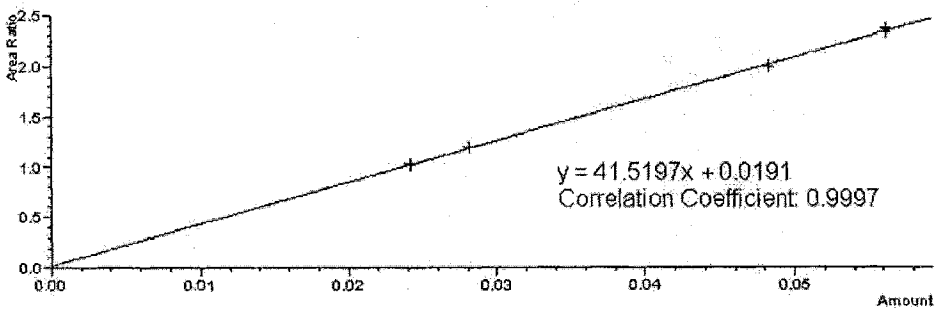

Figure 4 – Vehicle/Calibration Standard Curve for 1/24/2005 Analysis

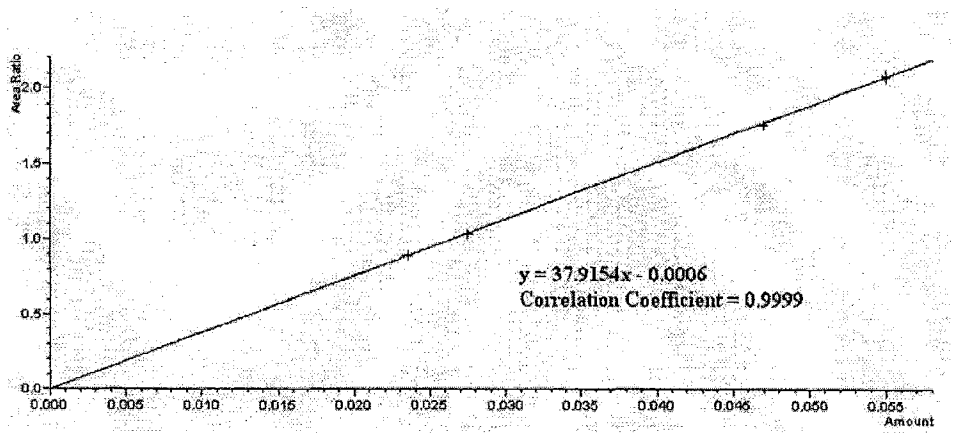

Figure 5 – Vehicle/Calibration Standard Curve for 6/30/2005 Analysis

The precision and accuracy results from a representative vehicle/calibration standard of January 24, 2005 analysis is shown in Table 3.

Table 3 –Vehicle/Calibration Standard 1/24/2005 Analysis Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	%RSD	%RE	Avg %RE
	55.75				-0.7	
56.13	56.44	56.28	0.00047	0.8	0.6	0.3
	56.66				0.9	
48.32	47.71	NA	NA	NA	-1.3	NA
28.06	28.08	NA	NA	NA	0.1	NA
	24.26				0.4	
24.16	24.13	24.20	0.000067	0.3	-0.1	0.2
	24.22				0.2	

The results of the formulation and formulation stability sample analysis are shown in Table 4 and 5. The formulation stability sample was the same formulation sample prepared and analyzed on January 24, 2005 that had been stored refrigerated for 59 days, protected from light in an amber glass bottle.

The results of the formulation analysis met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 4 – Formulation Analysis Results

Batch	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1-AG-1	23.03	22.99	22.81	22.94	-1.1	0.5
2-AG-1	22.48	22.00	22.34	22.27	-4.0	1.1

The formulation stability sample analyzed on 3/24/2005 was within -7.8% of the Day 0 value (1/24/2005 analysis value) and met acceptance criteria ± 10 %.

Table 5 –Formulation Stability Analysis 3/24/2005 Results

Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % of Day 0 Conc ± s
21.14	21.14	21.16	21.15	92.2 ± 0.1

4.7 Conclusions

The average concentration of the stock formulation and its percent relative standard deviation were within acceptance criteria. Therefore, the formulation was suitable for use.

The aminoglutethimide formulation at a target concentration of 23.2 mg/mL (0.1M) in DMSO was stable for 59 days when stored refrigerated and protected from light.

5 ACKNOWLEDGMENTS

Analytical support for this work was provided by Sandy Runyon. The report was written by Louise Baughman and Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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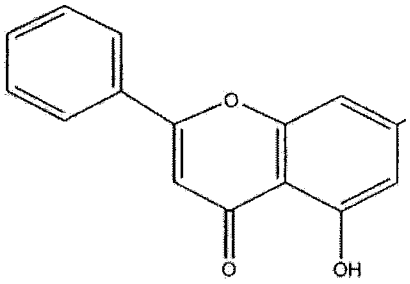
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

CHRYSIN

CAS No.: 480-40-0	Lot No.: 10101DC (Sigma Aldrich)
Receipt Date: 10/26/04	Amount Received: 25 g
Appearance: Solid	Vendor Purity: 98.20% by HPLC
Storage Conditions (@ Battelle): Room temperature (~25°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	254.24 g/mol	$C_{15}H_{10}O_4$

Prepared By:

Denise A. Contos, M.S. 12/22/05

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Test substance receipt	10/26/04	10/26/04
Dispensing*	12/2/04	12/2/04
Formulation analysis*	12/2/04	12/2/04
Formulation preparation*	12/2/04	12/2/04
Audit analytical report	8/9/05	8/9/05
Audit study file	8/9/05	8/9/05
Audit analytical report	12/14/05	12/14/05
Audit study file	12/14/05	12/14/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — Date: 12-22-05

Battelle

EXECUTIVE SUMMARY

The title compound, chrysin (CHRY), was analyzed in support of the EPA Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

The solubility of CHRY was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze CHRY in DMSO at a target concentration of 2.54 mg/mL (0.01 M). This method was used to analyze samples from both the formulation and formulation storage stability studies.

The storage stability study indicated that a 2.54 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 100 days at approximately 5°C.

The stock formulations prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom) 9

1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for Chrysin (CHRY) on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO)
- Developing and validating a formulation analysis method
- Conducting a storage stability study
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 60-mL amber glass bottle of CHRY, 10101DC, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 26, 2004. The label amount indicated 25 grams was sent. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1, which states that purity was 98.20% based on high performance liquid chromatography (HPLC).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Chrysin
Product Number	C8,010-5
Product Brand	ALDRICH
CAS Number	480-40-0
Molecular Formula	$C_{15}H_{10}O_4$
Molecular Weight	254.24

TEST	SPECIFICATION	LOT 10101DC RESULTS
APPEARANCE	YELLOW TO YELLOW GREEN TO TAN POWDER	YELLOW POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD.
UV-VISIBLE SPECTRUM	C IN 0.1N NAOH	0.01 G/L,0.01N NAOH
	E(348 +/- 2NM) = > 8,000	E348= 8,500
	E(282 +/- 2NM) = >22,000	E282=23,400
	E(263 +/- 2NM) = >20,000	E264=20,400
	E(224 +/- 2NM) = >27,000	E224=28,000
MISCELLANEOUS ASSAYS	97.0% - 103.0% (WITH TBAH)	101.4 % (WITH TBAH)
TITRATION	97.0% - 103.0% (WITH NAOH)	99.3 % (WITH NAOH)
HIGH PRESSURE LIQUID CHROMATOGRAPHY	CONSISTENT WITH CONTROL	98.20 %
SOLUBILITY	50 MG/ML PYRIDINE; CLEAR TO SLIGHT HAZY,	5%, PYRIDINE; CLEAR, YELLOW SOLUTION
QUALITY CONTROL ACCEPTANCE DATE		APRIL, 2004

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of CHRY in 100% DMSO, at a concentration of at least 2.54 mg/mL. CHRY (0.50848 ± 0.05085 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the CHRY dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well. The CHRY went readily into solution. Although the solution was prepared at approximately 50 mg/mL, higher than the target concentration, CHRY was readily soluble and would therefore, be soluble at the target concentration 2.54 mg/mL. This experiment showed that DMSO was an acceptable solvent for the 2.54 mg/mL formulation (0.01M).

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of CHRY in DMSO at a target concentration of 2.54 mg/mL (0.01 M) for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time and peak shape. The detection method chosen was HPLC with ultraviolet (UV) detection with the wavelength set at the absorbance maximum above 270 nm.

4.2 Method

The HPLC parameters for CHRY are presented in Table 1.

Table 1 – HPLC System

Instrument System	Waters (Milford, MA) and Agilent (Palo Alto, CA)
Column	Supelcosil LC-ABZ, 5 µm particle size, 150 mm × 4.6 mm (ID) (Supelco, Bellefonte, PA)
Mobile Phase	70:30 (v/v) Methanol:0.15% Ammonium Acetate, Isocratic
Flow Rate	1.0 mL/minute
Injection Volume	10 µL
Detector Type	UV
Detector Wavelength	270 nm
Run Time	~15 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and quantitation (LOQ). Triplicate vehicle/calibration blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Mobile Phase

A 0.15% ammonium acetate solution was prepared by weighing approximately 1.5 grams of ammonium acetate into a 1-L volumetric flask. The content of the flask was diluted to volume with Milli-Q water, sealed, and mixed well.

The mobile phase was prepared by mixing 700 mL of methanol and 300 mL of 0.15% ammonium acetate.

4.3.2 Preparation of Standards and Blanks

4.3.2.1 Internal Standard (IS)

Fifty (50) ± 2 mg of terconazole was added to a 25-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.2.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 25 ± 1 mg of CHRY each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 1000 µg/mL each.

4.3.2.3 Working Standards

Working standards were prepared as shown in Table 2. The content of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. One standard was prepared for each concentration.

Table 2 – Preparation of Working Standards

Working Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Final Volume (mL)
WS1	500	A	5	10
WS2	400	B	4	10
WS3	200	A	2	10
WS4	100	B	1	10

4.3.2.4 Vehicle/Calibration Standards

These standards were prepared in as shown in Table 3. The content of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 3 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Internal Std (mL)	DMSO (mL)	Final Volume (mL)
VS1	50	WS1	0.5	1	0.1	5
VS2	40	WS2	0.5	1	0.1	5
VS3	20	WS3	0.5	1	0.1	5
VS4	10	WS4	0.5	1	0.1	5

4.3.2.5 Triplicate Blanks without and with IS

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 5-mL volumetric flasks and adding 1 mL of methanol to each. The content of the flasks was diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 5-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.3.3 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autosampler vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.4 Calculations

The integration of the CHRY and IS peaks by the chromatography data system were evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of chrysin divided by the IS (y) to the concentration of the vehicle/ calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.5 Results

Specificity is shown by representative overlaid chromatograms from high and low vehicle/calibration standards, blank with IS, and a blank from the method validation are presented in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the CHRY or IS peaks.

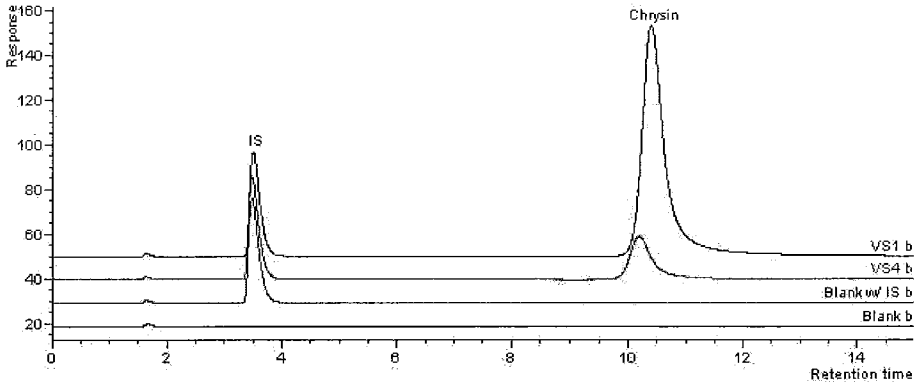

Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

The regression analysis results from the standard curve are linear and are shown in Table 4.

Table 4 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.1034	-0.0463	0.9999	0.0219

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 5.

Table 5 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	%RSD	Avg %RE
50.08	50.06	50.06	0.36	0.7	0.0	0.0
	49.70				-0.8	
	50.42				0.7	
40.14	40.20	NA	NA	NA	0.1	NA
20.03	20.08	NA	NA	NA	0.2	NA
10.04	10.09	10.02	0.06	0.6	0.5	-0.1
	10.00				-0.4	
	9.978				-0.6	

For the method validation the LOD is 0.18 µg/mL and is defined as three times the standard deviation of the lowest vehicle/calibration standard. This is equivalent to a formulation concentration of 18 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ is 0.59 µg/mL and is defined as ten times the standard deviation of the lowest vehicle/calibration standard because there was no blank response. This is equivalent to a

formulation concentration of 59 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest vehicle/calibration standard with acceptable accuracy and precision, was 10.04 µg/mL.

4.3.6 Conclusions

The method met all acceptance criteria for precision, accuracy, linearity, sensitivity, and specificity. The method was suitable for the stability study and subsequent formulation analyses for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a concentration of 2.54 mg/mL in DMSO for 100 days (approximately 14 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A single sample was analyzed on the day of preparation (Day 0), Days 14, 71, and 100. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on Day 0 of the storage stability study at a target concentration of 2.54 mg/mL in DMSO by accurately weighing 63.5 ± 6 mg into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with DMSO. The flask was sealed and manually shaken to mix the contents. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four 8-mL amber glass vials that were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After the desired storage period, a vial was removed from storage, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.2) of this report.

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One-half (0.5) mL of the diluted formulation and 1-mL of the IS were pipetted into individual 5-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. An appropriate volume of each was transferred to an autosampler vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 6 and presented in control chart format in Figure 3.

Table 6 – Formulation Storage Stability Results (2.54 mg/mL)

Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Avg Conc ± s
0	2.475	2.454	2.454	2.461 ± 0.012	100.0 ± 0.5
14	2.582	2.521	2.563	2.555 ± 0.031	103.8 ± 1.3
71	2.529	2.553	2.502	2.528 ± 0.025	102.7 ± 1.0
100	2.448	2.447	2.427	2.441 ± 0.012	99.2 ± 0.5

The pooled RSD of the analytical method was 0.987%. This means that there would have to be a difference of more than 2.24% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

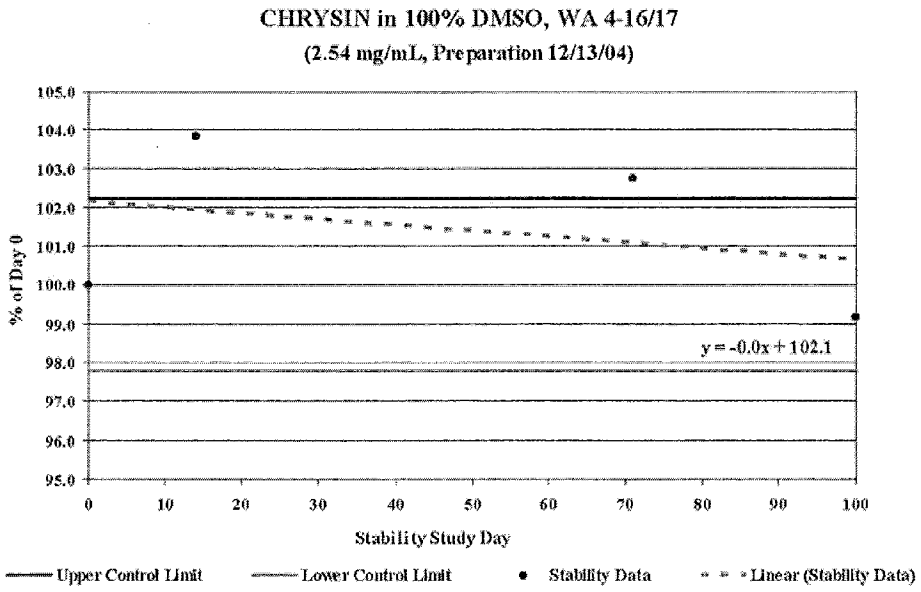

Figure 3 – Control Chart for Storage Stability Analysis

5.5 Discussion and Conclusions

The Day 0 determined value for the formulation was approximately 3.6% below nominal (the calculated concentration based on the weight of the chemical). There was no statistical difference between the Day 0 and Day 100 samples. However, Days 14 and 71 were above the upper significance level due to the tight precision of the assay but were within 3.8% and 2.7%, respectively, of the Day 0 value. These data indicate that the formulation was stable when protected from light at approximately 5°C for 100 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on January 25, 2005 and August 9, 2005 according to SOP COMSPEC.II-028-00, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Chrysin (CHRY) in 100% Dimethylsulfoxide (DMSO)." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

CHRY (127.00 ± 5.00 mg) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the CHRY dissolved. The content of the flask was diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.2 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One-half (0.5) mL of the diluted formulation and 0.5 mL of the IS were pipetted into individual 5-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

6.4 Analysis

Autosampler vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the HPLC conditions from the validation (Table 1). Representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with IS, and a blank are shown in Figure 4.

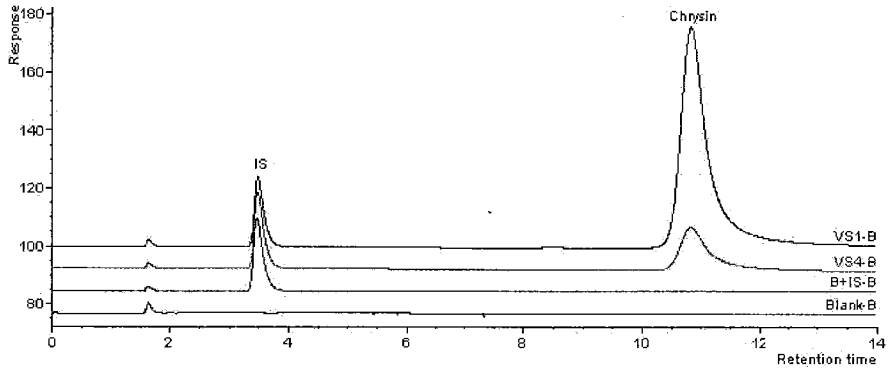

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

The peaks for CHRY and the IS were integrated for each injection by the chromatography data system. Any peak with inconsistent integration was manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio (CHRY/IS) (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation(s), and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

The results of the formulation analysis are shown in Table 7. The results of the standard curve regression analysis are shown in Table 8. The formulation met acceptance criteria (RE within 10% of target and RSD of $\leq 10\%$).

Table 7 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1-CHRY-1	1/25/05	2.592	2.409	2.395	2.466	-2.9	4.5
2-CHRY-1	8/9/05	2.479	2.531	2.487	2.499	-1.6	1.1

Table 8 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient
0.1852	-0.3622	0.9999
0.0970	-0.0921	0.9997

6.7 Conclusions

The average concentration of the stock formulations and its percent RSDs were within acceptance criteria. Therefore, the formulations were suitable for use.

7 ACKNOWLEDGMENTS.

Analytical support for this work was provided by Melinda Pauff, Sandy Runyon, Tudor Fernando, and Ron Haney. The report was written by Louise Glazier and Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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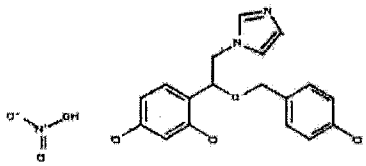
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

ECONAZOLE

CAS No.: 24169-02-6	Lot No.: 123K1220 (Sigma Aldrich)
Receipt Date: 10/26/04 and 12/4/04	Amount Received: 10 g
Appearance: Solid/White Powder	Vendor Purity: 98% by TLC
Storage Conditions (@ Battelle): Room temperature (~25°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	444.70 g/mol	$C_{18}H_{15}Cl_3N_2O \cdot HNO_3$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit study file	1/3/2006	1/3/2006
Audit analytical report	1/3/2006	1/3/2006

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 1-5-06 Date

EXECUTIVE SUMMARY

The title compound, econazole, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

The solubility of econazole was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze econazole in DMSO at a concentration of 44.47 mg/mL (0.1 M). This method was used to analyze samples from both formulation and formulation storage stability studies at 44.47 mg/mL.

The storage stability study indicated that a 44.47 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 56 days at approximately 5°C.

The stock formulations prepared for shipment to the testing laboratory were determined to meet the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for econazole on the Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

Two 15-mL amber glass bottles of econazole, Lot No. 123K1220, were received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA (one each on October 26, 2004 and December 4, 2004). The label amount indicated 5 grams was sent at each shipment. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 98% based on thin layer chromatography.

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Econazole nitrate salt,
Product Number	E4632
Product Brand	SIGMA
CAS Number	24169-02-6
Molecular Formula	$C_{18}H_{15}Cl_3N_2O \cdot HNO_3$
Molecular Weight	444.70

TEST	SPECIFICATION	LOT 123K1220 RESULTS
APPEARANCE	WHITE TO OFF WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION WITH SOME INSOLUBLES AT 25MG/ML IN CHLOROFORM:METHANOL (1:1)	CLEAR COLORLESS
WATER BY KARL FISCHER	NMT 0.5%	0.0%
ELEMENTAL ANALYSIS	47.6 TO 49.6% CARBON	48.6%
	9.2 TO 9.8% NITROGEN	9.4%
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS (SUPPLIER DATA)
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 98%	98%
QC ACCEPTANCE DATE		JANUARY 2004

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of econazole in 100% DMSO, at a concentration of at least 44.47 mg/mL (0.1 M). Econazole (0.44470 ± 0.04447 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the econazole dissolved. The contents of flask were diluted to volume with DMSO, sealed, and mixed well. The econazole went readily into solution. This experiment showed that DMSO was an acceptable solvent for the 44.47 mg/mL formulation.

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of econazole in DMSO at a target concentration of 44.47 mg/mL for the stability study, the results and the conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time and peak shape. The detection method chosen was high pressure liquid chromatography with ultraviolet detection (HPLC/UV) with the wavelength set at the absorbance maximum of 271 nm.

4.2 Method

The HPLC parameters for econazole are shown in Table 1.

Table 1 – HPLC System

Instrument System	Agilent (Palo Alto, CA), Waters (Milford, MA)
Column	Supelcosil LC-ABZ, 150 mm × 4.6 mm (ID) (Supelco, Bellefonte, PA)
Guard Column	C-18 guard column
Mobile Phase	70:30 (v/v) Methanol:0.15% Ammonium Acetate, Isocratic
Flow Rate	1.0 mL/minute
Injection Volume	50 µL
Detector Type	UV
Detector Wavelength	271 nm
Run Time	~20 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of

detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle/calibration blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Mobile Phase

A 0.15% ammonium acetate solution was prepared by weighing approximately 1.5 grams of ammonium acetate into a 1-L volumetric flask. The flask was diluted to volume with Milli-Q water, sealed, and mixed well.

The mobile phase was prepared by mixing 700 mL of methanol and 300 mL of 0.15% ammonium acetate.

4.3.2 Preparation of Standards and Blanks

4.3.2.1 Internal Standard (IS)

An IS solution was prepared by weighing 100 ± 4 mg of terconazole into a 50-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.2.2 Stock Standards

Two stock standards (A and B) were prepared by accurately weighing 25 ± 1 mg of econazole each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with HPLC mobile phase. This produced stocks A and B with target concentrations of 1 mg/mL each.

4.3.2.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/ Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	IS (mL)	DMSO (mL)	Final Volume (mL)
VS1	500	A	5	1	0.1	10
VS2	400	B	4	1	0.1	10
VS3	200	A	2	1	0.1	10
VS4	100	B	1	1	0.1	10

4.3.2.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.3.3 Analysis

A portion of each vehicle/calibration standard, blank and sample were transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.4 Calculations

The integration of the econazole and IS peaks by the chromatography data system was evaluated to assure it was correct in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of econazole divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.5 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank from the validation as indicated in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the econazole or IS peaks. The regression analysis results from the standard curve indicate linearity and are shown in Table 3.

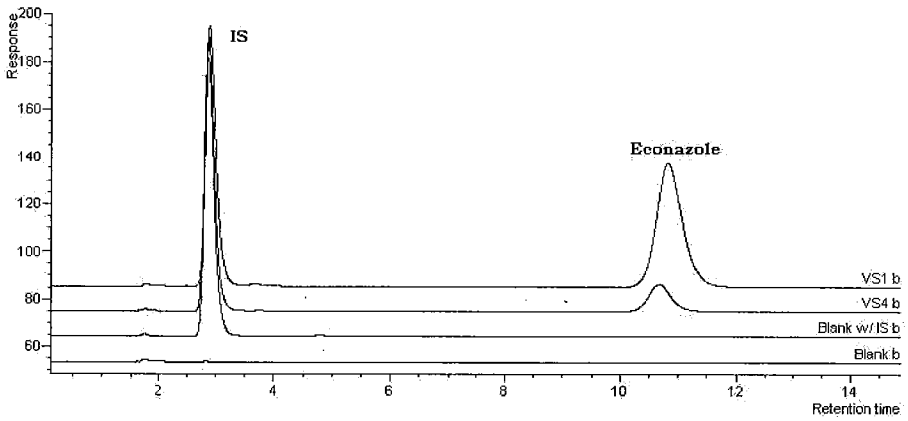


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0023	0.0088	1.000	0.0021

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	% RE	Avg % RE
512.6	513.2	512.5	0.8	0.1	0.1	0.0
	512.7				0.0	
	511.7				-0.2	
406.9	406.7	NA	NA	NA	0.0	NA
205.0	206.8	NA	NA	NA	0.9	NA
101.7	101.3	101.3	0.1	0.1	-0.4	-0.4
	101.2				-0.5	
	101.3				-0.4	

The method validation sensitivity was 0.1732 µg/mL, the LOD, which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 20 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 0.5774

µg/mL. This is equivalent to a formulation concentration of 60 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest vehicle/calibration standard with acceptable accuracy and precision, was 101.7 µg/mL.

4.3.6 Conclusions

The method met all acceptance criteria for specificity, linearity, precision, accuracy and sensitivity. The method was suitable for the stability study and subsequent formulation analysis for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 44.47 mg/mL in DMSO for 56 days (8 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A formulation sample was analyzed on the day of preparation (Day 0) and Day 14. A second formulation sample was analyzed on the day of preparation (Day 0), Week 4 and Week 8. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on November 19, 2004, Day 0 of the storage stability study at a target concentration of 44.47 mg/mL in DMSO by accurately weighing 1110 ± 10 mg into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with DMSO. The flask was sealed and sonicated for approximately 5 minutes to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After 14 days of storage, a vial was removed from the refrigerator, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation was prepared on February 7, 2005, Day 0, at a target concentration of 44.47 mg/mL in DMSO by accurately weighing 2.22350 ± 0.08894 g into a 50-mL volumetric flask. The flask was diluted to approximately 80% volume with DMSO, sealed and sonicated for approximately 5 minutes. The content of the flask was inverted 10 times, and diluted to volume with DMSO and shaken to mix well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Day 0, Weeks 4 and 8 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.2) of this report.

In triplicate, 1 mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

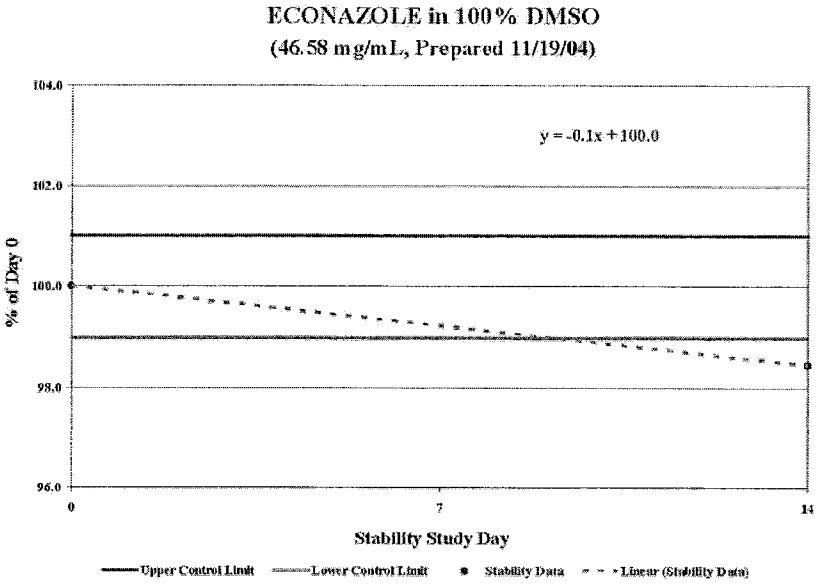
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (44.47 mg/mL)

Preparation Date	Analysis Date	Day	Def'd Conc (mg/mL)			Avg Def'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
11/19/04	11/19/04	0	46.91	46.37	46.47	46.58 ± 0.29	100.0 ± 0.6
11/19/04	12/3/04	14	45.91	45.88	45.80	45.86 ± 0.06	98.5 ± 0.1
2/7/05	2/7/05	0	46.24	46.08	45.89	46.07 ± 0.17	100.0 ± 0.4
2/7/05	3/7/05	28	45.03	44.74	44.66	44.81 ± 0.19	97.3 ± 0.4
2/7/05	4/4/05	56	42.95	42.92	41.94	42.60 ± 0.58	92.5 ± 1.3

For the formulation stability sample prepared on November 19, 2004, the pooled RSD of the analytical method was 0.44%. This means that there would have to be a difference of more than 1.01% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation stability sample prepared on February 7, 2005, the pooled RSD of the analytical method was 0.79%. This means that there would have to be a difference of more than 1.80% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.



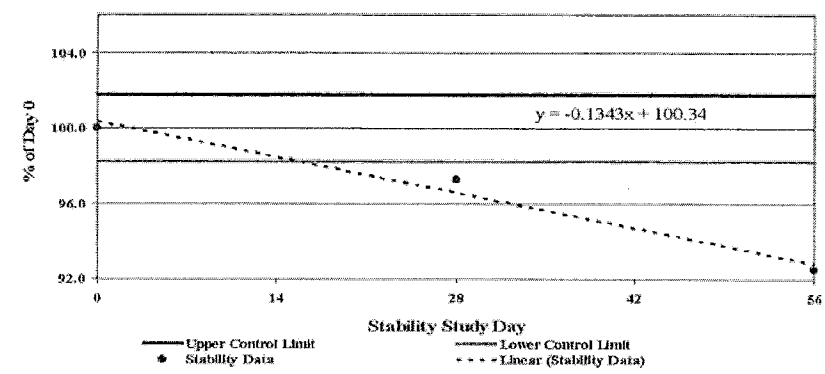


Figure 3 – Control Charts for the Storage Stability Analysis

5.5 Discussion and Conclusions

The Day 0 determined value for the November 19, 2004 formulation was approximately 4.7% above the nominal value (the calculated concentration based on the weight of the chemical). The concentration of the sample stored at approximately 5°C protected from light in amber glass vial for Day 14 was below the lower significance level due to the tight precision of the assay but was within 1.5% of the Day 0 value (November 19, 2004) and met acceptance criteria ± 10%.

The Day 0 determined value for the February 7, 2005 formulation was approximately 3.5% above the nominal value (the calculated concentration based on the weight of the chemical). The concentrations of the sample stored at approximately 5°C protected from light in an amber glass vial for Days 28 and 56 was below the lower significance level due to the tight precision of the assay but was within 2.7 and 7.5%, respectively of the Day 0 value (February 7, 2005) and met acceptance criteria ± 10%.

These data indicate the formulation was stable when protected from light at approximately 5°C for 56 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on February 7, 2005 and August 8, 2005 according to SOP COMSPEC.II-031, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Econazole in 100% DMSO." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Econazole (2.22350 ± 0.08894 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were sonicated for approximately 5 minutes until the econazole dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.2 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One (1) mL of the diluted formulation and 1 mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

6.4 Analysis

Autoinjector vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the HPLC conditions from the validation (Table 1).

6.5 Calculations

The integration of the econazole and IS peaks by the chromatography data system was evaluated to assure it was correct in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of econazole divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. The percent RE for each vehicle/calibration standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation (s), and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

Specificity is shown by the representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with working IS and a blank are presented in Figure 4.

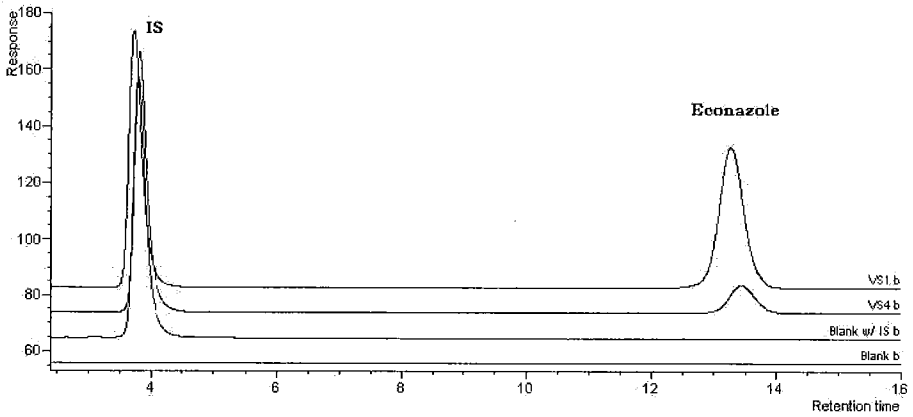


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

The vehicle/calibration standard curve and the results of the regression analysis indicated linearity and are shown in Figure 5.

The results of the formulation analysis are shown in Table 7. Formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 7 – Formulation Analysis Results

Formulation Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1/24/05	30.02	29.88	29.93	29.95	3.0	0.2
3/21/05	29.23	29.67	29.20	29.37	1.0	0.9
7/1/05	29.32	29.26	29.63	29.40	1.1	0.7

6.7 Conclusions

The average concentration of the formulations and its percent RSD were within acceptance criteria. Therefore the formulation was suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Barb Harritos, Darren Brown, John Kelly, Christina Zielinski, Jim Hoskinson, Melinda Pauff, Tudor Fernando, and Sandy Runyon. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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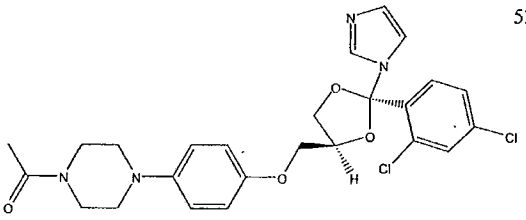
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

KETOCONAZOLE

CAS No.: 65277-42-1
Receipt Date: 10/26/04
Appearance: Solid
Storage Conditions (@ Battelle): Refrigerated (~5°C)

Lot No.: 121H0524 (Sigma Aldrich)
Amount Received: 2.7 g
Vendor Purity: > 99% by TLC

STRUCTURE:



Mol. Wt.: 531.43 g/mol

Mol. Formula: $C_{26}H_{28}Cl_2N_4O_4$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit study file	8/23/2005	8/23/2005
Audit analytical report	8/23/2005	8/23/2005
Audit study file	12/16/2005	12/16/2005
Audit analytical report	12/16/2005	12/16/2005

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 12-27-05 Date

EXECUTIVE SUMMARY

The title compound, ketoconazole, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

Solubility of ketoconazole was determined to be acceptable in dimethylsulfoxide (DMSO) at a concentration of 5.31 mg/mL (0.01 M).

A ketoconazole formulation analysis method was validated on the previous EPA WA 3-10 study. The method was modified by including an additional 1:10 dilution of the formulation which resulted in a 1:200 final dilution of the 5.31 mg/mL (0.01 M) formulation prior to analysis. This modified method was used to analyze both stability and formulation analysis samples.

Storage stability was previously determined (EPA WA 3-10 study, Analytical Chemistry Activities Report, Ketoconazole, 2004) as 28 days when stored at approximately 5°C and protected from light at a target formulation concentration of 0.532 mg/mL in DMSO. In the current study, a formulation sample with a target concentration of 5.31 mg/mL in DMSO was stable when stored refrigerated and protected from light for 60 days.

The stock formulations prepared for shipment to the testing laboratory were analyzed and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for ketoconazole on the Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Preparing and analyzing a stock formulation and a formulation stability sample.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle of ketoconazole, Lot No. 121H0524, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 26, 2004. The label amount indicated 2.7 grams was sent. The chemical was received and subsequently stored refrigerated.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was greater than 99% based on thin layer chromatography.

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Ketoconazole
Product Number	K1003
Product Brand	SIGMA
CAS Number	65277-42-1
Molecular Formula	$C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight	531.43

TEST	SPECIFICATION	LOT 121H0524 RESULTS
APPEARANCE	WHITE TO YELLOW WITH A LIGHT TAN CAST POWDER	WHITE POWDER WITH A LIGHT YELLOW CAST
SOLUBILITY	CLEAR FAINT YELLOW TO YELLOW SOLUTION AT 50MG/ML IN METHANOL	CLEAR FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF METHANOL
ELEMENTAL ANALYSIS	57.6 TO 59.9% CARBON	58.6% CARBON 10.5% NITROGEN
SPECIFIC ROTATION	+1 TO -1 DEG (C=4 IN METHANOL AT 20DEGCENTIGRADE)	+0.08 DEG (C = 3.8 IN METHANOL AT 20 DEG CENTIGRADE)
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 98%	GREATER THAN 99%
SHELF LIFE	3 YEARS	MARCH 2005
QC ACCEPTANCE DATE		MARCH 2004

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 - Certificate of Analysis

3 SOLUBILITY STUDY

A solubility study was conducted to determine the solubility of ketoconazole in either 95% ethanol or 100% DMSO. Initially, both 95% ethanol and DMSO were used to prepare a 53.14 mg/mL (0.1 M) ketoconazole solution. Neither of the solvents dissolved the ketoconazole at the 0.1 M concentration. At the direction of the Task Leader, a 5.31 mg/mL ketoconazole solution (0.01 M) in 100% DMSO was prepared by weighing 0.05314 ± 0.00531 g into a

10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was capped and contents were mixed. The content of the flask was diluted to volume with DMSO, sealed, mixed and sonicated. The ketoconazole went into solution with minimal shaking and sonication. This experiment showed that DMSO was an acceptable solvent for a 5.31 mg/mL (0.01 M) formulation.

4 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on February 4, 2005 and June 29, 2005 according to SOP COMSPEC.II-018-02, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Ketoconazole in 100% DMSO." In addition, the February 4, 2005 formulation was re-analyzed to determine stability on April 5, 2005, 60 days after storage at approximately 5°C and protected from light. This section describes the method, results, and conclusions.

4.1 Preparation of Formulation

A ketoconazole formulation with a target concentration of 5.31 mg/mL (0.01 M) in DMSO was prepared on February 4, 2005 by accurately weighing 265.5 ± 5 mg of ketoconazole into a tarred 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and sonicated for approximately 10 minutes then inverted ten times. The content of the flask was diluted to volume with DMSO, sealed, and mixed well by inverting at least ten times.

4.2 Preparation of High Performance Liquid Chromatography (HPLC) Mobile Phase

An accurate amount of ammonium acetate (3.0 g) was weighed into a 2000-mL HPLC mobile phase bottle. A 600 mL volume of Milli-Q water was added to the bottle and the contents were mixed well. A final volume of 1400 mL of methanol and 2.8 mL of diethanolamine were added to the bottle and the contents were mixed well. This produced a HPLC mobile phase containing 70:30:0.14 (v:v:v) of methanol:0.5% ammonium acetate: diethanolamine.

4.3 Preparation of Standards and Blanks

4.3.1 Internal Standard (IS)

Fifteen (15) ± 2 mg terconazole was added to a 50-mL volumetric flask. The content of the flask was diluted to volume with HPLC mobile phase, sealed, and mixed well. This produced a solution with a target concentration of 300 µg/mL.

4.3.2 Stock Standards

Two stock standards were prepared by accurately weighing 30 ± 3.0 mg of ketoconazole into two individual 200-mL volumetric flasks and dissolving in and diluting to volume with HPLC mobile phase. This produced stocks A and B with target concentrations of 150 µg/mL each.

4.3.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 1. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 1 – Preparation of Vehicle/Calibration Standards

Vehicle Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Internal Std (mL)	DMSO (mL)	Final Volume (mL)
VS1	60	A	4	1	0.05	10
VS2	45	B	3	1	0.05	10
VS3	30	A	2	1	0.05	10
VS4	15	B	1	1	0.05	10

4.3.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.05 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.05 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.4 Preparation of Formulation and Formulation Stability Samples

Triplicate 1 mL aliquots of the formulation were pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. A 0.5 mL aliquot of the diluted formulation and 1 mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.5 Analysis

A portion of each vehicle/calibration standard, blank and sample were transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the HPLC parameters for ketoconazole which are presented in Table 2.

Table 2 – HPLC System

Instrument System	Agilent (Palo Alto, CA); Waters (Milford, MA)
Column	Supelcosil LC-ABZ, 5 µm particle size, 250 mm × 4.6 mm (ID) (Supelco, St. Louis, MO)
Guard	C_{18} guard cartridge
Mobile Phase	70:30:0.14 (v/v/v) Methanol:0.5% Ammonium Acetate:Diethanolamine, Isocratic
Flow Rate	1.5 mL/minute
Injection Volume	20 µL
Detector Type	Ultraviolet (UV)
Detector Wavelength	245 nm

4.6 Calculations

The integration of the ketoconazole and the IS peaks by the chromatography data system were evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation weighted 1/x was calculated relating the response ratio of ketoconazole/IS (y) to the concentration of the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each vehicle/calibration standard and formulation sample. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.7 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/ calibration standard, a blank with IS, and a blank as presented in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the ketoconazole or IS peaks.

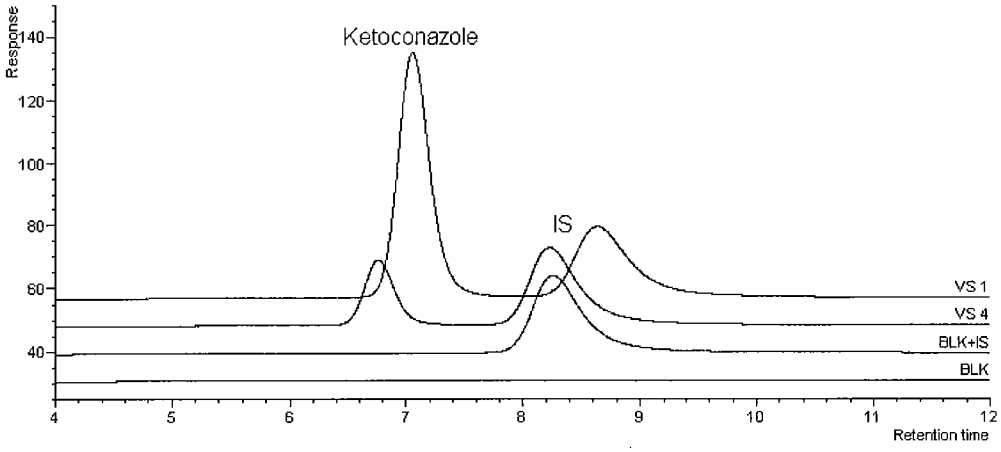

Figure 2 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from 2/4/2005 Analysis (Shown Top to Bottom)

The regression analysis results from the standard curve for February 4, 2005 analysis indicate linearity and are shown in Figure 3.

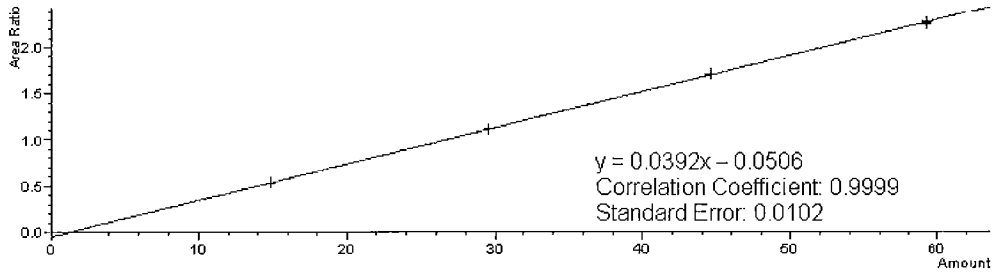

Figure 3 – Vehicle/Calibration Standard Curve for 2/4/2005 Analysis

The precision and accuracy of the vehicle/calibration standard results from February 4, 2005 analysis are shown in Table 3.

Table 3 –Vehicle/Calibration Standard Results for 2/4/2005 Analysis

Nominal Std Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s (µg/mL)	% RSD	% RE	Avg % RE
59.20	59.15	59.08	0.30	0.5	-0.1	-0.2
	59.33				0.2	
	58.75				-0.8	
44.60	44.98	NA	NA	NA	0.9	NA
29.60	29.64	NA	NA	NA	0.1	NA
14.87	14.69	14.85	0.14	0.9	-1.2	-0.1
	14.92				0.4	
	14.94				0.5	

The results of the formulation and formulation stability sample analysis are shown in Table 4 and 5. The formulation stability sample was the same formulation sample prepared and analyzed on February 4, 2005 that had been stored refrigerated for 60 days, protected from light in an amber glass bottle.

Table 4 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	RSD (%)
1-KET-1	2/4/2005	5.136	5.122	5.134	5.131	-3.4	0.1
2-KET-1	6/29/2005	5.458	5.487	5.464	5.470	3.0	0.3

The formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 5 –Formulation Stability Analysis Results

Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % of Day 0 Conc ± s
4/5/2005	5.316	5.359	5.369	5.348	104.2 ± 0.5

The formulation stability sample analyzed on April 5, 2005 was within 4.2% of the Day 0 value (February 4, 2005 analysis value) and met acceptance criteria + 10 %.

4.8 Conclusions

The average concentration of the stock formulation and its percent relative standard deviation were within acceptance criteria. Therefore the formulations were suitable for use.

The ketoconazole formulation at a target concentration of 5.31 mg/mL in DMSO was stable for 60 days when stored refrigerated and protected from light.

5 ACKNOWLEDGMENTS

Analytical support for this work was provided by Jim Hoskinson and Tudor Fernando. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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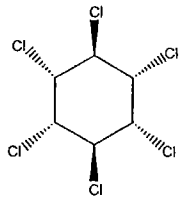
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

LINDANE

CAS No.: 58-89-9	Lot No.: 14419EB (Sigma Aldrich)
Receipt Date: 1/6/05	Amount Received: 10 g
Appearance: White Solid	Vendor Purity: 99.6% by GC
Storage Conditions (@ Battelle): Room temperature (~25°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	290.83 g/mol	$C_6H_6Cl_6$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

Battelle Study No. WA 4-16/17

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit analytical report	12/22/2005	12/22/2005
Audit study file	12/22/2005	12/22/2005

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Kathleen C. Reed 1-6-06

Quality Assurance Unit Date

EXECUTIVE SUMMARY

The title compound, lindane, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation Work, Work Assignment 4-16/17.

Solubility of lindane was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze lindane in DMSO at a concentration of 29.08 mg/mL (0.1M). This method was used to analyze samples from both formulation and formulation storage stability studies at 29.08 mg/mL.

Storage stability study indicated that a 29.08 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 168 days at approximately 5°C.

The formulations prepared for shipment to the testing laboratory were determined and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for lindane on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 20-mL amber glass bottle of lindane, 14419EB, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on January 6, 2005. The label amount indicated 10 grams was sent. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99.6% based on gas chromatography (GC).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Lindane
Product Number	23,339-0
Product Brand	ALDRICH
CAS Number	58-89-9
Molecular Formula	$C_6H_6Cl_6$
Molecular Weight	290.83

TEST	SPECIFICATION	LOT 14419EB RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD
GAS LIQUID CHROMATOGRAPHY	96.5% (MINIMUM)	99.6%
QUALITY CONTROL ACCEPTANCE DATE		MAY, 2003

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of lindane in 100% DMSO, at a concentration of at least 29.08 mg/mL. Lindane (0.29080 ± 0.02908 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well. The lindane went readily into solution. This experiment showed that DMSO was an acceptable solvent for the 29.08 mg/mL formulation.

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of lindane in DMSO at a target concentration of 29.08 mg/mL for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time for the major peak, apparent resolution of significant impurities and acceptable peak shape. The detection method chosen was gas chromatography with flame ionization detection (FID).

4.2 Method

The GC parameters for lindane are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5, 30 m × 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at ~2 mL/minute
Oven Temperature	150°C, hold for ~2 minutes, increase at 20°C/minute to 300°C; hold for 2 minutes
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at ~30 mL/minute; Air at ~380 mL/minute
Detector Temperature	320°C
Injector Temperature	285°C
Injection Volume	1 µL
Injection Mode	Split 5:1
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle/calibration blanks with and without working internal standard (WIS) were used to assess the specificity of the method.

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Approximately 25 ± 1 mg of phenanthrene was added to a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

The IS was prepared by pipetting 10 mL of stock IS into a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards were prepared by accurately weighing 50 ± 2 mg of lindane each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 2000 µg/mL each.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with methanol and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two middle concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	WIS (mL)	DMSO (mL)	Final Volume (mL)
VS1	800	A	4	1	0.1	10
VS2	600	B	3	1	0.1	10
VS3	400	A	2	1	0.1	10
VS4	200	B	1	1	0.1	10

4.3.1.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

4.3.2 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development as shown in Table 1.

4.3.3 Calculations

The integration of the lindane and IS peaks by the chromatography data system was evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of lindane divided by the IS (y) to the concentration of

the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.4 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank from the validation as indicated in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the lindane or IS peaks. The regression analysis results from the standard curve indicate the linearity and are shown in Table 3.

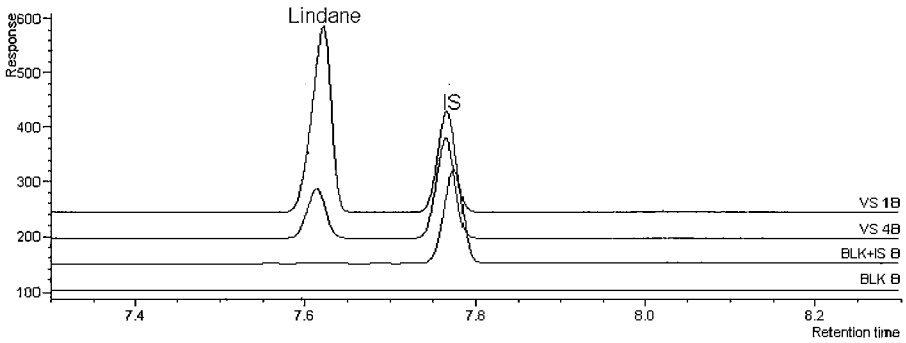


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Method Validation Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0027	-0.0351	1.000	0.0047

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	%RSD	%RE	Avg %RE
	777.3				0.1	
776.3	777.6	776.8	1.1	0.1	0.2	0.1
	775.6				-0.1	
600.2	598.4	NA	NA	NA	-0.3	NA
388.2	387.0	NA	NA	NA	-0.3	NA
	202.8				1.4	
200.1	200.1	200.5	2.1	1.1	0.0	0.2
	198.6				-0.7	

The sensitivity of the method resulted in 6.4 µg/mL LOD which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 640 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 21.3 µg/mL. This is equivalent to a formulation concentration of 2130 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 200.1 µg/mL.

4.3.5 Conclusions

The method met all acceptance criteria for specificity, linearity, precision, accuracy, and sensitivity. The method was suitable for the stability study and subsequent formulation analyses.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 29.08 mg/mL in DMSO for 168 days (24 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A single sample was analyzed on the day of preparation (Day 0), Day 14, Weeks 4, 8 and 12. A second formulation sample was prepared and analyzed on January 24, 2005 (Day 0) and on Week 24. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on January 13, 2005, Day 0 of the storage stability study at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 727 ± 7 mg of lindane into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with

DMSO. The flask was sealed and manually shaken to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four, 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After the desired storage period, a vial was removed from storage, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation (Batch 1-LIN-1) was prepared on January 24, 2005 (Day 0) at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 1.45400 ± 0.058 g into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with DMSO, sealed and mixed well. The contents of the flask was diluted to volume with DMSO and mixed well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0 and 168 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report.

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of IS were pipetted into 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (29.08 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
1/13/05	1/13/05	0	29.38	29.48	29.18	29.35 ± 0.15	100 ± 0.5
1/13/05	1/27/05	14	28.56	28.56	28.67	28.60 ± 0.06	97.4 ± 0.2
1/13/05	2/10/05	28	31.36	31.30	31.64	31.43 ± 0.18	107 ± 0.6
1/13/05	3/10/05	56	28.77	28.76	28.65	28.73 ± 0.07	97.9 ± 0.2
1/13/05	4/7/05	84	29.22	29.67	29.47	29.45 ± 0.23	100 ± 0.8
1/24/05	1/24/05	0	30.02	29.88	29.93	29.95 ± 0.07	100 ± 0.2
1/24/05	7/11/05	168	29.64	29.72	29.95	29.77 ± 0.16	99.4 ± 0.5

For the formulation sample prepared on January 13, 2005, the pooled relative standard deviation of the analytical method was 0.5%. This means that there would have to be a difference of more than 1.2% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation sample prepared on January 24, 2005, the pooled RSD of the analytical method was 0.6%. This means that there would have to be a difference of more than 1.3% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

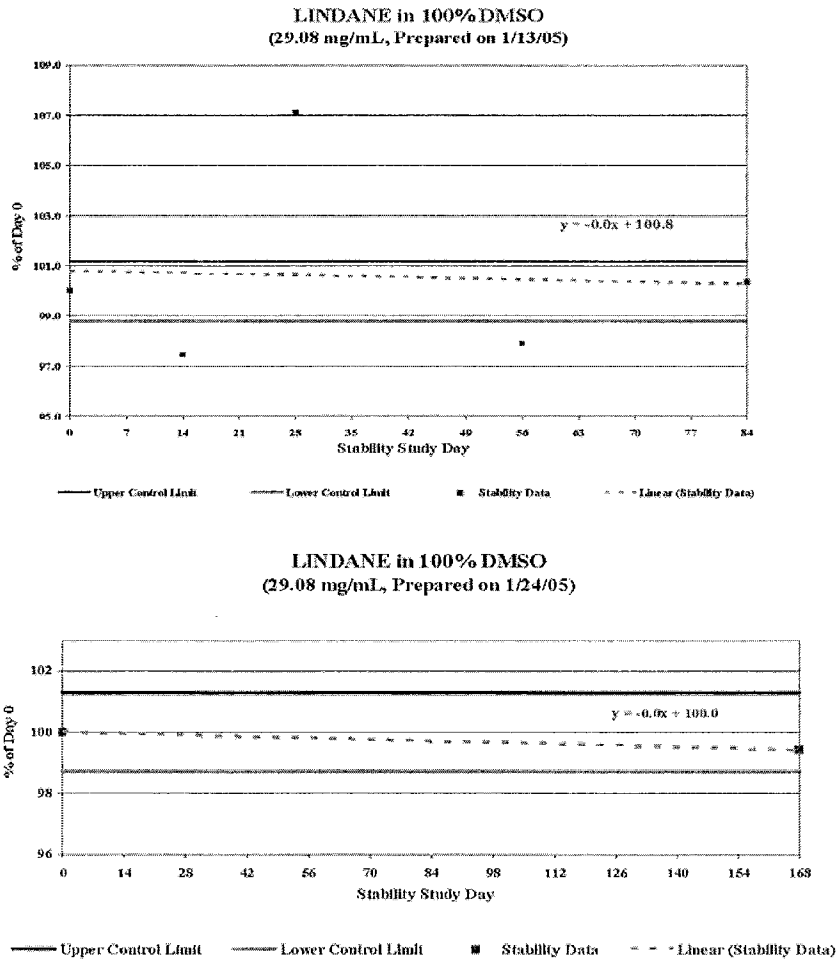


Figure 3 – Control Chart for the Storage Stability Study

5.5 Discussion and Conclusions

The Day 0 determined value for the formulation prepared on January 13, 2005 was approximately 1.0% above nominal (the calculated concentration based on the weight of the chemical). The concentrations of the samples stored at approximately 5°C protected from light in amber glass vials for Days 14 and 56 were below the lower significance level and for Day 28 it was above the upper significance level due to the tight precision of the assay. The average concentrations of the samples were within 2.6% (Day 14), 7.1% (Day 28), 2.1% (Day 56), and 0.4% (Day 84) of the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C for 84 days.

The formulation stability sample prepared on January 24, 2005 (Day 0) and analyzed on Day 0 and Day 168 (July 11, 2005) was approximately 3.0% above nominal for Day 0 (the calculated concentration based on the weight of the chemical) and for Day 168, 0.6% below the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C protected from light for 168 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on January 24, 2005, March 21, 2005 and July 1, 2005, according to SOP COMSPEC.II-029, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Lindane in 100% Dimethylsulfoxide (DMSO)." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Lindane (1.45400 ± 0.058 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.1 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

6.4 Analysis

Autosampler vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the GC conditions from the validation (Table 1). Representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with IS, and a blank are shown in Figure 4.

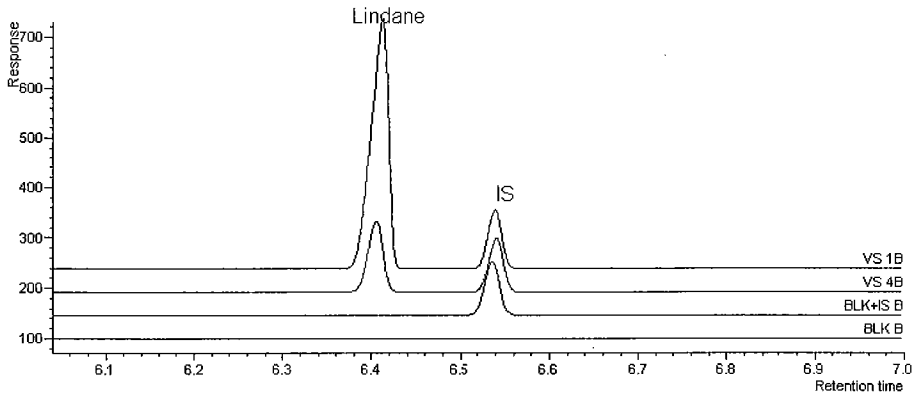


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

The peaks for lindane and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation was calculated relating the response ratio (lindane/IS) to the concentration of the vehicle/calibration standards. This regression equation and the response ratios were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

The regression analysis results of the vehicle/calibration standard curves indicated linearity and are shown in Table 6.

Table 6 – Formulation Regression Analysis Results

Formulation Date	Slope	y-Intercept	Correlation Coefficient
1/24/05	6.8029	-0.0081	1.000
3/21/05	7.2898	-0.0197	1.000
7/1/05	6.8477	-0.1022	1.000

The results of the formulation analysis are shown in Table 7. Formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 7 – Formulation Analysis Results

Formulation Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1/24/05	30.02	29.88	29.93	29.95	3.0	0.2
3/21/05	29.23	29.67	29.20	29.37	1.0	0.9
7/1/05	29.32	29.26	29.63	29.40	1.1	0.7

6.7 Conclusions

The average concentration of the formulations and its percent RSD were within acceptance criteria. Therefore the formulation was suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Barb Harritos, Darren Brown, John Kelly, Christina Zielinski, Jim Hoskinson, Melinda Pauff, Tudor Fernando, and Sandy Runyon. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

APPENDIX E

Spreadsheets – Include the Aromatase Activity Calculation Page of the Spreadsheet for Each Replicate. E-1

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0198	21418	1081717	
2	0.0195	21856	1120821	
3	0.0194	21642	1115567	
4	0.0196	23344	1191020	
5	0.0196	21838	1114184	
			Average DPM/g soln	1124662
			SD	40171
			CV	3.57
			µCi/g soln	0.507

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.6	10		1060.00
Dilution A			100	10.60
Dilution B			10	1.06

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0284 g
Mass of dilution B used in substrate prep	4.5145 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.596055 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00573 µg/g soln.
µg/g soln.

a. µCi/g soln 0.507
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.596055 + 0.00573
= 0.601790 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.842 µCi/µg ASDN

535242 dpm/nmol

Assay Date	2/2/2005	Test Chemical ID	1A	# Concentrations tested	8				
Technician ID	TD	Replicate #	1	Microsome type	placental	Microsome ID	11343-7		

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.14	Blank	25	20	210000238
	0.265	0.221	0.178	0.124	0.079	0.056	0.000			
	0.260	0.234	0.172	0.125	0.066	0.081	0.000			
	0.266	0.215	0.195	0.130	0.078	0.075	0.000			
Samples	11343-7									
	0.072									
	0.072									
	0.072									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{corr}	A_{std}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.270	0.270	0.0247	m, b	0.103	-0.003
0.8	14.3	25	0.00080	25	0.0200	0.223	0.223	0.0199	ss_m, ss_b	0.002	0.000
0.6	10.7	25	0.00060	25	0.0150	0.182	0.182	0.0157	r^2, ss_y	0.998	0.000
0.4	7.1	25	0.00040	25	0.0099	0.126	0.126	0.0100	F, df	1672	4
0.2	3.6	25	0.00020	25	0.0050	0.074	0.074	0.0046	ss_{reg}, ss_{resc}	0.000	0.000
0.14	2.5	25	0.00014	25	0.0035	0.064	0.064	0.0036			

Regression results are calculated using the function LINEST

Blank: 0.000

R^2= 0.998
m= 0.103
b= -0.003

	A_{corr}	A_{std}	mg protein measured	µL diluted µSOMES	Vol usome prep (µL)	Final vol Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL	
11343-7	0.072	0.072	0.004	25	100	5000	0.009	0.009	8.841	
11343-7	0.072	0.072	0.004	25	100	5000	0.009			
11343-7	0.072	0.072	0.004	25	100	5000	0.009			

Assay Date	2/2/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	8.841
Protein Concentration (dilution added to assay, mg/mL):	0.017682

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	1.00E-06
5	1.00E-07
6	1.00E-08
7	1.00E-09
8	1.00E-10

Assay Date	2/5/2005	Test Chemical ID	146	# Concentrations tested	6	% Methanol in final	placebo	Master comp ID	11343-7	Technician ID	TD	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % Extraction			Calculate nmol ^3H_2O formed					
Sample type	Replicate Level	Incubation time (hr)	Aliq Volume (mL)	Aliq #	DPM/Aliq 1	DPM/Aliq 2	Ave DPM/Aliq	Total DPM	Volume of substrate solution in scintillation tube (mL)	total DPM in assay tube (dpm)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation Time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3222.9	6449.8	6449.1	130186.2	0.1	112466	11.48	12731	0.6295	1	0.009	15	0.0697
			0.5	2	3226.7	6075.3			0.1					1		15	
	2	2	0.5	1	3223.7	6038.4	6049.1	130 389.2	0.1	112466	11.59	13211	0.6347	1	0.009	15	0.0691
			0.5	2	3558.4	6712.9			0.1					1		15	
	3	2	0.5	1	3267.2	6614.4	4073.3	87 449.8	0.1	112466	7.24	6054	0.6153	1	0.009	15	0.0659
			0.5	1	2066.1	4082.2			0.1					1		15	
	4	2	0.5	1	2061.7	4170.4	4119.6	88 229.8	0.1	112466	7.33	6152	0.6152	1	0.009	15	0.0674
			0.5	2	2058.1	4116.2			0.1					1		15	
Boiled blank control	1	2	0.5	1	24.47	47.94	45.39	96.13	0.1	112466	0.09	12	0.6090	1	0.009	15	0.0002
			0.5	2	20.09	42.99			0.1					1		15	
	2	2	0.5	1	17.66	34.05	37.28	74.52	0.1	112466	0.07	-10	0.6090	1	0.009	15	-0.0002
			0.5	2	20.21	40.44			0.1					1		15	
	3	2	0.5	1	21.51	41.23	48.27	96.54	0.1	112466	0.09	3	0.6090	1	0.009	15	0.0001
			0.5	1	27.75	43.57			0.1					1		15	
	4	2	0.5	1	24.19	48.39	39.96	79.92	0.1	112466	0.07	-8	0.6090	1	0.009	15	-0.0001
			0.5	2	15.8	31.6			0.1					1		15	
Positive control	1	2	0.5	1	1180.3	2357.5	2405.6	4811.2	0.1	112466	1.28	4734	0.6090	1	0.009	15	0.0123
			0.5	2	1206.3	2413.8			0.1					1		15	
	2	2	0.5	1	1182.3	2354.8	2376.1	4753.2	0.1	112466	4.23	4695	0.6087	1	0.009	15	0.0329
			0.5	2	1193.9	2397.4			0.1					1		15	
	3	2	0.5	1	1403.1	2804.2	2865.2	5973.4	0.1	112466	5.31	5883	0.6110	1	0.009	15	0.0414
			0.5	2	1525.1	3048.2			0.1					1		15	
	4	2	0.5	1	1470.4	2946.8	2896.6	5793.2	0.1	112466	5.16	5736	0.6107	1	0.009	15	0.0402
			0.5	2	1426.2	2852.4			0.1					1		15	
Negative Control	1	2	0.5	1	2918.5	5837.2	5931.9	118 638.6	0.1	112466	10.53	11725	0.6200	1	0.009	15	0.0829
			0.5	2	3012.7	6026.4			0.1					1		15	
	2	2	0.5	1	4561.1	9187.4	6588.6	131 772.2	0.1	112466	17.72	19690	0.6745	1	0.009	15	0.0923
			0.5	1	2987.4	4014.9			0.1					1		15	
	3	2	0.5	1	2953.9	4811.9	4103.6	82 072.3	0.1	112466	7.30	6129	0.6152	1	0.009	15	0.0572
			0.5	2	2047.7	4095.4			0.1					1		15	
	4	2	0.5	1	1924.5	3849.1	3965.1	7930.2	0.1	112466	7.06	7843	0.6147	1	0.009	15	0.0552
			0.5	1	1980.9	3961			0.1					1		15	
Blk	1-1	2	0.5	1	29.43	58.86	55.02	110.04	0.1	112466	0.10	25	0.6090	1	0.009	15	0.0002
			0.5	1	20.83	41.66			0.1					1		15	
	1-2	2	0.5	1	34.92	69.84	73.25	146.49	0.1	112466	0.13	59	0.6091	1	0.009	15	0.0004
			0.5	2	38.68	77.36			0.1					1		15	
	1-3	2	0.5	1	27.61	55.64	55.97	111.94	0.1	112466	0.10	26	0.6090	1	0.009	15	0.0002
			0.5	1	28.31	56.3			0.1					1		15	
	2-1	2	0.5	1	101.09	202.64	208.69	417.38	0.1	112466	0.37	326	0.6095	1	0.009	15	0.0012
			0.5	2	105.67	211.34			0.1					1		15	
	2-2	2	0.5	1	104.24	208.48	220.56	441.1	0.1	112466	0.39	354	0.6096	1	0.009	15	0.0013
			0.5	1	133.38	232.63			0.1					1		15	
	2-3	2	0.5	1	99.08	188.16	209.45	418.93	0.1	112466	0.37	321	0.6096	1	0.009	15	0.0011
			0.5	2	110.93	222.86			0.1					1		15	
	3-1	2	0.5	1	691.82	1381.64	1393.85	2786.19	0.1	112466	2.48	2589	0.6150	1	0.009	15	0.0130
			0.5	2	705.87	1405.24			0.1					1		15	
	3-2	2	0.5	1	697.77	1397.54	1395.34	2790.69	0.1	112466	2.24	2585	0.6145	1	0.009	15	0.0134
			0.5	2	655.67	1307.14			0.1					1		15	
	3-3	2	0.5	1	647.27	1293.68	1384	2768	0.1	112466	2.32	2630	0.6147	1	0.009	15	0.0179
			0.5	1	656.73	1281.46			0.1					1		15	
	4-1	2	0.5	1	2042.8	4085.6	4067	8064	0.1	112466	7.24	8095	0.6150	1	0.009	15	0.0564
			0.5	1	2004	4048.4			0.1					1		15	
	4-2	2	0.5	1	2114.4	4230.8	4125.7	8311.4	0.1	112466	7.35	8226	0.6154	1	0.009	15	0.0579
			0.5	2	2042.9	4084.6			0.1					1		15	
	4-3	2	0.5	1	2113	4226	4243.9	8487.8	0.1	112466	7.55	8439	0.6157	1	0.009	15	0.0593
			0.5	2	2130.9	4261.8			0.1					1		15	
	5-1	2	0.5	1	2859	5718	5748.9	11497.8	0.1	112466	10.22	11410	0.6210	1	0.009	15	0.0804
			0.5	2	2889.9	5781.8			0.1					1		15	
	5-2	2	0.5	1	2736.8	5473.6	5449.8	10901.2	0.1	112466	9.83	10954	0.6202	1	0.009	15	0.0762
			0.5	2	2724.3	5419.6			0.1					1		15	
	5-3	2	0.5	1	2463.8	4927.6	4902.1	9804.2	0.1	112466	8.77	9777	0.6185	1	0.009	15	0.0686
			0.5	1	2438.3	4876.6			0.1					1		15	
	6-1	2	0.5	1	2746.7	5493.4	5443.6	10887.2	0.1	112466	9.75	10860	0.6201	1	0.009	15	0.0760
			0.5	2	2730.9	5477.2			0.1					1		15	
	6-2	2	0.5	1	2787.9	5575.2	5601.9	11203.8	0.1	112466	9.94	11116	0.6206	1	0.009	15	0.0780
			0.5	2	2814.3	5628.6			0.1					1		15	
	6-3	2	0.5	1	2729.5	5479	5499.9	10999.8	0.1	112466	9.75	10905	0.6204	1	0.009	15	0.0758
			0.5	2	2757.3	5514.8			0.1					1		15	
	7-1	2	0.5	1	2693.3	5386.6	5469.9	10939.8	0.1	112466	9.73	10882	0.6200	1	0.009	15	0.0754
			0.5	1	2746.5	5493.2			0.1					1		15	
	7-2	2	0.5	1	2729.7	5459	5425	10850	0.1	112466	9.64	10782	0.6193	1	0.009	15	0.0746
			0.5	2	2726.5	5477			0.1					1		15	
	7-3	2	0.5	1	2615.8	5231.8	5125	10250	0.1	112466	9.11	10162	0.6180	1	0.009	15	0.0708
			0.5	1	2548.1	5096.3			0.1					1		15	
	8-1	2	0.5	1	2129.2	4258.4	4299.7	8599.4	0.1	112466	7.64	8480	0.6155	1	0.009	15	0.0589
			0.5	2	2170.1	4340.2			0.1					1		15	
	8-2	2	0.5	1	2082.1	4164.2	4102.8	8205.6	0.1	112466	7.30	8102	0.6152	1	0.009	15	0.0562
			0.5	1	2019.3	4038.4			0.1					1		15	
	8-3	2	0.5	1	2011.2	4021.6	4003.2	8006.4	0.1	112466	7.11	7901	0.6149	1	0.009	15	0.0551
			0.5	1	2017.4	4035.6			0.1					1		15	

Assay Date	2/2/2005	Test Chemical ID	1/A	# Concentrations tested		Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0914	0.0024
Full activity	End	0.0571	0.0005
Full activity	Overall	0.0742	0.0198
Background	Beginning	0.0000	0.000122531
Background	End	0.0000	8.27834E-05
Background	Overall	0.0000	8.55681E-05
Positive	Beginning	0.0331	0.0003
Positive	End	0.0408	0.0009
Positive	Overall	0.0369	0.0045
Negative	Beginning	0.0876	0.0065
Negative	End	0.0562	0.0014
Negative	Overall	0.0719	0.0185

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/A	1	1	1.00E-03	-3.00	0.0002
1/A	1	2	1.00E-03	-3.00	0.0004
1/A	1	3	1.00E-03	-3.00	0.0002
1/A	2	1	1.00E-04	-4.00	0.0023
1/A	2	2	1.00E-04	-4.00	0.0025
1/A	2	3	1.00E-04	-4.00	0.0023
1/A	3	1	1.00E-05	-5.00	0.0190
1/A	3	2	1.00E-05	-5.00	0.0184
1/A	3	3	1.00E-05	-5.00	0.0178
1/A	4	1	1.00E-06	-6.00	0.0564
1/A	4	2	1.00E-06	-6.00	0.0579
1/A	4	3	1.00E-06	-6.00	0.0592
1/A	5	1	1.00E-07	-7.00	0.0804
1/A	5	2	1.00E-07	-7.00	0.0760
1/A	5	3	1.00E-07	-7.00	0.0689
1/A	6	1	1.00E-08	-8.00	0.0766
1/A	6	2	1.00E-08	-8.00	0.0783
1/A	6	3	1.00E-08	-8.00	0.0768
1/A	7	1	1.00E-09	-9.00	0.0764
1/A	7	2	1.00E-09	-9.00	0.0766
1/A	7	3	1.00E-09	-9.00	0.0716
1/A	8	1	1.00E-10	-10.00	0.0599
1/A	8	2	1.00E-10	-10.00	0.0572
1/A	8	3	1.00E-10	-10.00	0.0561

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-3.00	0.22	0.56	0.23
2	-4.00	3.10	3.36	3.14
3	-5.00	25.61	24.72	23.92
4	-6.00	75.98	78.06	79.71
5	-7.00	108.28	102.43	92.77
6	-8.00	103.24	105.49	103.49
7	-9.00	102.86	103.13	96.44
8	-10.00	80.75	77.04	75.64

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	25944	1330462	
2	0.0194	26839	1383454	
3	0.0194	26340	1357732	
4	0.0195	26159	1341487	
5	0.0195	26980	1383590	
			Average DPM/g soln	1359345
			SD	24108
			CV	1.77
			µCi/g soln	0.612

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.5	10		1050.00
Dilution A			100	10.50
Dilution B			10	1.05

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0412 g
Mass of dilution B used in substrate prep	4.5223 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.590511 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00693 µg/g soln.
µg/g soln.

a. µCi/g soln	0.612
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.590511 + 0.00693
= 0.597442 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.025 µCi/µg ASDN

651638 dpm/nmol

Aromatase results 02-05.xls; 1/9/2006;
Substrate Specific Activity 8:52 AM

Assay Date	2/4/2005	Test Chemical ID	1/A	# Concentrations tested	8		
Technician ID	TD	Replicate #	2	Microsome type	Placental	Microsome ID	11343-7

Standards:	1	0.8	0.6	0.4	0.2	0.14	Blank
	0.308	0.243	0.187	0.133	0.070	0.056	0.000
	0.293	0.246	0.168	0.130	0.065	0.050	0.000
	0.302	0.249	0.184	0.141	0.048	0.054	0.000

Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
28	20	21000023B

Samples:

0.094
0.083
0.093

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.301	0.301	0.0250
0.8	14.3	25	0.00080	25	0.0200	0.246	0.246	0.0202
0.6	10.7	25	0.00060	25	0.0150	0.180	0.180	0.0145
0.4	7.1	25	0.00040	25	0.0099	0.135	0.135	0.0106
0.2	3.6	25	0.00020	25	0.0050	0.067	0.067	0.0048
0.14	2.5	25	0.00014	25	0.0035	0.053	0.053	0.0035

Blank 0.000

r^2= 0.996
m= 0.087
b= -0.001

Variables	Regression results	
m, b	0.087	-0.001
se_m, se_b	0.002	0.000
r^2, se_y	0.998	0.000
F, df	1913	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.
0.094	0.094	0.007	25	120	6000	0.014
0.083	0.083	0.006	25	120	6000	0.012
0.093	0.093	0.007	25	120	6000	0.014

average mg/µL: 0.013
mg/mL: 13.384

Assay Date	2/4/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Microsome Dilution Details		
Dilution A	0.12	mL microsome Stock used
	6	mL total volume
	50	dilution factor
Dilution B	5	mL microsome Dilution A used
	45	mL total volume
	9	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	450	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	13.384
Protein Concentration (dilution added to assay, mg/mL):	0.029742

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	5.00E-06
5	2.50E-06
6	1.00E-06
7	1.00E-07
8	1.00E-08

Assay Date	2/4/2005	Test Chemical ID	1/A	# Concentrations tested		# Microsome type	Placental	Microsome ID	113437	Technician ID	TD	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3409	6818	6735	13470	0.1	135934	9.91	13348	0.0206	1	0.015	15	0.0459
			0.5	2	3306	6652											
	2	2	0.5	1	3293	6586	6503	13006	0.1	135934	9.57	12884	0.0198	1	0.015	15	0.0440
			0.5	2	3250	6500											
	3	2	0.5	1	3071	6142	6175	12350	0.1	135934	9.09	12228	0.0188	1	0.015	15	0.0421
			0.5	2	3104	6208											
	4	2	0.5	1	2997	5994	6080	12160	0.1	135934	8.96	12038	0.0185	1	0.015	15	0.0414
			0.5	2	3083	6166											
Background control	1	2	0.5	1	31	62	59	118	0.1	135934	0.09	-4	0.0000	1	0.015	15	0.0000
			0.5	2	26	56											
	2	2	0.5	1	30	60	63	126	0.1	135934	0.09	4	0.0000	1	0.015	15	0.0000
			0.5	2	33	66											
	3	2	0.5	1	36	72	64	128	0.1	135934	0.09	6	0.0000	1	0.015	15	0.0000
			0.5	2	28	56											
	4	2	0.5	1	27	54	58	116	0.1	135934	0.09	-6	0.0000	1	0.015	15	0.0000
			0.5	2	31	62											
Positive control	1	2	0.5	1	1732	3464	3478	6956	0.1	135934	5.12	6834	0.0105	1	0.015	15	0.0238
			0.5	2	1746	3492											
	2	2	0.5	1	1719	3438	3415	6830	0.1	135934	5.02	6708	0.0103	1	0.015	15	0.0231
			0.5	2	1696	3392											
	3	2	0.5	1	1653	3306	3295	6590	0.1	135934	4.85	6468	0.0099	1	0.015	15	0.0222
			0.5	2	1642	3284											
	4	2	0.5	1	1604	3208	3194	6388	0.1	135934	4.70	6266	0.0096	1	0.015	15	0.0216
			0.5	2	1590	3180											
Negative Control	1	2	0.5	1	3178	6356	6454	12908	0.1	135934	9.50	12786	0.0196	1	0.015	15	0.0440
			0.5	2	3276	6552											
	2	2	0.5	1	2338	4676	4525	9050	0.1	135934	6.66	8928	0.0137	1	0.015	15	0.0307
			0.5	2	2267	4574											
	3	2	0.5	1	3033	6070	6186	12373	0.1	135934	9.10	12250	0.0188	1	0.015	15	0.0421
			0.5	2	3151	6302											
	4	2	0.5	1	3106	6211	6214	12428	0.1	135934	9.14	12306	0.0189	1	0.015	15	0.0423
			0.5	2	3105	6210											
1/A	1-1	2	0.5	1	36	72	74	148	0.1	135934	0.11	26	0.0000	1	0.015	15	0.0001
			0.5	2	38	76											
	1-2	2	0.5	1	32	64	68	136	0.1	135934	0.10	14	0.0000	1	0.015	15	0.0000
			0.5	2	36	72											
	1-3	2	0.5	1	43	86	77	154	0.1	135934	0.11	32	0.0000	1	0.015	15	0.0001
			0.5	2	34	68											
	2-1	2	0.5	1	110	220	212	424	0.1	135934	0.31	302	0.0005	1	0.015	15	0.0010
			0.5	2	102	204											
	2-2	2	0.5	1	101	202	208	416	0.1	135934	0.31	294	0.0005	1	0.015	15	0.0010
			0.5	2	107	214											
	2-3	2	0.5	1	105	210	211	422	0.1	135934	0.31	300	0.0005	1	0.015	15	0.0010
			0.5	2	106	212											
	3-1	2	0.5	1	645	1290	1280	2560	0.1	135934	1.86	2438	0.0037	1	0.015	15	0.0084
			0.5	2	635	1270											
	3-2	2	0.5	1	690	1380	1268	2532	0.1	135934	1.86	2410	0.0037	1	0.015	15	0.0083
			0.5	2	596	1272											
	3-3	2	0.5	1	603	1206	1217	2434	0.1	135934	1.79	2312	0.0035	1	0.015	15	0.0080
			0.5	2	614	1228											
	4-1	2	0.5	1	1014	2028	2060	4120	0.1	135934	3.03	3998	0.0061	1	0.015	15	0.0139
			0.5	2	1046	2092											
	4-2	2	0.5	1	1025	2050	2044	4088	0.1	135934	3.01	3966	0.0061	1	0.015	15	0.0136
			0.5	2	1019	2038											
	4-3	2	0.5	1	1027	2054	2033	4066	0.1	135934	2.99	3944	0.0061	1	0.015	15	0.0136
			0.5	2	1006	2012											
	5-1	2	0.5	1	1507	3014	3025	6050	0.1	135934	4.45	5928	0.0091	1	0.015	15	0.0254
			0.5	2	1518	3036											
	5-2	2	0.5	1	1503	3006	2982	5964	0.1	135934	4.40	5842	0.0090	1	0.015	15	0.0250
			0.5	2	1469	2938											
	5-3	2	0.5	1	1479	2958	2966	5932	0.1	135934	4.36	5810	0.0089	1	0.015	15	0.0250
			0.5	2	1487	2974											
	6-1	2	0.5	1	2153	4306	4247	8494	0.1	135934	6.25	8372	0.0128	1	0.015	15	0.0358
			0.5	2	2094	4188											
	6-2	2	0.5	1	2117	4234	4311	8622	0.1	135934	6.34	8500	0.0130	1	0.015	15	0.0292
			0.5	2	2194	4388											
	6-3	2	0.5	1	2116	4232	4241	8482	0.1	135934	6.24	8360	0.0128	1	0.015	15	0.0286
			0.5	2	2126	4252											
	7-1	2	0.5	1	2853	5706	5708	11416	0.1	135934	8.40	11294	0.0173	1	0.015	15	0.0396
			0.5	2	2845	5690											
	7-2	2	0.5	1	2895	5790	5774	11548	0.1	135934	8.50	11426	0.0175	1	0.015	15	0.0393
			0.5	2	2879	5758											
	7-3	2	0.5	1	2956	5912	5879	11758	0.1	135934	8.65	11636	0.0179	1	0.015	15	0.0400
			0.5	2	2923	5846											
	8-1	2	0.5	1	3061	6122	6041	12082	0.1	135934	8.85	11960	0.0184	1	0.015	15	0.0411
			0.5	2	2980	5960											
	8-2	2	0.5	1	2987	5974	6029	12058	0.1	135934	8.87	11934	0.0183	1	0.015	15	0.0411
			0.5	2	3047	6062											
	8-3	2	0.5	1	3045	6089	6151	12302	0.1	135934	9.05	12180	0.0187	1	0.015	15	0.0419
			0.5	2	3102	6204											

Assay Date	2/4/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0451	0.0011
Full activity	End	0.0417	0.0005
Full activity	Overall	0.0434	0.0021
Background	Beginning	0.0000	1.94582E-05
Background	End	0.0000	2.91874E-05
Background	Overall	0.0000	2.02528E-05
Positive	Beginning	0.0233	0.0003
Positive	End	0.0219	0.0005
Positive	Overall	0.0226	0.0009
Negative	Beginning	0.0373	0.0094
Negative	End	0.0422	0.0001
Negative	Overall	0.0398	0.0061

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/A	1	1	1.00E-03	-3.00	0.0001
1/A	1	2	1.00E-03	-3.00	0.0000
1/A	1	3	1.00E-03	-3.00	0.0001
1/A	2	1	1.00E-04	-4.00	0.0010
1/A	2	2	1.00E-04	-4.00	0.0010
1/A	2	3	1.00E-04	-4.00	0.0010
1/A	3	1	1.00E-05	-5.00	0.0084
1/A	3	2	1.00E-05	-5.00	0.0083
1/A	3	3	1.00E-05	-5.00	0.0080
1/A	4	1	5.00E-06	-5.30	0.0138
1/A	4	2	5.00E-06	-5.30	0.0136
1/A	4	3	5.00E-06	-5.30	0.0136
1/A	5	1	2.50E-06	-5.60	0.0204
1/A	5	2	2.50E-06	-5.60	0.0202
1/A	5	3	2.50E-06	-5.60	0.0200
1/A	6	1	1.00E-06	-6.00	0.0298
1/A	6	2	1.00E-06	-6.00	0.0292
1/A	6	3	1.00E-06	-6.00	0.0288
1/A	7	1	1.00E-07	-7.00	0.0388
1/A	7	2	1.00E-07	-7.00	0.0393
1/A	7	3	1.00E-07	-7.00	0.0400
1/A	8	1	1.00E-08	-8.00	0.0411
1/A	8	2	1.00E-08	-8.00	0.0411
1/A	8	3	1.00E-08	-8.00	0.0419

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-3.00	0.21	0.11	0.25
2	-4.00	2.39	2.33	2.38
3	-5.00	19.31	19.09	18.31
4	-5.30	31.67	31.42	31.24
5	-5.60	46.96	46.43	46.02
6	-6.00	66.32	67.33	66.22
7	-7.00	89.46	90.51	92.17
8	-8.00	94.74	94.53	95.48

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	25696	1317744	
2	0.0195	26112	1339077	
3	0.0194	27378	1411237	
4	0.0195	28198	1446051	
5	0.0194	27411	1412938	
			Average DPM/g soln	1385409
			SD	54378
			CV	3.93
			µCi/g soln	0.624

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.5	10		1050.00
Dilution A			100	10.50
Dilution B			10	1.05

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0441 g
Mass of dilution B used in substrate prep	4.5243 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.590559 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00706 µg/g soln.
µg/g soln.

a. µCi/g soln	0.624
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.590559 + 0.00706
= 0.597623 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.044 µCi/µg ASDN

663932 dpm/nmol

Aromatase results 02-07.xls; 1/9/2006;
Substrate Specific Activity 8:55 AM

Assay Date	2/7/2005	Test Chemical ID	1/A	# Concentrations tested	8						
Technician ID	TD	Replicate #	3	Microsome type	Placental	Microsome ID	11343-7				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.14	Blank	28	20	210000238
	0.304	0.238	0.188	0.135	0.077	0.053	0.000			
	0.303	0.226	0.188	0.131	0.074	0.060	0.000			
	0.318	0.248	0.179	0.124	0.106	0.058	0.000			

Samples:

0.074
0.073
0.080

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.308	0.308	0.0257	m, b	0.089	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.237	0.237	0.0193	se_m, se_b	0.003	0.001
0.6	10.7	25	0.00060	25	0.0150	0.185	0.185	0.0147	r^2, se_y	0.996	0.001
0.4	7.1	25	0.00040	25	0.0099	0.130	0.130	0.0098	F, df	895	4
0.2	3.6	25	0.00020	25	0.0050	0.086	0.086	0.0058	ss_{reg}, ss_{resid}	0.000	0.000
0.14	2.5	25	0.00014	25	0.0035	0.057	0.057	0.0033			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.996		
		m=	0.089		
		b=	-0.002		

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.074	0.074	0.005	25	120	6000	0.009	0.010	9.801
0.073	0.073	0.005	25	120	6000	0.009		
0.080	0.080	0.005	25	120	6000	0.011		

Assay Date	2/7/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Microsome Dilution Details		
Dilution A	0.12	mL microsome Stock used
	6	mL total volume
	50	dilution factor
Dilution B	5	mL microsome Dilution A used
	50	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	9.801
Protein Concentration (dilution added to assay, mg/mL):	0.019602

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	5.00E-06
5	2.50E-06
6	1.00E-06
7	1.00E-07
8	1.00E-08

Assay Date	2/7/2006	Test Chemical ID	n/a	# Concentrations tested		b Microsome type	Placental	Microsome ID	11343-7	Technician ID	ID	Replicate #	3

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed						
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	total DPM in assay tube (total)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2691	5382	5367	10784	0.1	138541	7.79	10656	0.0161	1	0.010	15	0.0646
			0.5	2	2736	5472											
	2	2	0.5	1	2474	4948	4921	9842	0.1	138541	7.10	9714	0.0146	1	0.010	15	0.0586
			0.5	2	2467	4934											
	3	2	0.5	1	2371	4742	4666	9332	0.1	138541	6.74	9204	0.0139	1	0.010	15	0.0421
			0.5	2	2295	4590											
	4	2	0.5	1	2195	4390	4384	8768	0.1	138541	6.33	8640	0.0130	1	0.010	15	0.0445
			0.5	2	2219	4438											
Background control	1	2	0.5	1	34	68	70	140	0.1	138541	0.10	12	0.0000	1	0.010	15	0.0001
			0.5	2	36	72											
	2	2	0.5	1	40	80	75	150	0.1	138541	0.11	22	0.0000	1	0.010	15	0.0001
			0.5	2	36	72											
	3	2	0.5	1	35	70	66	132	0.1	138541	0.10	4	0.0000	1	0.010	15	0.0000
			0.5	2	31	62											
	4	2	0.5	1	28	56	46	92	0.1	138541	0.07	-37	-0.0001	1	0.010	15	-0.0002
			0.5	2	18	36											
Positive control	1	2	0.5	1	1353	2706	2659	5318	0.1	138541	3.84	5190	0.0078	1	0.010	15	0.0296
			0.5	2	1306	2612											
	2	2	0.5	1	1514	3028	3020	6040	0.1	138541	4.37	5912	0.0089	1	0.010	15	0.0304
			0.5	2	1518	3032											
	3	2	0.5	1	1380	2760	2773	5546	0.1	138541	4.00	5418	0.0082	1	0.010	15	0.0278
			0.5	2	1393	2786											
	4	2	0.5	1	1442	2884	2842	5684	0.1	138541	4.10	5556	0.0084	1	0.010	15	0.0285
			0.5	2	1400	2800											
Negative Control	1	2	0.5	1	2642	5284	5042	10084	0.1	138541	7.28	9956	0.0150	1	0.010	15	0.0510
			0.5	2	2400	4800											
	2	2	0.5	1	2568	5136	5067	10134	0.1	138541	7.31	10006	0.0151	1	0.010	15	0.0513
			0.5	2	2499	4998											
	3	2	0.5	1	2093	4186	4224	8448	0.1	138541	6.10	8320	0.0125	1	0.010	15	0.0426
			0.5	2	2131	4262											
	4	2	0.5	1	2305	4610	4611	9222	0.1	138541	6.66	9094	0.0137	1	0.010	15	0.0466
			0.5	2	2302	4604											
n/a	1-1	2	0.5	1	39	78	78	156	0.1	138541	0.11	78	0.0000	1	0.010	15	0.0001
			0.5	2	39	78											
	1-2	2	0.5	1	47	94	94	188	0.1	138541	0.14	60	0.0001	1	0.010	15	0.0003
			0.5	2	47	94											
	1-3	2	0.5	1	40	80	80	160	0.1	138541	0.12	32	0.0000	1	0.010	15	0.0002
			0.5	2	40	80											
	2-1	2	0.5	1	111	222	242	484	0.1	138541	0.34	366	0.0006	1	0.010	15	0.0019
			0.5	2	130	260											
	2-2	2	0.5	1	127	254	248	496	0.1	138541	0.36	368	0.0006	1	0.010	15	0.0019
			0.5	2	121	242											
	2-3	2	0.5	1	142	284	284	568	0.1	138541	0.41	440	0.0007	1	0.010	15	0.0023
			0.5	2	142	284											
	3-1	2	0.5	1	653	1306	1301	2602	0.1	138541	1.88	2474	0.0037	1	0.010	15	0.0127
			0.5	2	648	1296											
	3-2	2	0.5	1	680	1370	1349	2698	0.1	138541	1.95	2570	0.0039	1	0.010	15	0.0132
			0.5	2	664	1328											
	3-3	2	0.5	1	667	1334	1354	2708	0.1	138541	1.95	2582	0.0039	1	0.010	15	0.0133
			0.5	2	687	1374											
	4-1	2	0.5	1	1063	2126	2164	4328	0.1	138541	3.04	4200	0.0061	1	0.010	15	0.0209
			0.5	2	1041	2082											
	4-2	2	0.5	1	1322	2644	2627	4254	0.1	138541	2.91	3926	0.0059	1	0.010	15	0.0201
			0.5	2	1305	2610											
	4-3	2	0.5	1	1051	2102	2093	4186	0.1	138541	3.02	4058	0.0061	1	0.010	15	0.0208
			0.5	2	1042	2084											
	5-1	2	0.5	1	1474	2948	2976	5952	0.1	138541	4.30	5824	0.0088	1	0.010	15	0.0298
			0.5	2	1520	3040											
	5-2	2	0.5	1	1405	2810	2800	5600	0.1	138541	4.04	5472	0.0082	1	0.010	15	0.0280
			0.5	2	1395	2790											
	5-3	2	0.5	1	1442	2884	2888	5776	0.1	138541	4.17	5648	0.0085	1	0.010	15	0.0289
			0.5	2	1446	2892											
	6-1	2	0.5	1	1972	3944	3941	7882	0.1	138541	5.69	7754	0.0117	1	0.010	15	0.0397
			0.5	2	1969	3938											
	6-2	2	0.5	1	1971	3942	3955	7910	0.1	138541	5.71	7782	0.0117	1	0.010	15	0.0398
			0.5	2	1984	3968											
	6-3	2	0.5	1	1822	3644	3823	7646	0.1	138541	5.52	7518	0.0113	1	0.010	15	0.0385
			0.5	2	1901	3802											
	7-1	2	0.5	1	1216	2432	2422	4844	0.1	138541	3.50	4716	0.0071	1	0.010	15	0.0242
			0.5	2	1206	2412											
	7-2	2	0.5	1	2322	4644	4598	9196	0.1	138541	6.64	9068	0.0137	1	0.010	15	0.0469
			0.5	2	2276	4552											
	7-3	2	0.5	1	2173	4346	4344	8688	0.1	138541	6.27	8560	0.0129	1	0.010	15	0.0438
			0.5	2	2171	4342											
	8-1	2	0.5	1	2455	4910	4900	9800	0.1	138541	7.07	9672	0.0146	1	0.010	15	0.0495
			0.5	2	2445	4890											
	8-2	2	0.5	1	2492	4984	4967	9934	0.1	138541	7.17	9806	0.0148	1	0.010	15	0.0502
			0.5	2	2470	4940											
	8-3	2	0.5	1	2537	5074	5010	10020	0.1	138541	7.23	9892	0.0149	1	0.010	15	0.0507
			0.5	2	2487	4974											

Assay Date	2/7/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0522	0.0034
Full activity	End	0.0457	0.0020
Full activity	Overall	0.0489	0.0044
Background	Beginning	0.0001	3.62218E-05
Background	End	-0.0001	0.000144887
Background	Overall	0.0000	0.000130231
Positive	Beginning	0.0285	0.0027
Positive	End	0.0281	0.0005
Positive	Overall	0.0283	0.0016
Negative	Beginning	0.0511	0.0002
Negative	End	0.0446	0.0028
Negative	Overall	0.0479	0.0041

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/A	1	1	1.00E-03	-3.00	0.0001
1/A	1	2	1.00E-03	-3.00	0.0003
1/A	1	3	1.00E-03	-3.00	0.0002
1/A	2	1	1.00E-04	-4.00	0.0019
1/A	2	2	1.00E-04	-4.00	0.0019
1/A	2	3	1.00E-04	-4.00	0.0023
1/A	3	1	1.00E-05	-5.00	0.0127
1/A	3	2	1.00E-05	-5.00	0.0132
1/A	3	3	1.00E-05	-5.00	0.0132
1/A	4	1	5.00E-06	-5.30	0.0209
1/A	4	2	5.00E-06	-5.30	0.0201
1/A	4	3	5.00E-06	-5.30	0.0208
1/A	5	1	2.50E-06	-5.60	0.0298
1/A	5	2	2.50E-06	-5.60	0.0280
1/A	5	3	2.50E-06	-5.60	0.0289
1/A	6	1	1.00E-06	-6.00	0.0397
1/A	6	2	1.00E-06	-6.00	0.0399
1/A	6	3	1.00E-06	-6.00	0.0385
1/A	7	1	1.00E-07	-7.00	0.0242
1/A	7	2	1.00E-07	-7.00	0.0464
1/A	7	3	1.00E-07	-7.00	0.0438
1/A	8	1	1.00E-08	-8.00	0.0495
1/A	8	2	1.00E-08	-8.00	0.0502
1/A	8	3	1.00E-08	-8.00	0.0507

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-3.00	0.29	0.62	0.33
2	-4.00	3.83	3.85	4.60
3	-5.00	25.89	26.89	26.99
4	-5.30	42.69	41.08	42.46
5	-5.60	60.94	57.26	59.10
6	-6.00	81.14	81.43	78.67
7	-7.00	49.35	94.89	89.58
8	-8.00	101.21	102.62	103.52

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	24616	1262359	
2	0.0190	26805	1410789	
3	0.0192	26144	1361667	
4	0.0193	27026	1400311	
5	0.0196	27472	1401633	
			Average DPM/g soln	1367352
			SD	61653
			CV	4.51
			µCi/g soln	0.616

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.7	10		1070.00
Dilution A			100	10.70
Dilution B			10	1.07

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0304 g
Mass of dilution B used in substrate prep	4.5165 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.601795 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00697 µg/g soln.
µg/g soln.

a. µCi/g soln	0.616
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.601795 + 0.00697
= 0.608767 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.012 µCi/µg ASDN

643283 dpm/nmol

Aromatase results 03-14-05.xls; 1/9/2006;
Substrate Specific Activity 8:57 AM

Assay Date	3/14/2005	Test Chemical ID	1/A		# Concentrations tested		8				
Technician ID	TD	Replicate #	4	Microsome type	Placental	Microsome ID	11343-7				

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	25	20	210000236
	0.293	0.247	0.191	0.107	0.076	0.048	0.000			
	0.285	0.232	0.195	0.131	0.083	0.042	0.000			
	0.281	0.222	0.198	0.143	0.075	0.056	0.000			
Samples:										
	0.081	0.038	0.155	0.286						
	0.085	0.041	0.160	0.303						
	0.095									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.286	0.286	0.0247	m, b	0.094	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.234	0.234	0.0197	se_m, se_b	0.003	0.001
0.6	10.7	25	0.00060	25	0.0150	0.195	0.195	0.0161	r^2, se_y	0.996	0.001
0.4	7.1	25	0.00040	25	0.0099	0.127	0.127	0.0098	F, df	927	4
0.2	3.6	25	0.00020	25	0.0050	0.078	0.078	0.0052	ss_{reg}, ss_{resid}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.049	0.049	0.0025			
										Regression results are calculated using the function LINEST	
			Blank	0.000	r^2=	0.996					
					m=	0.094					
					b=	-0.002					

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.081	0.081	0.005	25	100	5000	0.011	0.012	12.004
0.085	0.085	0.006	25	100	5000	0.012		
0.095	0.095	0.007	25	100	5000	0.014		
0.038	0.038	0.001	25	1	1	0.000	0.000	0.062
0.041	0.041	0.002	25	1	1	0.000		
0.155	0.155	0.012	25	1	1	0.000	0.001	0.505
0.160	0.160	0.013	25	1	1	0.001		
0.286	0.286	0.025	25	1	1	0.001	0.001	1.016
0.303	0.303	0.026	25	1	1	0.001		

Assay Date	3/14/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	4

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.004
Protein Concentration (dilution added to assay, mg/mL):	0.024008

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	5.00E-06
5	2.50E-06
6	1.00E-06
7	1.00E-07
8	1.00E-08

Assay Date	3/14/2005	Test Chemical ID	n/a	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TS	Replicate #	4

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
Fxt activity control	1	3	0.5	1	1947	3894	3933	7866	0.1	136735	5.75	7727	0.0120	1	0.012	15	0.0334
			0.5	2	1986	3972											
	2	2	0.5	1	1375	2750	2758	5492	0.1	136735	3.99	5313	0.0083	1	0.012	15	0.0229
			0.5	2	1301	2602											
	3	3	0.5	1	1735	3470	3446	6896	0.1	136735	5.04	6759	0.0105	1	0.012	15	0.0292
			0.5	2	1714	3428											
	4	2	0.5	1	1788	3576	3583	7166	0.1	136735	5.24	7027	0.0109	1	0.012	15	0.0303
			0.5	2	1795	3590											
Background control	1	3	0.5	1	36	72	71	142	0.1	136735	0.10	-5	0.0000	1	0.012	15	0.0000
			0.5	2	35	70											
	2	2	0.5	1	32	64	67	134	0.1	136735	0.10	-6	0.0000	1	0.012	15	0.0000
			0.5	2	33	66											
	3	3	0.5	1	32	64	73	146	0.1	136735	0.11	7	0.0000	1	0.012	15	0.0000
			0.5	2	41	82											
	4	2	0.5	1	34	68	68	136	0.1	136735	0.10	-4	0.0000	1	0.012	15	0.0000
			0.5	2	34	68											
Positive control	1	2	0.5	1	1239	2478	2433	4865	0.1	136735	3.56	4721	0.0073	1	0.012	15	0.0204
			0.5	2	1191	2382											
	2	3	0.5	1	1195	2390	2414	4828	0.1	136735	3.53	4689	0.0073	1	0.012	15	0.0202
			0.5	2	1219	2438											
	3	2	0.5	1	1128	2256	2263	4526	0.1	136735	3.31	4387	0.0068	1	0.012	15	0.0189
			0.5	2	1135	2270											
	4	2	0.5	1	1106	2212	2218	4436	0.1	136735	3.24	4293	0.0067	1	0.012	15	0.0185
			0.5	2	1109	2218											
Negative Control	1	2	0.5	1	1790	3580	3597	7194	0.1	136735	5.26	7055	0.0110	1	0.012	15	0.0305
			0.5	2	1807	3614											
	2	3	0.5	1	1697	3394	3433	6866	0.1	136735	5.02	6727	0.0105	1	0.012	15	0.0290
			0.5	2	1736	3472											
	3	2	0.5	1	1535	3070	3119	6238	0.1	136735	4.58	6099	0.0095	1	0.012	15	0.0263
			0.5	2	1584	3168											
	4	2	0.5	1	1638	3276	3263	6526	0.1	136735	4.77	6387	0.0099	1	0.012	15	0.0276
			0.5	2	1625	3250											
1/4	1-1	2	0.5	1	46	92	92	184	0.1	136735	0.13	-45	0.0001	1	0.012	15	0.0003
			0.5	2	46	92											
	1-2	2	0.5	1	41	82	77	154	0.1	136735	0.11	-19	0.0000	1	0.012	15	0.0001
			0.5	2	36	72											
	1-3	3	0.5	1	44	88	88	176	0.1	136735	0.13	-37	0.0001	1	0.012	15	0.0002
			0.5	2	44	88											
	2-1	2	0.5	1	139	278	296	592	0.1	136735	0.43	452	0.0007	1	0.012	15	0.0020
			0.5	2	157	314											
	2-2	3	0.5	1	153	306	309	618	0.1	136735	0.45	479	0.0007	1	0.012	15	0.0021
			0.5	2	156	312											
	2-3	2	0.5	1	168	336	325	650	0.1	136735	0.48	511	0.0008	1	0.012	15	0.0022
			0.5	2	157	314											
	3-1	2	0.5	1	709	1418	1427	2854	0.1	136735	2.09	2715	0.0043	1	0.012	15	0.0117
			0.5	2	718	1436											
	3-2	2	0.5	1	751	1502	1505	3010	0.1	136735	2.20	2871	0.0045	1	0.012	15	0.0124
			0.5	2	754	1508											
	3-3	2	0.5	1	771	1542	1562	3124	0.1	136735	2.27	2965	0.0048	1	0.012	15	0.0128
			0.5	2	781	1562											
	4-1	2	0.5	1	998	1996	1998	3992	0.1	136735	2.88	3793	0.0060	1	0.012	15	0.0164
			0.5	2	998	1996											
	4-2	2	0.5	1	959	1918	1916	3832	0.1	136735	2.80	3693	0.0057	1	0.012	15	0.0159
			0.5	2	958	1916											
	4-3	2	0.5	1	1029	2058	2060	4120	0.1	136735	3.01	3981	0.0062	1	0.012	15	0.0172
			0.5	2	1031	2062											
	5-1	2	0.5	1	1314	2628	2605	5210	0.1	136735	3.81	5071	0.0079	1	0.012	15	0.0219
			0.5	2	1291	2582											
	5-2	2	0.5	1	1314	2628	2629	5258	0.1	136735	3.85	5119	0.0080	1	0.012	15	0.0221
			0.5	2	1315	2630											
	5-3	2	0.5	1	1273	2546	2534	5068	0.1	136735	3.71	4929	0.0077	1	0.012	15	0.0213
			0.5	2	1261	2522											
	6-1	2	0.5	1	1357	2714	2765	5530	0.1	136735	4.04	5391	0.0084	1	0.012	15	0.0233
			0.5	2	1408	2816											
	6-2	2	0.5	1	1439	2878	2868	5736	0.1	136735	4.20	5637	0.0088	1	0.012	15	0.0243
			0.5	2	1449	2898											
	6-3	2	0.5	1	1431	2862	2832	5664	0.1	136735	4.14	5525	0.0086	1	0.012	15	0.0238
			0.5	2	1401	2802											
	7-1	2	0.5	1	1579	3158	3153	6306	0.1	136735	4.62	6247	0.0097	1	0.012	15	0.0270
			0.5	2	1584	3168											
	7-2	2	0.5	1	1660	3320	3315	6630	0.1	136735	4.84	6481	0.0101	1	0.012	15	0.0280
			0.5	2	1650	3300											
	7-3	2	0.5	1	1654	3308	3302	6604	0.1	136735	4.83	6446	0.0100	1	0.012	15	0.0278
			0.5	2	1638	3276											
	8-1	2	0.5	1	1603	3206	3181	6362	0.1	136735	4.65	6203	0.0097	1	0.012	15	0.0268
			0.5	2	1578	3156											
	8-2	2	0.5	1	1719	3438	3467	6934	0.1	136735	5.07	6795	0.0106	1	0.012	15	0.0293
			0.5	2	1748	3496											
	8-3	2	0.5	1	1564	3128	3148	6296	0.1	136735	4.60	6157	0.0096	1	0.012	15	0.0266
			0.5	2	1584	3168											

Assay Date	3/14/2005	Test Chemical ID	1/A	# Concentrations tested		Microsome S type	Placental	Microsome ID	11343-7	Technician ID	TO	Replicate #	4

Control Type	Portion	Average	SD
Full activity	Beginning	0.0281	0.0074
Full activity	End	0.0298	0.0008
Full activity	Overall	0.0289	0.0044
Background	Beginning	0.0000	2.44189E-05
Background	End	0.0000	3.05236E-05
Background	Overall	0.0000	2.37745E-05
Positive	Beginning	0.0203	0.0001
Positive	End	0.0187	0.0003
Positive	Overall	0.0195	0.0009
Negative	Beginning	0.0297	0.0010
Negative	End	0.0269	0.0009
Negative	Overall	0.0283	0.0018

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/A	1	1	1.00E-03	-3.00	0.0002
1/A	1	2	1.00E-03	-3.00	0.0001
1/A	1	3	1.00E-03	-3.00	0.0002
1/A	2	1	1.00E-04	-4.00	0.0020
1/A	2	2	1.00E-04	-4.00	0.0021
1/A	2	3	1.00E-04	-4.00	0.0022
1/A	3	1	1.00E-05	-5.00	0.0117
1/A	3	2	1.00E-05	-5.00	0.0124
1/A	3	3	1.00E-05	-5.00	0.0128
1/A	4	1	5.00E-06	-5.30	0.0164
1/A	4	2	5.00E-06	-5.30	0.0159
1/A	4	3	5.00E-06	-5.30	0.0172
1/A	5	1	2.50E-06	-5.60	0.0219
1/A	5	2	2.50E-06	-5.60	0.0221
1/A	5	3	2.50E-06	-5.60	0.0213
1/A	6	1	1.00E-06	-6.00	0.0233
1/A	6	2	1.00E-06	-6.00	0.0243
1/A	6	3	1.00E-06	-6.00	0.0238
1/A	7	1	1.00E-07	-7.00	0.0270
1/A	7	2	1.00E-07	-7.00	0.0280
1/A	7	3	1.00E-07	-7.00	0.0278
1/A	8	1	1.00E-08	-8.00	0.0269
1/A	8	2	1.00E-08	-8.00	0.0293
1/A	8	3	1.00E-08	-8.00	0.0266

		Percent of control values		
	Log[test	Replicate		
Level	substance]	1	2	3
1	-3.00	0.66	0.22	0.54
2	-4.00	6.75	7.14	7.61
3	-5.00	40.48	42.80	44.21
4	-5.30	56.55	55.06	59.36
5	-5.60	75.61	76.33	73.49
6	-6.00	80.38	84.05	82.38
7	-7.00	93.15	96.64	96.10
8	-8.00	92.79	101.32	91.81

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0188	25317	1346649	
2	0.0193	27282	1413575	
3	0.0193	28268	1464663	
4	0.0189	27854	1473757	
5	0.0189	27695	1465344	
			Average DPM/g soln	1432798
			SD	53720
			CV	3.75
			µCi/g soln	0.645

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	11.4	10		1140.00
Dilution A			100	11.40
Dilution B			10	1.14

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0268 g
Mass of dilution B used in substrate prep	4.5134 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.641012 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00731 µg/g soln.
µg/g soln.

a. µCi/g soln	0.645
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.641012 + 0.00731
= 0.648318 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.996 µCi/µg ASDN

632950 dpm/nmol

Aromatase results 03-16-05.xls; 1/9/2006;
Substrate Specific Activity 8:59 AM

Assay Date	3/16/2005	Test Chemical ID	1/A	# Concentrations tested	8		
Technician ID	TD	Replicate #	5	Microsome type	Placental	Microsome ID	11343-7

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000238
	0.295	0.255	0.198	0.137	0.065	0.026	0.000			
	0.302	0.251	0.202	0.138	0.081	0.057	0.000			
	0.292	0.273	0.205	0.140	0.089	0.056	0.000			
Samples:										
	0.077	0.042	0.159	0.287						
	0.079	0.045	0.170	0.300						
	0.081									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.296	0.296	0.0239	m, b	0.086	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.260	0.260	0.0207	se_m, se_b	0.004	0.001
0.6	10.7	25	0.00060	25	0.0150	0.201	0.201	0.0157	r^2, se_y	0.993	0.001
0.4	7.1	25	0.00040	25	0.0099	0.138	0.138	0.0102	F, df	547	4
0.2	3.6	25	0.00020	25	0.0050	0.078	0.078	0.0050	ss_{reg}, ss_{resd}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.046	0.046	0.0023			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.993	
		m=	0.086	
		b=	-0.002	

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.077	0.077	0.005	25	100	5000	0.010	0.010	10.192
0.079	0.079	0.005	25	100	5000	0.010		
0.081	0.081	0.005	25	100	5000	0.011		
0.042	0.042	0.002	25	1	1	0.000	0.000	0.081
0.045	0.045	0.002	25	1	1	0.000		
0.159	0.159	0.012	25	1	1	0.000	0.001	0.501
0.170	0.170	0.013	25	1	1	0.001		
0.287	0.287	0.023	25	1	1	0.001	0.001	0.945
0.300	0.300	0.024	25	1	1	0.001		

Assay Date	3/16/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	5

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	10.192
Protein Concentration (dilution added to assay, mg/mL):	0.020384

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	5.00E-06
5	2.50E-06
6	1.00E-06
7	1.00E-07
8	1.00E-08

Assay Date	3/16/2006	Test Chemical ID	1A	# Concentrations tested		# Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	5

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tube=0	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay tube (P, mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min)
Full activity control	1	2	0.5	1	2243	4486	4475	8950	0.1	143280	6.25	6805	0.0139	1	0.910	15	0.5455
			0.5	2	2232	4464											
	2	2	0.5	1	2053	4106	4156	8275	0.1	143280	5.77	6125	0.0128	1	0.910	15	0.5020
			0.5	2	2002	4154											
	3	2	0.5	1	1969	3976	3995	7990	0.1	143280	5.58	7645	0.0124	1	0.910	15	0.5405
			0.5	2	2005	4012											
	4	2	0.5	1	2108	4218	4209	8418	0.1	143280	5.88	8273	0.0131	1	0.910	15	0.5427
			0.5	2	2101	4202											
Background control	1	2	0.5	1	46	92	79	158	0.1	143280	0.11	13	0.0000	1	0.910	15	0.0001
			0.5	2	33	66											
	2	2	0.5	1	43	84	91	182	0.1	143280	0.11	12	0.0000	1	0.910	15	0.0001
			0.5	2	39	78											
	3	2	0.5	1	31	62	66	132	0.1	143280	0.09	-13	0.0000	1	0.910	15	-0.0001
			0.5	2	35	70											
	4	2	0.5	1	37	74	64	128	0.1	143280	0.09	-17	0.0000	1	0.910	15	-0.0001
			0.5	2	27	54											
Positive control	1	2	0.5	1	1905	3810	3815	7630	0.1	143280	5.45	5085	0.0099	1	0.910	15	0.0363
			0.5	2	1910	3820											
	2	2	0.5	1	1391	2781	2774	5548	0.1	143280	3.97	3403	0.0065	1	0.910	15	0.0229
			0.5	2	1383	2768											
	3	2	0.5	1	1235	2470	2430	4860	0.1	143280	3.39	4715	0.0074	1	0.910	15	0.0244
			0.5	2	1202	2404											
	4	2	0.5	1	1167	2334	2369	4736	0.1	143280	3.31	4568	0.0073	1	0.910	15	0.0237
			0.5	2	1202	2404											
Negative Control	1	2	0.5	1	2235	4472	4470	8942	0.1	143280	6.24	8795	0.0139	1	0.910	15	0.0454
			0.5	2	2234	4468											
	2	2	0.5	1	2160	4320	4318	8636	0.1	143280	6.03	8491	0.0134	1	0.910	15	0.0439
			0.5	2	2158	4316											
	3	2	0.5	1	2024	4048	4103	8206	0.1	143280	5.73	8063	0.0127	1	0.910	15	0.0417
			0.5	2	2079	4158											
	4	2	0.5	1	1949	3898	3947	7894	0.1	143280	5.37	7540	0.0119	1	0.910	15	0.0390
			0.5	2	1998	3996											
1A	1-1	2	0.5	1	45	90	93	186	0.1	143280	0.13	41	0.0001	1	0.910	15	0.0005
			0.5	2	48	96											
	1-2	2	0.5	1	50	100	94	188	0.1	143280	0.13	43	0.0001	1	0.910	15	0.0005
			0.5	2	44	88											
	1-3	2	0.5	1	50	100	97	194	0.1	143280	0.14	49	0.0001	1	0.910	15	0.0003
			0.5	2	47	94											
	2-1	2	0.5	1	210	420	415	830	0.1	143280	0.58	685	0.0011	1	0.910	15	0.0035
			0.5	2	205	410											
	2-2	2	0.5	1	201	402	400	800	0.1	143280	0.56	658	0.0010	1	0.910	15	0.0034
			0.5	2	199	398											
	2-3	2	0.5	1	194	388	386	772	0.1	143280	0.54	627	0.0010	1	0.910	15	0.0032
			0.5	2	192	384											
	3-1	2	0.5	1	884	1768	1735	3470	0.1	143280	2.42	3325	0.0053	1	0.910	15	0.0170
			0.5	2	851	1702											
	3-2	2	0.5	1	843	1686	1667	3334	0.1	143280	2.33	3189	0.0050	1	0.910	15	0.0163
			0.5	2	824	1648											
	3-3	2	0.5	1	868	1736	1718	3438	0.1	143280	2.40	3291	0.0052	1	0.910	15	0.0170
			0.5	2	850	1700											
	4-1	2	0.5	1	1178	2356	2325	4650	0.1	143280	3.25	4508	0.0071	1	0.910	15	0.0233
			0.5	2	1149	2298											
	4-2	2	0.5	1	1204	2408	2433	4866	0.1	143280	3.40	4721	0.0075	1	0.910	15	0.0244
			0.5	2	1225	2450											
	4-3	2	0.5	1	1213	2426	2415	4830	0.1	143280	3.37	4685	0.0074	1	0.910	15	0.0242
			0.5	2	1202	2404											
	5-1	2	0.5	1	1591	3182	3149	6298	0.1	143280	4.40	6153	0.0097	1	0.910	15	0.0318
			0.5	2	1558	3116											
	5-2	2	0.5	1	1502	3052	3054	6108	0.1	143280	4.26	5963	0.0094	1	0.910	15	0.0308
			0.5	2	1526	3056											
	5-3	2	0.5	1	1406	2812	2848	5696	0.1	143280	3.98	5581	0.0088	1	0.910	15	0.0287
			0.5	2	1442	2884											
	6-1	2	0.5	1	1901	3802	3821	7642	0.1	143280	5.33	7497	0.0118	1	0.910	15	0.0387
			0.5	2	1920	3840											
	6-2	2	0.5	1	1857	3714	3716	7432	0.1	143280	5.19	7287	0.0115	1	0.910	15	0.0377
			0.5	2	1859	3718											
	6-3	2	0.5	1	1781	3562	3605	7210	0.1	143280	5.03	7065	0.0112	1	0.910	15	0.0365
			0.5	2	1824	3648											
	7-1	2	0.5	1	2106	4212	4212	8424	0.1	143280	5.88	8279	0.0131	1	0.910	15	0.0428
			0.5	2	2106	4212											
	7-2	2	0.5	1	1904	3808	3843	7686	0.1	143280	5.36	7541	0.0119	1	0.910	15	0.0390
			0.5	2	1939	3878											
	7-3	2	0.5	1	2038	4076	4046	8092	0.1	143280	5.65	7947	0.0126	1	0.910	15	0.0411
			0.5	2	2018	4036											
	8-1	2	0.5	1	2126	4252	4173	8346	0.1	143280	5.82	8201	0.0130	1	0.910	15	0.0424
			0.5	2	2067	4134											
	8-2	2	0.5	1	2216	4432	4445	8890	0.1	143280	6.20	8745	0.0138	1	0.910	15	0.0452
			0.5	2	2230	4460											
	8-3	2	0.5	1	2077	4154	4172	8344	0.1	143280	5.82	8199	0.0130	1	0.910	15	0.0424
			0.5	2	2095	4190											

Assay Date	3/16/2005	Test Chemical ID	1/A	# Concentrations tested	8	Microsome type	Placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	5

Control Type	Portion	Average	SD
Full activity	Beginning	0.0437	0.0025
Full activity	End	0.0416	0.0016
Full activity	Overall	0.0427	0.0021
Background	Beginning	0.0001	1.46149E-05
Background	End	-0.0001	1.46149E-05
Background	Overall	0.0000	9.02894E-05
Positive	Beginning	0.0271	0.0012
Positive	End	0.0240	0.0004
Positive	Overall	0.0256	0.0019
Negative	Beginning	0.0447	0.0011
Negative	End	0.0403	0.0019
Negative	Overall	0.0425	0.0028

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/A	1	1	1.00E-03	-3.00	0.0002
1/A	1	2	1.00E-03	-3.00	0.0002
1/A	1	3	1.00E-03	-3.00	0.0003
1/A	2	1	1.00E-04	-4.00	0.0035
1/A	2	2	1.00E-04	-4.00	0.0034
1/A	2	3	1.00E-04	-4.00	0.0032
1/A	3	1	1.00E-05	-5.00	0.0172
1/A	3	2	1.00E-05	-5.00	0.0165
1/A	3	3	1.00E-05	-5.00	0.0170
1/A	4	1	5.00E-06	-5.30	0.0233
1/A	4	2	5.00E-06	-5.30	0.0244
1/A	4	3	5.00E-06	-5.30	0.0242
1/A	5	1	2.50E-06	-5.60	0.0318
1/A	5	2	2.50E-06	-5.60	0.0308
1/A	5	3	2.50E-06	-5.60	0.0287
1/A	6	1	1.00E-06	-6.00	0.0387
1/A	6	2	1.00E-06	-6.00	0.0377
1/A	6	3	1.00E-06	-6.00	0.0365
1/A	7	1	1.00E-07	-7.00	0.0428
1/A	7	2	1.00E-07	-7.00	0.0390
1/A	7	3	1.00E-07	-7.00	0.0411
1/A	8	1	1.00E-08	-8.00	0.0424
1/A	8	2	1.00E-08	-8.00	0.0452
1/A	8	3	1.00E-08	-8.00	0.0424

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-3.00	0.50	0.52	0.59
2	-4.00	8.29	7.93	7.59
3	-5.00	40.24	38.60	39.83
4	-5.30	54.53	57.14	56.71
5	-5.60	74.47	72.17	67.19
6	-6.00	90.74	88.20	85.51
7	-7.00	100.21	91.27	96.19
8	-8.00	99.26	105.85	99.24

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	24353	1236193	
2	0.0198	25900	1308081	
3	0.0196	25840	1318367	
4	0.0197	26631	1351827	
5	0.0198	27299	1378737	
			Average DPM/g soln	1318641
			SD	53903
			CV	4.09
			µCi/g soln	0.594

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.8	10		1080.00
Dilution A			100	10.80
Dilution B			10	1.08

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0422 g
Mass of dilution B used in substrate prep	4.5255 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.607737 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00672 µg/g soln.
µg/g soln.

a. µCi/g soln 0.594
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.607737 + 0.00672
= 0.614461 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.967 µCi/µg ASDN

614618 dpm/nmol

Assay Date	2/15/2005	Test Chemical ID	2/B	# Concentrations tested	8
Technician ID	TD	Replicate #	1	Microsome type	placental

Microsome ID 11343-7

	1	0.8	0.6	0.4	0.2	0.14	Blank	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.291	0.231	0.186	0.142	0.089	0.064	0.000	26	20	210000238
	0.298	0.242	0.218	0.153	0.074	0.066	0.000			
	0.282	0.235	0.201	0.164	0.074	0.067	0.000			

	11343-7
Samples:	0.091
	0.087
	0.099

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.291	0.291	0.0245	m, b	0.095	-0.003
0.8	14.3	25	0.00080	25	0.0200	0.236	0.236	0.0193	se_m, se_b	0.005	0.001
0.6	10.7	25	0.00060	25	0.0150	0.202	0.202	0.0160	r^2, se_y	0.987	0.001
0.4	7.1	25	0.00040	25	0.0099	0.152	0.153	0.0114	F, df	301	4
0.2	3.6	25	0.00020	25	0.0050	0.078	0.079	0.0043	ss_{reg}, ss_{resid}	0.000	0.000
0.14	2.5	25	0.00014	25	0.0035	0.065	0.065	0.0031			

Regression results are calculated using the function LINEST

				r^2=	0.987
Blank	0.000			m=	0.095
				b=	-0.003

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	Vol usomes prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL	
11343-7	0.091	0.091	0.005	25	100	5000	0.011	0.011	11.241	
11343-7	0.087	0.087	0.005	25	100	5000	0.010			
11343-7	0.099	0.099	0.006	25	100	5000	0.013			

Assay Date	2015/2/05	Test Chemical ID	2B	# Concentrations tested	8	Microbiome type	placenta	Microbiome ID	11343-T	Individual ID	TP	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed				
Sample type	Replicate/Treatment	Nominal test volume (mL)	Alkl. Volume(mL)	Alkl. #	DPM/mL	DPM/uL	Ave DPM/mL	Total DPM	Volume of substrate used on vesicle test tube (mL)	Total DPM in assay tube (DPM)	% conversion to product	Total DPM corrected for background (Background Tubes)	Input ^3H_2O formed	Volume diluted microbiome used in assay tube (mL)	Final (protein) in assay (mg/mL)	Incubation time (min)	Aromatase activity (fmol estrogen formed/mg protein/hr)
Full activity control	1	2	0.5	1	3758	7516	7493	14986	0.1	1.31864	11.35	14912	0.0241	1	0.011	15	0.0715
			0.5	2	3733	7466			0.1					1		15	
	2	2	0.5	1	3574	7148	7031	14062	0.1	1.31864	10.66	13988	0.0226	1	0.011	15	0.0671
			0.5	2	3452	6914			0.1					1		15	
	3	2	0.5	1	3920	7840	8947	17894	0.1	1.31864	9.47	11948	0.0194	1	0.011	15	0.0578
			0.5	2	4974	9954			0.1					1		15	
	4	2	0.5	1	3117	6234	6245	12489	0.1	1.31864	9.42	11938	0.0194	1	0.011	15	0.0696
			0.5	2	3129	6258			0.1					1		15	
Background control	1	2	0.5	1	38	76	76	152	0.1	1.31864	0.11	-4	0.0000	1	0.011	15	0.0000
			0.5	2	37	74			0.1					1		15	
	2	2	0.5	1	37	74	81	162	0.1	1.31864	0.12	5	0.0000	1	0.011	15	0.0000
			0.5	2	44	88			0.1					1		15	
	3	2	0.5	1	43	86	76	152	0.1	1.31864	0.11	-4	0.0000	1	0.011	15	0.0000
			0.5	2	38	76			0.1					1		15	
	4	2	0.5	1					0.1	1.31864		#VALUE!	#VALUE!	1	0.011	15	#VALUE!
			0.5	2					0.1					1		15	
Positive control	1	2	0.5	1	8920	17840	3915	7829	0.1	1.31864	2.93	7832	0.0122	1	0.011	15	0.0383
			0.5	2	8923	17846			0.1					1		15	
	2	2	0.5	1	3897	7794	3809	7618	0.1	1.31864	2.78	7454	0.0121	1	0.011	15	0.0368
			0.5	2	3741	7482			0.1					1		15	
	3	2	0.5	1	1722	3444	1653	3306	0.1	1.31864	0.33	887	0.0113	1	0.011	15	0.0092
			0.5	2	1761	3522			0.1					1		15	
	4	2	0.5	1	1923	3846	3939	7878	0.1	1.31864	5.82	7834	0.0120	1	0.011	15	0.0353
			0.5	2	1981	3962			0.1					1		15	
Negative Control	1	2	0.5	1	3606	7212	7159	14318	0.1	1.31864	10.50	14222	0.0220	1	0.011	15	0.0666
			0.5	2	3553	7106			0.1					1		15	
	2	2	0.5	1	3737	7474	7409	14818	0.1	1.31864	11.29	14864	0.0232	1	0.011	15	0.0717
			0.5	2	3772	7544			0.1					1		15	
	3	2	0.5	1	3678	7356	7351	14702	0.1	1.31864	11.16	14712	0.0224	1	0.011	15	0.0693
			0.5	2	3649	7298			0.1					1		15	
	4	2	0.5	1	3401	6802	6929	13859	0.1	1.31864	10.01	13704	0.0203	1	0.011	15	0.0650
			0.5	2	3458	6916			0.1					1		15	
2B	1-1	2	0.5	1	887	1774	1732	3464	0.1	1.31864	2.61	3290	0.0064	1	0.011	15	0.0155
			0.5	2	869	1738			0.1					1		15	
	1-2	2	0.5	1	854	1708	1713	3426	0.1	1.31864	2.60	3272	0.0063	1	0.011	15	0.0156
			0.5	2	858	1716			0.1					1		15	
	1-3	2	0.5	1	991	1982	2001	4002	0.1	1.31864	3.07	3948	0.0063	1	0.011	15	0.0188
			0.5	2	1019	2038			0.1					1		15	
	2-1	2	0.5	1	1048	1696	1050	2140	0.1	1.31864	1.62	1948	0.0030	1	0.011	15	0.0094
			0.5	2	525	1050			0.1					1		15	
	2-2	2	0.5	1	599	1198	1159	2318	0.1	1.31864	1.77	2184	0.0026	1	0.011	15	0.0105
			0.5	2	559	1118			0.1					1		15	
	2-3	2	0.5	1	553	1106	1112	2224	0.1	1.31864	1.68	2070	0.0024	1	0.011	15	0.0100
			0.5	2	557	1114			0.1					1		15	
	3-1	2	0.5	1	2153	4306	4196	8392	0.1	1.31864	6.39	8338	0.0135	1	0.011	15	0.0412
			0.5	2	2086	4172			0.1					1		15	
	3-2	2	0.5	1	2059	4118	4118	8236	0.1	1.31864	6.27	8065	0.0131	1	0.011	15	0.0389
			0.5	2	2059	4118			0.1					1		15	
	3-3	2	0.5	1	1842	3684	3771	7542	0.1	1.31864	5.64	7280	0.0119	1	0.011	15	0.0356
			0.5	2	1929	3858			0.1					1		15	
	4-1	2	0.5	1	2634	5268	5387	10844	0.1	1.31864	8.24	10660	0.0170	1	0.011	15	0.0506
			0.5	2	2673	5346			0.1					1		15	
	4-2	2	0.5	1	2506	5012	5435	10870	0.1	1.31864	8.35	10722	0.0174	1	0.011	15	0.0517
			0.5	2	2729	5458			0.1					1		15	
	4-3	2	0.5	1	2191	4382	4235	8470	0.1	1.31864	6.46	8326	0.0135	1	0.011	15	0.0399
			0.5	2	2044	4088			0.1					1		15	
	5-1	2	0.5	1	2052	4104	4212	8424	0.1	1.31864	6.42	8278	0.0130	1	0.011	15	0.0392
			0.5	2	2160	4320			0.1					1		15	
	5-2	2	0.5	1	1979	3958	4051	8102	0.1	1.31864	6.19	7966	0.0128	1	0.011	15	0.0383
			0.5	2	2123	4246			0.1					1		15	
	5-3	2	0.5	1	2133	4266	4172	8344	0.1	1.31864	6.36	8202	0.0132	1	0.011	15	0.0396
			0.5	2	2078	4156			0.1					1		15	
	6-1	2	0.5	1	2139	4278	4244	8488	0.1	1.31864	6.48	8344	0.0134	1	0.011	15	0.0402
			0.5	2	2105	4210			0.1					1		15	
	6-2	2	0.5	1	2200	4400	4496	8992	0.1	1.31864	6.87	8848	0.0142	1	0.011	15	0.0426
			0.5	2	2296	4592			0.1					1		15	
	6-3	2	0.5	1	2163	4326	4247	8494	0.1	1.31864	6.49	8350	0.0134	1	0.011	15	0.0402
			0.5	2	2168	4336			0.1					1		15	
	7-1	2	0.5	1	2492	4984	5307	10614	0.1	1.31864	8.12	10466	0.0168	1	0.011	15	0.0498
			0.5	2	2815	5630			0.1					1		15	
	7-2	2	0.5	1	2446	4892	5307	10614	0.1	1.31864	8.12	10466	0.0168	1	0.011	15	0.0498
			0.5	2	2815	5630			0.1					1		15	
	7-3	2	0.5	1	2446	4892	5307	10614	0.1	1.31864	8.12	10466	0.0168	1	0.011	15	0.0498
			0.5	2	2815	5630			0.1					1		15	
	8-1	2	0.5	1	2144	4288	4301	8602	0.1	1.31864	6.58	8458	0.0136	1	0.011	15	0.0407
			0.5	2	2158	4316			0.1					1		15	
	8-2	2	0.5	1	2266	4532	4532	9064	0.1	1.31864	6.94	8920	0.0144	1	0.011	15	0.0429
			0.5	2	2266	4532			0.1					1		15	
	8-3	2	0.5	1	2263	4526	4509	9018	0.1	1.31864	6.91	8874	0.0143	1	0.011	15	0.0426
			0.5	2	2263	4526			0.1					1		15	

Assay Date	2/15/2005	Test Chemical ID	2/B	# Concentrations tested		Microsome B type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0693	0.0031
Full activity	End	0.0586	0.0014
Full activity	Overall	0.0639	0.0065
Background	Beginning	0.0000	4.09387E-05
Background	End	#VALUE!	#VALUE!
Background	Overall	#VALUE!	#VALUE!
Positive	Beginning	0.0362	0.0002
Positive	End	0.0347	0.0022
Positive	Overall	0.0355	0.0015
Negative	Beginning	0.0702	0.0022
Negative	End	0.0677	0.0023
Negative	Overall	0.0689	0.0023

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/B	1	1	1.00E-04	-4.00	0.0159
2/B	1	2	1.00E-04	-4.00	0.0156
2/B	1	3	1.00E-04	-4.00	0.0186
2/B	2	1	1.00E-05	-5.00	0.0094
2/B	2	2	1.00E-05	-5.00	0.0105
2/B	2	3	1.00E-05	-5.00	0.0100
2/B	3	1	1.00E-06	-6.00	0.0412
2/B	3	2	1.00E-06	-6.00	0.0389
2/B	3	3	1.00E-06	-6.00	0.0351
2/B	4	1	2.50E-07	-6.60	0.0505
2/B	4	2	2.50E-07	-6.60	0.0517
2/B	4	3	2.50E-07	-6.60	0.0594
2/B	5	1	1.00E-07	-7.00	0.0592
2/B	5	2	1.00E-07	-7.00	0.0603
2/B	5	3	1.00E-07	-7.00	0.0576
2/B	6	1	1.00E-08	-8.00	0.0610
2/B	6	2	1.00E-08	-8.00	0.0616
2/B	6	3	1.00E-08	-8.00	0.0617
2/B	7	1	1.00E-09	-9.00	0.0603
2/B	7	2	1.00E-09	-9.00	0.0613
2/B	7	3	1.00E-09	-9.00	0.0608
2/B	8	1	1.00E-10	-10.00	0.0608
2/B	8	2	1.00E-10	-10.00	0.0602
2/B	8	3	1.00E-10	-10.00	0.0625

Percent of control values				
	Log[test substance]	Replicate		
Level		1	2	3
1	-4.00	24.83	24.70	29.04
2	-5.00	14.69	16.48	15.62
3	-6.00	64.44	60.88	54.95
4	-6.60	78.95	80.92	92.95
5	-7.00	92.61	94.25	90.06
6	-8.00	95.45	96.44	96.53
7	-9.00	94.25	95.90	95.14
8	-10.00	95.10	94.10	97.71

Assay Date	2/15/2005	Test Chemical ID	2/B	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #

Microsome Dilution Details

Dilution A	0.12	mL microsome Stock used
	6	mL total volume
	50	dilution factor
Dilution B	5	mL microsome Dilution A used
	50	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	11.241
Protein Concentration (dilution added to assay, mg/mL):	0.022482

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	1.00E-06
4	2.50E-07
5	1.00E-07
6	1.00E-08
7	1.00E-09
8	1.00E-10

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0196	24428	1246327	
2	0.0197	24786	1258173	
3	0.0197	26630	1351777	
4	0.0196	27209	1388214	
5	0.0197	27164	1378883	
			Average DPM/g soln	1324675
			SD	67586
			CV	5.10
			µCi/g soln	0.597

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	11.4	10		1140.00
Dilution A			100	11.40
Dilution B			10	1.14

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0473 g
Mass of dilution B used in substrate prep	4.5275 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.641377 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00675 µg/g soln.
µg/g soln.

a. µCi/g soln 0.597
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.641377 + 0.00675
= 0.648131 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.921 µCi/µg ASDN

585355 dpm/nmol

Assay Date 2/16/2005 Test Chemical ID 2/8 # Concentrations tested 8

Technician ID TD Replicate # 2 Microsome type placental Microsome ID 11343-7

	1	0.8	0.6	0.4	0.2	0.14	Blank	Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	0.304	0.245	0.191	0.143	0.042	0.043	0.000	28	20	21000238
	0.307	0.252	0.195	0.142	0.079	0.058	0.000			
	0.300	0.254	0.199	0.140	0.082	0.061	0.000			

Samples: 11343-7
0.078
0.083
0.082

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL
1	17.9	25	0.00100
0.8	14.3	25	0.00080
0.6	10.7	25	0.00060
0.4	7.1	25	0.00040
0.2	3.6	25	0.00020
0.14	2.5	25	0.00014

	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
	25	0.0251	0.304	0.304	0.0246
	25	0.0200	0.250	0.250	0.0200
	25	0.0150	0.195	0.195	0.0153
	25	0.0099	0.142	0.142	0.0108
	25	0.0050	0.067	0.067	0.0045
	25	0.0035	0.054	0.054	0.0033
Blank	0.000				

Variables	Regression results	
m, b	0.085	-0.001
se_m, se_b	0.003	0.001
r^2, se_y	0.996	0.001
F, df	977	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2_{adj} = 0.996
m = 0.085
b = -0.001

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usomes prep. (µL)	Final vol Diluted usomes (µL)	mg protein/µL Prep	average mg/µL	mg/mL
11343-7	0.078	0.078	0.005	25	100	5000	0.011	0.011	11.273
11343-7	0.083	0.083	0.006	25	100	5000	0.012		
11343-7	0.082	0.082	0.006	25	100	5000	0.011		

Assay Date	2/16/2005	Test Chemical ID	2/B	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	11.273
Protein Concentration (dilution added to assay, mg/mL):	0.022546

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date:	2/18/2004	Test Chemical ID: 298	# Concentrations tested:	# Microbeams/ tube:	Microbeads ID:	11312-7	Technician ID:	TD	Replicate #:	2

Sample ID		Calculate DPM in aqueous portion after extraction								Calculate % turnover		Calculate nmol ^3H_2O formed					
Sample type	Replicate Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (dilute)	% conversion to product	Total DPM corrected for background (background subtracted)	nmol ^3H_2O formed	Volume diluted microbeads in tube after assay (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Normalized activity (nmol hydrogen formed/mg protein/min)
Full activity control	1	2	0.5	1	2963	5926	5961	11923	0.1	1.32467	9.00	11794	0.6191	1	0.011	15	0.0559
			0.5	2	2979	5958			0.1					1		15	
	2	2	0.5	1	2999	5998	5965	11929	0.1	1.32467	9.01	11802	0.6202	1	0.011	15	0.0599
			0.5	2	3006	6012			0.1					1		15	
	3	2	0.5	1	2333	4666	4695	9872	0.1	1.32467	7.07	9743	0.5120	1	0.011	15	0.0467
			0.5	2	2354	4708			0.1					1		15	
	4	2	0.5	1	2546	5092	5051	10865	0.1	1.32467	7.98	10735	0.5643	1	0.011	15	0.0504
			0.5	2	2495	4971			0.1					1		15	
Background control	1	2	0.5	1	30	59	38	146	0.1	1.32467	0.11	17	0.0089	1	0.011	15	0.0007
			0.5	2	22	45			0.1					1		15	
	2	2	0.5	1	24	48	42	124	0.1	1.32467	0.09	-6	0.0030	1	0.011	15	0.0003
			0.5	2	33	66			0.1					1		15	
	3	2	0.5	1	26	50	67	134	0.1	1.32467	0.09	-15	0.0080	1	0.011	15	-0.0001
			0.5	2	32	64			0.1					1		15	
	4	2	0.5	1	36	72	68	136	0.1	1.32467	0.09	3	0.0006	1	0.011	15	0.0000
			0.5	2	32	64			0.1					1		15	
4-carbon control	1	2	0.5	1	1467	2934	2482	5873	0.1	1.32467	4.43	3742	0.1990	1	0.011	15	0.0249
			0.5	2	1479	2958			0.1					1		15	
	2	2	0.5	1	1643	3286	3082	8408	0.1	1.32467	4.89	5286	0.2810	1	0.011	15	0.0305
			0.5	2	1557	3114			0.1					1		15	
	3	2	0.5	1	1454	2908	2816	5809	0.1	1.32467	4.09	3692	0.1963	1	0.011	15	0.0238
			0.5	2	1478	2956			0.1					1		15	
	4	2	0.5	1	1407	2814	2825	5800	0.1	1.32467	4.07	3523	0.1954	1	0.011	15	0.0279
			0.5	2	1419	2837			0.1					1		15	
Negative Control	1	2	0.5	1	1755	3509	3124	11852	0.1	1.32467	8.34	10024	0.5187	1	0.011	15	0.0553
			0.5	2	2734	5468			0.1					1		15	
	2	2	0.5	1	2894	5788	5176	10353	0.1	1.32467	7.81	10026	0.5175	1	0.011	15	0.0519
			0.5	2	2077	5144			0.1					1		15	
	3	2	0.5	1	2035	4069	4035	9869	0.1	1.32467	7.09	9252	0.5116	1	0.011	15	0.0468
			0.5	2	2355	4710			0.1					1		15	
	4	2	0.5	1	2113	5000	5027	13014	0.1	1.32467	7.65	9926	0.5170	1	0.011	15	0.0506
			0.5	2	2913	5826			0.1					1		15	
2-B	1-1	2	0.5	1	654	1308	1278	3558	0.1	1.32467	1.89	2435	0.2541	1	0.011	15	0.0125
			0.5	2	626	1252			0.1					1		15	
	1-3	2	0.5	1	769	1538	1425	3968	0.1	1.32467	2.31	3038	0.2950	1	0.011	15	0.0148
			0.5	2	741	1482			0.1					1		15	
	1-3	2	0.5	1	843	1686	1667	2164	0.1	1.32467	1.63	2096	0.2035	1	0.011	15	0.0100
			0.5	2	829	1658			0.1					1		15	
	2-2	2	0.5	1	753	1506	1479	3963	0.1	1.32467	2.28	2930	0.2846	1	0.011	15	0.0143
			0.5	2	726	1452			0.1					1		15	
	2-2	2	0.5	1	756	1470	1462	2924	0.1	1.32467	2.21	2796	0.2049	1	0.011	15	0.0141
			0.5	2	727	1454			0.1					1		15	
	2-3	2	0.5	1	790	1580	1579	3140	0.1	1.32467	2.37	3012	0.2951	1	0.011	15	0.0152
			0.5	2	777	1554			0.1					1		15	
	3-1	2	0.5	1	1509	3018	3013	5813	0.1	1.32467	4.56	5510	0.5101	1	0.011	15	0.0299
			0.5	2	1519	3039			0.1					1		15	
	3-2	2	0.5	1	1538	3076	3071	6142	0.1	1.32467	4.64	5914	0.5193	1	0.011	15	0.0304
			0.5	2	1535	3070			0.1					1		15	
	3-3	2	0.5	1	1629	3259	3239	6960	0.1	1.32467	4.89	6432	0.5196	1	0.011	15	0.0325
			0.5	2	1651	3302			0.1					1		15	
	4-1	2	0.5	1	2063	4126	4061	9122	0.1	1.32467	6.13	7994	0.5143	1	0.011	15	0.0404
			0.5	2	1997	3994			0.1					1		15	
	4-2	2	0.5	1	2093	4186	4129	9214	0.1	1.32467	6.24	8086	0.5135	1	0.011	15	0.0409
			0.5	2	2015	4030			0.1					1		15	
	4-3	2	0.5	1	2038	4076	4129	9259	0.1	1.32467	6.25	8130	0.5155	1	0.011	15	0.0411
			0.5	2	2091	4182			0.1					1		15	
	5-1	2	0.5	1	2265	4530	4479	9848	0.1	1.32467	6.74	8839	0.5153	1	0.011	15	0.0448
			0.5	2	2214	4428			0.1					1		15	
	5-2	2	0.5	1	2088	4176	4097	9014	0.1	1.32467	6.19	8090	0.5152	1	0.011	15	0.0408
			0.5	2	2021	4042			0.1					1		15	
	5-3	2	0.5	1	2182	4364	4321	9464	0.1	1.32467	6.48	8506	0.5143	1	0.011	15	0.0428
			0.5	2	2139	4278			0.1					1		15	
	6-1	2	0.5	1	2432	4864	4880	9628	0.1	1.32467	7.24	9591	0.5184	1	0.011	15	0.0486
			0.5	2	2429	4846			0.1					1		15	
	6-2	2	0.5	1	2504	5008	5044	10088	0.1	1.32467	7.62	9960	0.5170	1	0.011	15	0.0503
			0.5	2	2580	5160			0.1					1		15	
	6-3	2	0.5	1	2450	4887	4914	9828	0.1	1.32467	7.42	9700	0.5186	1	0.011	15	0.0490
			0.5	2	2478	4956			0.1					1		15	
	7-1	2	0.5	1	2549	5098	5122	10244	0.1	1.32467	7.73	10116	0.5175	1	0.011	15	0.0511
			0.5	2	2573	5146			0.1					1		15	
	7-2	2	0.5	1	2359	4718	4912	9645	0.1	1.32467	7.31	9520	0.5181	1	0.011	15	0.0479
			0.5	2	2559	5118			0.1					1		15	
	7-3	2	0.5	1	2413	4826	4882	9656	0.1	1.32467	7.26	9476	0.5162	1	0.011	15	0.0479
			0.5	2	2391	4782			0.1					1		15	
	8-1	2	0.5	1	2733	5466	5547	11094	0.1	1.32467	8.37	10966	0.5187	1	0.011	15	0.0554
			0.5	2	2781	5562			0.1					1		15	
	8-2	2	0.5	1	2668	5336	5669	11339	0.1	1.32467	8.53	11172	0.5181	1	0.011	15	0.0564
			0.5	2	2841	5682			0.1					1		15	
	8-3	2	0.5	1	2603	5206	5449	10898	0.1	1.32467	8.23	10770	0.5164	1	0.011	15	0.0544
			0.5	2	2783	5565			0.1					1		15	

Assay Date	2/16/2005	Test Chemical ID	2/B	# Concentrations tested		Microsome S type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0596	0.0000
Full activity	End	0.0484	0.0025
Full activity	Overall	0.0540	0.0066
Background	Beginning	0.0000	5.71512E-05
Background	End	0.0000	7.85628E-05
Background	Overall	0.0000	5.96989E-05
Positive	Beginning	0.0297	0.0011
Positive	End	0.0283	0.0006
Postive	Overall	0.0290	0.0011
Negative	Beginning	0.0534	0.0025
Negative	End	0.0485	0.0024
Negative	Overall	0.0509	0.0035

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/B	1	1	1.00E-04	-4.00	0.0123
2/B	1	2	1.00E-04	-4.00	0.0148
2/B	1	3	1.00E-04	-4.00	0.0103
2/B	2	1	1.00E-05	-5.00	0.0143
2/B	2	2	1.00E-05	-5.00	0.0141
2/B	2	3	1.00E-05	-5.00	0.0152
2/B	3	1	2.50E-06	-5.60	0.0299
2/B	3	2	2.50E-06	-5.60	0.0304
2/B	3	3	2.50E-06	-5.60	0.0325
2/B	4	1	1.00E-06	-6.00	0.0404
2/B	4	2	1.00E-06	-6.00	0.0408
2/B	4	3	1.00E-06	-6.00	0.0411
2/B	5	1	5.00E-07	-6.30	0.0446
2/B	5	2	5.00E-07	-6.30	0.0468
2/B	5	3	5.00E-07	-6.30	0.0423
2/B	6	1	2.50E-07	-6.60	0.0485
2/B	6	2	2.50E-07	-6.60	0.0503
2/B	6	3	2.50E-07	-6.60	0.0490
2/B	7	1	1.00E-07	-7.00	0.0511
2/B	7	2	1.00E-07	-7.00	0.0476
2/B	7	3	1.00E-07	-7.00	0.0479
2/B	8	1	1.00E-08	-8.00	0.0554
2/B	8	2	1.00E-08	-8.00	0.0564
2/B	8	3	1.00E-08	-8.00	0.0544

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-4.00	22.70	27.38	19.03
2	-5.00	26.46	26.14	28.16
3	-5.60	55.26	56.23	60.14
4	-6.00	74.75	75.61	76.02
5	-6.30	82.55	86.64	78.23
6	-6.60	89.69	93.13	90.70
7	-7.00	94.59	88.08	88.52
8	-8.00	102.54	104.47	100.71

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	23232	1191385	
2	0.0196	24291	1239337	
3	0.0196	25581	1305153	
4	0.0195	25944	1330462	
5	0.0195	25988	1331692	
			Average DPM/g soln	1279606
			SD	61961
			CV	4.84
			µCi/g soln	0.576

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.8	10		1080.00
Dilution A			100	10.80
Dilution B			10	1.08

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0326 g
Mass of dilution B used in substrate prep	4.5191 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.607603 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00652 µg/g soln.
µg/g soln.

a. µCi/g soln	0.576
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.607603 + 0.00652
= 0.614127 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.939 µCi/µg ASDN

596748 dpm/nmol

Assay Date	2/17/2005	Test Chemical ID	2/8	# Concentrations tested	8
Technician ID	TD	Replicate #	3	Microsome type	placental

Microsome ID 11343-7

	1	0.8	0.6	0.4	0.2	0.14	Blank
Standards	0.295	0.262	0.189	0.142	0.056	0.050	0.000
	0.348	0.248	0.197	0.143	0.081	0.055	0.000
	0.292	0.249	0.199	0.138	0.073	0.062	0.000
Samples	11343-7						
	0.074						
	0.079						
	0.076						

Protein stock	mg Total protein	Total volume of stock (mL)	Protein stock ID
BSA	28	20	210000238

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.312	0.312	0.0250
0.8	14.3	25	0.00080	25	0.0200	0.253	0.253	0.0200
0.6	10.7	25	0.00060	25	0.0150	0.195	0.195	0.0150
0.4	7.1	25	0.00040	25	0.0089	0.141	0.141	0.0104
0.2	3.6	25	0.00020	25	0.0050	0.072	0.072	0.0045
0.14	2.5	25	0.00014	25	0.0035	0.062	0.062	0.0037
			Blank	0.000				

Variables		Regression results
m, b	0.085	-0.002
se_m, se_b	0.002	0.000
r^2, se_y	0.999	0.000
F, df	2789	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2= 0.999
m= 0.085
b= -0.002

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.074	0.074	0.005	25	100	5000	0.009	0.010	9.813
11343-7	0.079	0.079	0.005	25	100	5000	0.010		
11343-7	0.076	0.076	0.005	25	100	5000	0.010		

Assay Date	2/17/2005	Test Chemical ID	2/B	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	9.813
Protein Concentration (dilution added to assay, mg/mL):	0.019626

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date:	2/12/2005	Test Chemical ID:	2B	# Concentrations Tested:	6	Microsome Batch:	Research	Microsome ID:	11343-7	Technician ID:	RD	Replicate #:	3

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate pmol ^3H_2O formed					
Sample type	Replicate # Level	Nominal total volume (mL)	Aliq. Volume (mL)	Aliq. #	DPM/Aliq.	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (dnt/tot)	% conversion to product	Total DPM corrected for background (Background Tubes)	pmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (pmol estrogen formed/mg protein/min)
Full activity control	1	2	0.5	1	2488	4976	4917	9834	0.1	122961	7.81	9826	0.0156	1	0.619	15	0.0252
			0.5	2	2503	5006			0.1					1		15	
	2	2	0.5	1	2323	4646	4485	8969	0.1	122961	7.03	8954	0.0148	1	0.619	15	0.0238
			0.5	2	2162	4324			0.1					1		15	
	3	2	0.5	1	2301	4602	4657	9314	0.1	122961	7.42	9298	0.0151	1	0.619	15	0.0227
			0.5	2	2299	4598			0.1					1		15	
	4	2	0.5	1	2261	4522	4507	9014	0.1	122961	7.04	8998	0.0149	1	0.619	15	0.0226
			0.5	2	2246	4492			0.1					1		15	
Background control	1	2	0.5	1	37	74	79	158	0.1	122961	0.12	36	0.0001	1	0.619	15	0.0002
			0.5	2	43	86			0.1					1		15	
	2	2	0.5	1	26	52	51	102	0.1	122961	0.08	-15	0.0000	1	0.619	15	-0.0001
			0.5	2	25	50			0.1					1		15	
	3	2	0.5	1	33	66	53	106	0.1	122961	0.08	-11	0.0000	1	0.619	15	-0.0001
			0.5	2	26	52			0.1					1		15	
	4	2	0.5	1	22	44	52	104	0.1	122961	0.08	-11	0.0000	1	0.619	15	-0.0001
			0.5	2	30	60			0.1					1		15	
Positive control	1	2	0.5	1	1901	3802	3923	7846	0.1	122961	4.71	7830	0.0094	1	0.619	15	0.0150
			0.5	2	2045	4090			0.1					1		15	
	2	2	0.5	1	1443	2886	2991	5981	0.1	122961	4.52	5966	0.0095	1	0.619	15	0.0152
			0.5	2	1542	3084			0.1					1		15	
	3	2	0.5	1	1437	2874	2963	5926	0.1	122961	4.51	5910	0.0095	1	0.619	15	0.0151
			0.5	2	1544	3088			0.1					1		15	
	4	2	0.5	1	1410	2820	2770	5540	0.1	122961	4.33	5524	0.0091	1	0.619	15	0.0139
			0.5	2	1360	2720			0.1					1		15	
Negative Control	1	2	0.5	1	2537	5074	5064	10128	0.1	122961	7.93	10034	0.0159	1	0.619	15	0.0171
			0.5	2	2527	5054			0.1					1		15	
	2	2	0.5	1	2580	5160	5156	10092	0.1	122961	8.12	10276	0.0163	1	0.619	15	0.0256
			0.5	2	2576	5152			0.1					1		15	
	3	2	0.5	1	2442	4884	4853	9706	0.1	122961	7.59	9590	0.0151	1	0.619	15	0.0240
			0.5	2	2411	4822			0.1					1		15	
	4	2	0.5	1	2220	4440	4484	8968	0.1	122961	6.97	8852	0.0137	1	0.619	15	0.0221
			0.5	2	2228	4456			0.1					1		15	
2B	1-1	2	0.5	1	762	1524	1505	3009	0.1	122961	2.35	2864	0.0045	1	0.619	15	0.0075
			0.5	2	743	1486			0.1					1		15	
	1-2	2	0.5	1	781	1562	1549	3095	0.1	122961	2.42	2950	0.0059	1	0.619	15	0.0116
			0.5	2	767	1534			0.1					1		15	
	1-3	2	0.5	1	868	1736	1387	2774	0.1	122961	2.01	2458	0.0041	1	0.619	15	0.0140
			0.5	2	529	1058			0.1					1		15	
	2-1	2	0.5	1	754	1508	1508	3016	0.1	122961	2.36	2900	0.0048	1	0.619	15	0.0185
			0.5	2	754	1508			0.1					1		15	
	2-2	2	0.5	1	733	1466	1480	2960	0.1	122961	2.32	2852	0.0048	1	0.619	15	0.0182
			0.5	2	751	1502			0.1					1		15	
	2-3	2	0.5	1	755	1510	1512	3024	0.1	122961	2.36	2908	0.0049	1	0.619	15	0.0186
			0.5	2	757	1514			0.1					1		15	
	3-1	2	0.5	1	1583	3166	3135	6269	0.1	122961	4.97	6240	0.0105	1	0.619	15	0.0355
			0.5	2	1552	3104			0.1					1		15	
	3-2	2	0.5	1	1394	2788	2984	5968	0.1	122961	4.68	5872	0.0098	1	0.619	15	0.0334
			0.5	2	1496	2992			0.1					1		15	
	3-3	2	0.5	1	1401	2802	2906	5812	0.1	122961	4.56	5696	0.0092	1	0.619	15	0.0312
			0.5	3	1405	2810			0.1					1		15	
	4-1	2	0.5	1	1874	3748	3722	7444	0.1	122961	5.85	7328	0.0123	1	0.619	15	0.0417
			0.5	2	1844	3688			0.1					1		15	
	4-2	2	0.5	1	1900	3800	3960	7920	0.1	122961	6.22	7984	0.0127	1	0.619	15	0.0432
			0.5	2	1900	3800			0.1					1		15	
	4-3	2	0.5	1	1908	3816	3846	7692	0.1	122961	6.01	7526	0.0121	1	0.619	15	0.0409
			0.5	2	1950	3900			0.1					1		15	
	5-1	2	0.5	1	2071	4142	4189	8377	0.1	122961	6.60	8196	0.0137	1	0.619	15	0.0463
			0.5	2	2095	4190			0.1					1		15	
	5-2	2	0.5	1	2302	4604	4509	8759	0.1	122961	6.96	8564	0.0145	1	0.619	15	0.0493
			0.5	2	2155	4310			0.1					1		15	
	5-3	2	0.5	1	2140	4280	4258	8516	0.1	122961	6.80	8400	0.0141	1	0.619	15	0.0472
			0.5	2	2130	4260			0.1					1		15	
	6-1	2	0.5	1	2157	4314	4263	8516	0.1	122961	6.73	8290	0.0142	1	0.619	15	0.0484
			0.5	2	2161	4322			0.1					1		15	
	6-2	2	0.5	1	2184	4368	4342	8684	0.1	122961	6.91	8570	0.0146	1	0.619	15	0.0417
			0.5	2	2158	4316			0.1					1		15	
	6-3	2	0.5	1	2220	4440	4439	8878	0.1	122961	6.92	8744	0.0147	1	0.619	15	0.0498
			0.5	2	2399	4498			0.1					1		15	
	7-1	2	0.5	1	2268	4536	4598	9197	0.1	122961	7.18	9086	0.0152	1	0.619	15	0.0517
			0.5	2	2606	4656			0.1					1		15	
	7-2	2	0.5	1	2320	4640	4801	9602	0.1	122961	7.79	9490	0.0163	1	0.619	15	0.0541
			0.5	2	2703	4555			0.1					1		15	
	7-3	2	0.5	1	2290	4476	4519	9037	0.1	122961	7.29	8956	0.0153	1	0.619	15	0.0510
			0.5	2	2561	4596			0.1					1		15	
	8-1	2	0.5	1	2363	4726	4720	9440	0.1	122961	7.65	9304	0.0157	1	0.619	15	0.0532
			0.5	2	2357	4714			0.1					1		15	
	8-2	2	0.5	1	2342	4684	4691	9382	0.1	122961	7.33	9246	0.0155	1	0.619	15	0.0527
			0.5	2	2349	4698			0.1					1		15	
	8-3	2	0.5	1	2374	4748	4681	9362	0.1	122961	7.16	9048	0.0152	1	0.619	15	0.0515
			0.5	2	2307	4614			0.1					1		15	

Assay Date	2/17/2005	Test Chemical ID	2/B	# Concentrations tested		Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0533	0.0041
Full activity	End	0.0509	0.0004
Full activity	Overall	0.0521	0.0028
Background	Beginning	0.0001	0.000201253
Background	End	-0.0001	0
Background	Overall	0.0000	0.000135144
Positive	Beginning	0.0330	0.0011
Positive	End	0.0315	0.0009
Positive	Overall	0.0323	0.0012
Negative	Beginning	0.0578	0.0010
Negative	End	0.0523	0.0032
Negative	Overall	0.0551	0.0037

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/B	1	1	1.00E-04	-4.00	0.0165
2/B	1	2	1.00E-04	-4.00	0.0170
2/B	1	3	1.00E-04	-4.00	0.0140
2/B	2	1	1.00E-05	-5.00	0.0165
2/B	2	2	1.00E-05	-5.00	0.0162
2/B	2	3	1.00E-05	-5.00	0.0166
2/B	3	1	2.50E-06	-5.60	0.0355
2/B	3	2	2.50E-06	-5.60	0.0334
2/B	3	3	2.50E-06	-5.60	0.0313
2/B	4	1	1.00E-06	-6.00	0.0417
2/B	4	2	1.00E-06	-6.00	0.0432
2/B	4	3	1.00E-06	-6.00	0.0431
2/B	5	1	5.00E-07	-6.30	0.0467
2/B	5	2	5.00E-07	-6.30	0.0493
2/B	5	3	5.00E-07	-6.30	0.0478
2/B	6	1	2.50E-07	-6.60	0.0484
2/B	6	2	2.50E-07	-6.60	0.0477
2/B	6	3	2.50E-07	-6.60	0.0498
2/B	7	1	1.00E-07	-7.00	0.0517
2/B	7	2	1.00E-07	-7.00	0.0517
2/B	7	3	1.00E-07	-7.00	0.0510
2/B	8	1	1.00E-08	-8.00	0.0532
2/B	8	2	1.00E-08	-8.00	0.0527
2/B	8	3	1.00E-08	-8.00	0.0515

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-4.00	31.60	32.54	26.84
2	-5.00	31.67	31.14	31.75
3	-5.60	66.14	64.12	60.02
4	-6.00	80.03	82.82	82.73
5	-6.30	89.50	94.62	91.73
6	-6.60	92.82	91.49	95.49
7	-7.00	99.12	99.22	97.80
8	-8.00	102.04	101.19	98.79

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	24454	1241320	
2	0.0197	24849	1261371	
3	0.0200	26226	1311300	
4	0.0198	26797	1353384	
5	0.0195	27276	1398769	
			Average DPM/g soln	1313229
			SD	64795
			CV	4.93
			µCi/g soln	0.592

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	11	10		1100.00
Dilution A			100	11.00
Dilution B			10	1.10

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0303 g
Mass of dilution B used in substrate prep	4.517 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.618744 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00670 µg/g soln.
µg/g soln.

a. µCi/g soln	0.592
b. Specific activity of [^{3}H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.618744 + 0.00670
= 0.625440 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.946 µCi/µg ASDN

601350 dpm/nmol

Aromatase_Master_Version1 2 (3-15-05).xls; 1/9/2006;
Substrate Specific Activity 9:08 AM

Assay Date	3/15/2005	Test Chemical ID	2/B	# Concentrations tested	8		
Technician ID	TD	Replicate #	4	Microsome type	placental	Microsome ID	11343-7

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000238
	0.305	0.260	0.195	0.127	0.083	0.039	0.000			
	0.296	0.250	0.198	0.144	0.087	0.060	0.000			
	0.309	0.254	0.197	0.142	0.120	0.056	0.000			

Samples:	11343-7	QC1	QC2	QC3
	0.087	0.039	0.165	0.284
	0.087	0.041	0.166	0.277
	0.090			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.303	0.303	0.0247	m, b	0.090	-0.003
0.8	14.3	25	0.00080	25	0.0200	0.254	0.254	0.0203	se_m, se_b	0.003	0.001
0.6	10.7	25	0.00060	25	0.0150	0.196	0.196	0.0151	r^2, se_y	0.995	0.001
0.4	7.1	25	0.00040	25	0.0099	0.138	0.138	0.0098	F, df	766	4
0.2	3.6	25	0.00020	25	0.0050	0.097	0.097	0.0061	SS_{reg}, SS_{total}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.052	0.052	0.0020			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.995					
		m=	0.090					
		b=	-0.003					

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.087	0.087	0.005	25	100	5000	0.010	0.011	10.537
11343-7	0.087	0.087	0.005	25	100	5000	0.010		
11343-7	0.090	0.090	0.005	25	100	5000	0.011		
QC1	0.039	0.039	0.001	25	1	1	0.000	0.000	0.037
QC1	0.041	0.041	0.001	25	1	1	0.000		
QC1									
QC2	0.165	0.165	0.012	25	1	1	0.000	0.000	0.490
QC2	0.166	0.166	0.012	25	1	1	0.000		
QC2									
QC3	0.284	0.284	0.023	25	1	1	0.001	0.001	0.906
QC3	0.277	0.277	0.022	25	1	1	0.001		
QC3									

Assay Date	3/15/2005	Test Chemical ID	2/8	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	4

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	10.537
Protein Concentration (dilution added to assay, mg/mL):	0.021074

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	2.50E-06
4	1.00E-06
5	5.00E-07
6	2.50E-07
7	1.00E-07
8	1.00E-08

Assay Date	3/15/2005	Test Chemical ID	2/5	# Cocktail tubes tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	4

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % transfer			Calculate $nmol^3H_2O$ formed						
Sample Type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM Blk	DPM/mL	Ave. DPM/mL	Total DPM	Volume of substrate added to assay tube (mL)	total DPM in assay tube (dpm)	% conversion to product	Total DPM corrected for background (background - Tube)	$nmol^3H_2O$ formed	Volume dilute microsomes used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (pmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	1809	3618	3640	7280	0.1	1313723	5.54	7189	0.0119	1	0.011	15	0.0377
			0.5	2	1831	3662			0.1					1		15	
	2	2	0.5	1	1787	3574	3684	7368	0.1	1313723	5.46	7047	0.0117	1	0.011	15	0.0371
			0.5	2	1797	3594			0.1					1		15	
	3	2	0.5	1	1731	3462	3506	7012	0.1	1313723	5.34	6891	0.0115	1	0.011	15	0.0363
			0.5	2	1775	3550			0.1					1		15	
	4	2	0.5	1	1708	3416	3417	6834	0.1	1313723	5.20	6713	0.0112	1	0.011	15	0.0353
			0.5	2	1709	3418			0.1					1		15	
Background control	1	2	0.5	1	32	64	66	132	0.1	1313723	0.10	11	0.0000	1	0.011	15	0.0001
			0.5	2	34	68			0.1					1		15	
	2	2	0.5	1	28	56	58	116	0.1	1313723	0.09	-6	0.0000	1	0.011	15	0.0000
			0.5	2	30	60			0.1					1		15	
	3	2	0.5	1	29	58	59	118	0.1	1313723	0.09	-3	0.0000	1	0.011	15	0.0000
			0.5	2	30	60			0.1					1		15	
	4	2	0.5	1	30	60	59	118	0.1	1313723	0.09	-3	0.0000	1	0.011	15	0.0000
			0.5	2	29	58			0.1					1		15	
Positive control	1	2	0.5	1	1208	2416	2421	4842	0.1	1313723	3.69	4721	0.0079	1	0.011	15	0.0245
			0.5	2	1213	2426			0.1					1		15	
	2	2	0.5	1	1222	2444	2436	4880	0.1	1313723	3.71	4749	0.0079	1	0.011	15	0.0246
			0.5	2	1218	2436			0.1					1		15	
	3	2	0.5	1	1142	2284	2276	4552	0.1	1313723	3.47	4431	0.0074	1	0.011	15	0.0230
			0.5	2	1134	2268			0.1					1		15	
	4	2	0.5	1	1409	2818	2772	5544	0.1	1313723	4.22	5423	0.0090	1	0.011	15	0.0280
			0.5	2	1363	2726			0.1					1		15	
Negative Control	1	2	0.5	1	1698	3396	3416	6832	0.1	1313723	5.20	6711	0.0112	1	0.011	15	0.0353
			0.5	2	1718	3436			0.1					1		15	
	2	2	0.5	1	1656	3312	3312	6624	0.1	1313723	5.04	6503	0.0108	1	0.011	15	0.0342
			0.5	2	1657	3314			0.1					1		15	
	3	2	0.5	1	1561	3122	3114	6228	0.1	1313723	4.74	6107	0.0102	1	0.011	15	0.0321
			0.5	2	1553	3106			0.1					1		15	
	4	2	0.5	1	1688	3376	3358	6716	0.1	1313723	5.11	6595	0.0110	1	0.011	15	0.0347
			0.5	2	1672	3340			0.1					1		15	
2/8	1-1	2	0.5	1	658	1316	1299	2598	0.1	1313723	1.98	2477	0.0041	1	0.011	15	0.0130
			0.5	2	641	1282			0.1					1		15	
	1-2	2	0.5	1	725	1450	1454	2908	0.1	1313723	2.21	2787	0.0046	1	0.011	15	0.0147
			0.5	2	728	1456			0.1					1		15	
	1-3	2	0.5	1	674	1350	1347	2694	0.1	1313723	2.05	2573	0.0043	1	0.011	15	0.0136
			0.5	2	672	1344			0.1					1		15	
	2-1	2	0.5	1	836	1672	1662	3324	0.1	1313723	2.53	3203	0.0053	1	0.011	15	0.0168
			0.5	2	826	1652			0.1					1		15	
	2-2	2	0.5	1	776	1552	1581	3162	0.1	1313723	2.41	3041	0.0051	1	0.011	15	0.0160
			0.5	2	805	1610			0.1					1		15	
	2-3	2	0.5	1	772	1544	1822	3644	0.1	1313723	2.32	2923	0.0049	1	0.011	15	0.0154
			0.5	2	749	1498			0.1					1		15	
	3-1	2	0.5	1	1291	2582	2619	5238	0.1	1313723	3.99	5117	0.0085	1	0.011	15	0.0269
			0.5	2	1328	2656			0.1					1		15	
	3-2	2	0.5	1	1298	2596	2663	5326	0.1	1313723	3.96	5086	0.0085	1	0.011	15	0.0268
			0.5	2	1365	2730			0.1					1		15	
	3-3	2	0.5	1	1291	2582	2615	5230	0.1	1313723	3.98	5109	0.0085	1	0.011	15	0.0269
			0.5	2	1324	2648			0.1					1		15	
	4-1	2	0.5	1	1693	3386	3181	6362	0.1	1313723	4.84	6241	0.0104	1	0.011	15	0.0328
			0.5	2	1588	3176			0.1					1		15	
	4-2	2	0.5	1	1555	3110	3097	6194	0.1	1313723	4.72	6073	0.0101	1	0.011	15	0.0319
			0.5	2	1542	3084			0.1					1		15	
	4-3	2	0.5	1	1472	2944	2959	5918	0.1	1313723	4.51	5797	0.0096	1	0.011	15	0.0305
			0.5	2	1487	2974			0.1					1		15	
	5-1	2	0.5	1	1553	3106	3117	6234	0.1	1313723	4.75	6113	0.0102	1	0.011	15	0.0322
			0.5	2	1564	3128			0.1					1		15	
	5-2	2	0.5	1	1610	3220	3231	6462	0.1	1313723	4.92	6341	0.0105	1	0.011	15	0.0334
			0.5	2	1621	3242			0.1					1		15	
	5-3	2	0.5	1	1624	3248	3248	6496	0.1	1313723	4.96	6375	0.0106	1	0.011	15	0.0336
			0.5	2	1614	3228			0.1					1		15	
	6-1	2	0.5	1	1416	2832	2367	5804	0.1	1313723	4.82	5812	0.0097	1	0.011	15	0.0306
			0.5	2	1491	2982			0.1					1		15	
	6-2	2	0.5	1	1595	3190	3221	6442	0.1	1313723	4.91	6321	0.0105	1	0.011	15	0.0333
			0.5	2	1626	3252			0.1					1		15	
	6-3	2	0.5	1	1665	3330	3375	6750	0.1	1313723	5.14	6629	0.0110	1	0.011	15	0.0349
			0.5	2	1710	3420			0.1					1		15	
	7-1	2	0.5	1	1680	3360	3371	6742	0.1	1313723	5.13	6621	0.0110	1	0.011	15	0.0348
			0.5	2	1691	3382			0.1					1		15	
	7-2	2	0.5	1	1569	3138	3121	6242	0.1	1313723	4.75	6121	0.0102	1	0.011	15	0.0322
			0.5	2	1552	3104			0.1					1		15	
	7-3	2	0.5	1	1655	3310	3306	6612	0.1	1313723	5.03	6491	0.0108	1	0.011	15	0.0341
			0.5	2	1651	3302			0.1					1		15	
	8-1	2	0.5	1	1681	3362	3369	6738	0.1	1313723	5.13	6617	0.0110	1	0.011	15	0.0348
			0.5	2	1684	3370			0.1					1		15	
	8-2	2	0.5	1	1677	3354	3395	6790	0.1	1313723	5.17	6669	0.0111	1	0.011	15	0.0351
			0.5	2	1718	3436			0.1					1		15	
	8-3	2	0.5	1	1767	3534	3510	7020	0.1	1313723	5.35	6899	0.0115	1	0.011	15	0.0363
			0.5	2	1743	3486			0.1					1		15	

Assay Date	3/15/2005	Test Chemical ID	2/B	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	4

Control Type	Portion	Average	SD
Full activity	Beginning	0.0374	0.0004
Full activity	End	0.0358	0.0007
Full activity	Overall	0.0366	0.0010
Background	Beginning	0.0000	5.95168E-05
Background	End	0.0000	0
Background	Overall	0.0000	3.86952E-05
Positive	Beginning	0.0249	0.0001
Positive	End	0.0259	0.0037
Positive	Overall	0.0254	0.0022
Negative	Beginning	0.0348	0.0008
Negative	End	0.0334	0.0018
Negative	Overall	0.0341	0.0014

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/B	1	1	1.00E-04	-4.00	0.0130
2/B	1	2	1.00E-04	-4.00	0.0147
2/B	1	3	1.00E-04	-4.00	0.0135
2/B	2	1	1.00E-05	-5.00	0.0168
2/B	2	2	1.00E-05	-5.00	0.0160
2/B	2	3	1.00E-05	-5.00	0.0154
2/B	3	1	2.50E-06	-5.60	0.0269
2/B	3	2	2.50E-06	-5.60	0.0268
2/B	3	3	2.50E-06	-5.60	0.0269
2/B	4	1	1.00E-06	-6.00	0.0328
2/B	4	2	1.00E-06	-6.00	0.0319
2/B	4	3	1.00E-06	-6.00	0.0305
2/B	5	1	5.00E-07	-6.30	0.0322
2/B	5	2	5.00E-07	-6.30	0.0334
2/B	5	3	5.00E-07	-6.30	0.0335
2/B	6	1	2.50E-07	-6.60	0.0306
2/B	6	2	2.50E-07	-6.60	0.0333
2/B	6	3	2.50E-07	-6.60	0.0349
2/B	7	1	1.00E-07	-7.00	0.0348
2/B	7	2	1.00E-07	-7.00	0.0322
2/B	7	3	1.00E-07	-7.00	0.0341
2/B	8	1	1.00E-08	-8.00	0.0348
2/B	8	2	1.00E-08	-8.00	0.0351
2/B	8	3	1.00E-08	-8.00	0.0363

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-4.00	35.63	40.09	37.01
2	-5.00	46.07	43.74	42.04
3	-5.60	73.60	73.14	73.48
4	-6.00	89.77	87.35	83.38
5	-6.30	87.93	91.20	91.69
6	-6.60	83.61	90.92	95.35
7	-7.00	95.23	88.04	93.36
8	-8.00	95.17	95.92	99.23

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0200	26198	1309900	
2	0.0204	28946	1418922	
3	0.0202	29667	1468663	
4	0.0203	29736	1464828	
5	0.0204	36406	1784608	
			Average DPM/g soln	1489384
			SD	177037
			CV	11.89
			µCi/g soln	0.671

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	11.4	10		1140.00
Dilution A			100	11.40
Dilution B			10	1.14

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0517 g
Mass of dilution B used in substrate prep	4.5188 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.639794 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00759 µg/g soln.
µg/g soln.

a. µCi/g soln 0.671
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 266.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.639794 + 0.00759
= 0.647389 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.036 µCi/µg ASDN

658892 dpm/nmol

Assay Date 2/22/2005 | Test Chemical ID 1/C | # Concentrations tested 8

Technician ID TD | Replicate # 1 | Microsome type placental | Microsome ID 11343-7

	1	0.8	0.6	0.4	0.2	0.14	Blank
Standards:	0.294	0.256	0.198	0.143	0.080	0.060	0.000
	0.299	0.258	0.201	0.149	0.072	0.054	0.000
	0.298	0.247	0.194	0.135	0.086	0.034	0.000

Protein stock (mg BSA): 25 | Total volume of stock (mL): 20 | Protein stock ID: 210000238

Samples: 11343-7
0.086
0.088
0.091

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.297	0.297	0.0242
0.8	14.3	25	0.00080	25	0.0200	0.252	0.253	0.0204
0.6	10.7	25	0.00060	25	0.0150	0.198	0.198	0.0165
0.4	7.1	25	0.00040	25	0.0099	0.142	0.142	0.0107
0.2	3.6	25	0.00020	25	0.0050	0.079	0.079	0.0052
0.14	2.5	25	0.00014	25	0.0035	0.049	0.049	0.0026
				Blank	0.000			

Variables	Regression results	
m, b	0.087	-0.002
se_m, se_b	0.004	0.001
r^2, se_y	0.993	0.001
F, df	551	4
ss_{reg}, ss_{resid}	0.000	0.000

r^2 = 0.993
m= 0.087
b= -0.002

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.086	0.086	0.008	25	100	5000	0.012	0.012	11.964
11343-7	0.088	0.088	0.008	25	100	5000	0.012		
11343-7	0.091	0.091	0.008	25	100	5000	0.012		

Assay Date	2/22/2005	Test Chemical ID	1/C	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	11.964
Protein Concentration (dilution added to assay, mg/mL):	0.023928

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	1.00E-06
5	1.00E-07
6	1.00E-08
7	1.00E-09
8	1.00E-10

Assay Date:	2/22/2006	Test Chemical ID: 1AC	# Concentrations tested:	8	Microsome type:	placental	Microsome ID:	113403-2	Technician ID:	SD	Replicate #:	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate Level	Nominal total volume (mL)	Aliq. Volume (mL)	Aliq. #	DPM/Aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Total DPM in a assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	Volume diluted microsomes added in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min)
Full activity control	1	2	0.5	1	2923	5846	5887	11764	0.1	148938	7.89	11596	0.0176	1	0.612	15	0.0480
			0.5	2	2954	5908			0.1					1		15	
	2	2	0.5	1	3066	6132	6124	12248	0.1	148938	8.27	12156	0.0184	1	0.612	15	0.0501
			0.5	2	3062	6124			0.1					1		15	
	3	2	0.5	1	2727	5455	5430	10860	0.1	148938	7.29	10768	0.0163	1	0.612	15	0.0443
			0.5	2	2687	5374			0.1					1		15	
	4	2	0.5	1	2636	5272	5352	10684	0.1	148938	6.98	10590	0.0159	1	0.612	15	0.0433
			0.5	2	2760	5519			0.1					1		15	
Background control	1	2	0.5	1	96	22	60	120	0.1	148938	0.11	2	0.0009	1	0.612	15	0.0000
			0.5	2	44	88			0.1					1		15	
	2	2	0.5	1	48	96	96	192	0.1	148938	0.15	48	0.0001	1	0.612	15	0.0002
			0.5	2	51	102			0.1					1		15	
	3	2	0.5	1	34	68	73	146	0.1	148938	0.16	-42	0.0000	1	0.612	15	-0.0001
			0.5	2	101	78			0.1					1		15	
	4	2	0.5	1	34	67	64	128	0.1	148938	0.09	-36	0.0000	1	0.612	15	-0.0001
			0.5	2	30	60			0.1					1		15	
Positive control	1	2	0.5	1	1611	3222	3154	6307	0.1	148938	4.25	6231	0.0095	1	0.612	15	0.0263
			0.5	2	1577	3154			0.1					1		15	
	2	2	0.5	1	1531	3062	3061	6122	0.1	148938	4.11	6964	0.0091	1	0.612	15	0.0382
			0.5	2	1540	3080			0.1					1		15	
	3	2	0.5	1	1518	3146	3168	6336	0.1	148938	4.25	6178	0.0094	1	0.612	15	0.0291
			0.5	2	1585	3190			0.1					1		15	
	4	2	0.5	1	1597	3194	3188	6376	0.1	148938	4.28	6218	0.0094	1	0.612	15	0.0290
			0.5	2	1591	3182			0.1					1		15	
Negative Control	1	2	0.5	1	4209	8418	5971	11942	0.1	148938	0.15	11884	0.0182	1	0.612	15	0.0497
			0.5	2	2961	5922			0.1					1		15	
	2	2	0.5	1	2880	5760	5740	11480	0.1	148938	7.71	11392	0.0175	1	0.612	15	0.0479
			0.5	2	2910	5820			0.1					1		15	
	3	2	0.5	1	2880	5760	5800	11380	0.1	148938	7.64	11322	0.0170	1	0.612	15	0.0475
			0.5	2	2916	5830			0.1					1		15	
	4	2	0.5	1	2764	5528	5551	11102	0.1	148938	7.65	11364	0.0167	1	0.612	15	0.0464
			0.5	2	2777	5554			0.1					1		15	
NC	1-1	2	0.5	1	30	60	64	128	0.1	148938	0.09	-36	0.0000	1	0.612	15	-0.0001
			0.5	2	34	68			0.1					1		15	
	1-2	2	0.5	1	34	68	66	132	0.1	148938	0.09	-36	0.0000	1	0.612	15	-0.0001
			0.5	2	33	66			0.1					1		15	
	1-3	2	0.5	1	32	64	66	132	0.1	148938	0.09	-36	0.0000	1	0.612	15	-0.0001
			0.5	2	36	72			0.1					1		15	
	2-1	2	0.5	1	31	62	64	128	0.1	148938	0.07	-38	-0.0001	1	0.612	15	-0.0002
			0.5	2	33	66			0.1					1		15	
	2-2	2	0.5	1	32	64	64	128	0.1	148938	0.09	-36	0.0000	1	0.612	15	-0.0001
			0.5	2	32	64			0.1					1		15	
	2-3	2	0.5	1	27	54	65	130	0.1	148938	0.09	-29	0.0000	1	0.612	15	-0.0001
			0.5	2	38	76			0.1					1		15	
	3-1	2	0.5	1	38	76	76	152	0.1	148938	0.10	-2	0.0000	1	0.612	15	0.0000
			0.5	2	38	76			0.1					1		15	
	3-2	2	0.5	1	30	60	59	118	0.1	148938	0.05	-49	-0.0001	1	0.612	15	-0.0002
			0.5	2	29	58			0.1					1		15	
	3-3	2	0.5	1	32	64	66	132	0.1	148938	0.09	-28	0.0000	1	0.612	15	-0.0001
			0.5	2	33	66			0.1					1		15	
	4-1	2	0.5	1	38	76	74	148	0.1	148938	0.10	-10	0.0000	1	0.612	15	-0.0001
			0.5	2	36	72			0.1					1		15	
	4-2	2	0.5	1	42	84	94	188	0.1	148938	0.11	10	0.0000	1	0.612	15	0.0000
			0.5	2	47	94			0.1					1		15	
	4-3	2	0.5	1	39	78	89	178	0.1	148938	0.12	25	0.0000	1	0.612	15	0.0001
			0.5	2	50	100			0.1					1		15	
	5-1	2	0.5	1	91	182	177	354	0.1	148938	0.24	186	0.0003	1	0.612	15	0.0008
			0.5	2	86	172			0.1					1		15	
	5-2	2	0.5	1	96	192	194	392	0.1	148938	0.26	234	0.0003	1	0.612	15	0.0009
			0.5	2	97	194			0.1					1		15	
	5-3	2	0.5	1	177	354	353	706	0.1	148938	0.47	545	0.0006	1	0.612	15	0.0018
			0.5	2	180	352			0.1					1		15	
	6-1	2	0.5	1	560	1120	1105	2210	0.1	148938	1.49	2054	0.0030	1	0.612	15	0.0077
			0.5	2	546	1092			0.1					1		15	
	6-2	2	0.5	1	502	1004	1005	2010	0.1	148938	1.34	1864	0.0028	1	0.612	15	0.0069
			0.5	2	509	1016			0.1					1		15	
	6-3	2	0.5	1	557	1114	1093	2186	0.1	148938	1.45	2008	0.0030	1	0.612	15	0.0075
			0.5	2	529	1058			0.1					1		15	
	7-1	2	0.5	1	2042	4084	4074	8148	0.1	148938	5.47	7910	0.0120	1	0.612	15	0.0304
			0.5	2	1982	3964			0.1					1		15	
	7-2	2	0.5	1	2049	4098	4080	8159	0.1	148938	5.48	7922	0.0120	1	0.612	15	0.0305
			0.5	2	2031	4062			0.1					1		15	
	7-3	2	0.5	1	2017	4034	4049	8098	0.1	148938	5.44	7940	0.0121	1	0.612	15	0.0302
			0.5	2	2032	4064			0.1					1		15	
	8-1	2	0.5	1	2844	5688	5551	11102	0.1	148938	7.10	10404	0.0158	1	0.612	15	0.0401
			0.5	2	2947	5894			0.1					1		15	
	8-2	2	0.5	1	2747	5494	5486	10932	0.1	148938	7.34	10774	0.0164	1	0.612	15	0.0466
			0.5	2	2719	5438			0.1					1		15	
	8-3	2	0.5	1	2806	5612	5561	11122	0.1	148938	7.47	10964	0.0166	1	0.612	15	0.0464
			0.5	2	2755	5510			0.1					1		15	

Assay Date	2/22/2005	Test Chemical ID	1/C	# Concentrations tested		Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0502	0.0017
Full activity	End	0.0443	0.0014
Full activity	Overall	0.0472	0.0037
Background	Beginning	0.0001	0.00011362
Background	End	-0.0001	5.38202E-05
Background	Overall	0.0000	0.000126628
Positive	Beginning	0.0258	0.0008
Positive	End	0.0262	0.0001
Positive	Overall	0.0260	0.0005
Negative	Beginning	0.0493	0.0020
Negative	End	0.0469	0.0007
Negative	Overall	0.0481	0.0018

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/C	1	1	1.00E-03	-3.00	-0.0001
1/C	1	2	1.00E-03	-3.00	-0.0001
1/C	1	3	1.00E-03	-3.00	-0.0001
1/C	2	1	1.00E-04	-4.00	-0.0002
1/C	2	2	1.00E-04	-4.00	-0.0001
1/C	2	3	1.00E-04	-4.00	-0.0001
1/C	3	1	1.00E-05	-5.00	0.0000
1/C	3	2	1.00E-05	-5.00	-0.0002
1/C	3	3	1.00E-05	-5.00	-0.0001
1/C	4	1	1.00E-06	-6.00	-0.0001
1/C	4	2	1.00E-06	-6.00	0.0000
1/C	4	3	1.00E-06	-6.00	0.0001
1/C	5	1	1.00E-07	-7.00	0.0008
1/C	5	2	1.00E-07	-7.00	0.0009
1/C	5	3	1.00E-07	-7.00	0.0010
1/C	6	1	1.00E-08	-8.00	0.0087
1/C	6	2	1.00E-08	-8.00	0.0084
1/C	6	3	1.00E-08	-8.00	0.0085
1/C	7	1	1.00E-09	-9.00	0.0334
1/C	7	2	1.00E-09	-9.00	0.0338
1/C	7	3	1.00E-09	-9.00	0.0336
1/C	8	1	1.00E-10	-10.00	0.0441
1/C	8	2	1.00E-10	-10.00	0.0456
1/C	8	3	1.00E-10	-10.00	0.0464

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-3.00	-0.27	-0.23	-0.23
2	-4.00	-0.45	-0.27	-0.25
3	-5.00	-0.02	-0.36	-0.25
4	-6.00	-0.14	0.09	0.16
5	-7.00	1.75	2.00	2.22
6	-8.00	18.38	17.85	17.97
7	-9.00	70.80	71.62	71.07
8	-10.00	93.30	96.43	98.13

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0198	26369	1331768	
2	0.0198	26359	1331263	
3	0.0196	28003	1428724	
4	0.0197	28101	1426447	
5	0.0197	29758	1510558	
			Average DPM/g soln	1405752
			SD	75767
			CV	5.39
			µCi/g soln	0.633

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.8	10		1080.00
Dilution A			100	10.80
Dilution B			10	1.08

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0114 g
Mass of dilution B used in substrate prep	4.4951 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.605975 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00717 µg/g soln.
µg/g soln.

a. µCi/g soln 0.633
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.605975 + 0.00717
= 0.613143 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.033 µCi/µg ASDN

656629 dpm/nmol

Assay Date	2/23/2005	Test Chemical ID	1/C	# Concentrations tested	8				
Technician ID	TD	Replicate #	2	Microsome type	placental	Microsome ID	11343-7		

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	
	0.285	0.250	0.188	0.140	0.070	0.028	0.000			
	0.300	0.248	0.198	0.146	0.080	0.017	0.000			
	0.294	0.250	0.199	0.143	0.074	0.057	0.000			
Samples:	11343-7	QC1	QC2	QC3						
	0.078	0.044	0.162	0.273						
	0.078	0.043	0.165	0.293						
	0.078									

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.293	0.293	0.0240	m, b	0.096	-0.001
0.8	14.3	25	0.00080	25	0.0200	0.249	0.249	0.0202	st_m, ss_e	0.004	0.001
0.6	10.7	25	0.00060	25	0.0150	0.195	0.195	0.0158	R^2, ss_y	0.989	0.001
0.4	7.1	25	0.00040	25	0.0099	0.143	0.143	0.0111	F, df	371	4
0.2	3.6	25	0.00020	25	0.0050	0.074	0.074	0.0052	ss_{reg}, ss_{resid}	0.060	0.000
0.11	2	25	0.00011	25	0.0028	0.034	0.034	0.0017			

Regression results are calculated using the function LINEST

Blank	0.000			R^2=	0.989								
				m=	0.096								
				b=	-0.001								

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.078	0.078	0.006	25	100	5000	0.011	0.011	11.050
11343-7	0.078	0.078	0.006	25	100	5000	0.011		
11343-7	0.078	0.078	0.006	25	100	5000	0.011		
QC1	0.044	0.044	0.003	25	1	1	0.000	0.000	0.101
QC1	0.043	0.043	0.003	25	1	1	0.000		
QC1									
QC2	0.162	0.162	0.013	25	1	1	0.001	0.001	0.515
QC2	0.165	0.165	0.013	25	1	1	0.001		
QC2									
QC3	0.273	0.273	0.022	25	1	1	0.001	0.001	0.925
QC3	0.293	0.293	0.024	25	1	1	0.001		
QC3									

Assay Date	2/23/2005	Test Chemical ID	1/C	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	11.05
Protein Concentration (dilution added to assay, mg/mL):	0.0221

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-06
3	1.00E-07
4	5.00E-08
5	2.50E-08
6	1.00E-08
7	2.50E-09
8	1.00E-09

Assay Date:	2/23/2005	Test Chemical(s):	HC	# Concentrations tested:	5	Microsome type:	placental	Microsome ID:	11343-2	Technician ID:	TD	Replicate #:	2

Example ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate Level	Nominal total volume(mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used in assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Taken)	nmol ^3H_2O formed	Volume diluted microsom es used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen form ed/ g protein/ h
Full activity control	1	2	0.5	1	2369	4738	5795	11592	0.1	140575	8.25	11595	0.0205	1	0.611	15	0.0837
			0.5	2	3390	6784			0.1					1		15	
	2	2	0.5	1	3567	7134	6901	13802	0.1	140575	9.82	13805	0.0211	1	0.611	15	0.0858
			0.5	2	3434	6868			0.1					1		15	
	3	2	0.5	1	2930	5860	5903	11804	0.1	140575	8.40	11807	0.0177	1	0.611	15	0.0535
			0.5	2	2972	5944			0.1					1		15	
	4	2	0.5	1	2795	5462	5265	10973	0.1	140575	7.65	10976	0.0163	1	0.611	15	0.0495
			0.5	2	2699	5318			0.1					1		15	
Background control	1	2	0.5	1	64	128	157	264	0.1	140575	0.13	77	0.0001	1	0.611	15	0.0004
			0.5	2	93	186			0.1					1		15	
	2	4	0.5	1	36	72	61	134	0.1	140575	0.10	-23	0.0000	1	0.611	15	-0.0001
			0.5	2	37	74			0.1					1		15	
	3	1	0.5	1	70	140	95	190	0.1	140575	0.03	-27	0.0000	1	0.611	15	-0.0001
			0.5	2	74	148			0.1					1		15	
	4	2	0.5	1	60	120	66	139	0.1	140575	0.05	-27	0.0000	1	0.611	15	-0.0001
			0.5	2	39	90			0.1					1		15	
Positive control	1	2	0.5	1	1751	3502	3440	6880	0.1	140575	4.87	6883	0.0154	1	0.611	15	0.0344
			0.5	2	1742	3484			0.1					1		15	
	2	2	0.5	1	1793	3586	3527	7054	0.1	140575	5.09	6997	0.0157	1	0.611	15	0.0351
			0.5	2	1736	3472			0.1					1		15	
	3	2	0.5	1	1751	3502	3624	7248	0.1	140575	5.01	7001	0.0156	1	0.611	15	0.0371
			0.5	2	1767	3534			0.1					1		15	
	4	2	0.5	1	1725	3450	3441	6882	0.1	140575	4.89	6725	0.0152	1	0.611	15	0.0339
			0.5	2	1706	3432			0.1					1		15	
Negative Control	1	2	0.5	1	2297	4594	4662	13984	0.1	140575	8.29	13987	0.0196	1	0.611	15	0.0507
			0.5	2	2330	4660			0.1					1		15	
	2	2	0.5	1	2342	4684	4601	13804	0.1	140575	8.26	13807	0.0196	1	0.611	15	0.0500
			0.5	2	2259	4518			0.1					1		15	
	3	2	0.5	1	2915	5830	5808	13952	0.1	140575	8.57	13955	0.0199	1	0.611	15	0.0506
			0.5	2	2894	5788			0.1					1		15	
	4	2	0.5	1	2112	4224	5242	12581	0.1	140575	8.55	12584	0.0198	1	0.611	15	0.0506
			0.5	2	3129	6258			0.1					1		15	
HC	1-1	2	0.5	1	42	84	84	168	0.1	140575	0.12	11	0.0000	1	0.611	15	0.0001
			0.5	2	42	84			0.1					1		15	
	1-2	2	0.5	1	37	74	72	144	0.1	140575	0.10	-13	0.0000	1	0.611	15	-0.0001
			0.5	2	35	70			0.1					1		15	
	1-3	2	0.5	1	41	82	84	168	0.1	140575	0.12	11	0.0000	1	0.611	15	0.0001
			0.5	2	43	86			0.1					1		15	
	2-1	2	0.5	1	59	118	112	224	0.1	140575	0.16	73	0.0001	1	0.611	15	0.0003
			0.5	2	56	112			0.1					1		15	
	2-2	2	0.5	1	66	132	102	204	0.1	140575	0.15	51	0.0000	1	0.611	15	0.0002
			0.5	2	51	102			0.1					1		15	
	2-3	2	0.5	1	49	98	93	186	0.1	140575	0.13	29	0.0000	1	0.611	15	0.0001
			0.5	2	44	88			0.1					1		15	
	3-1	2	0.5	1	95	190	190	380	0.1	140575	0.26	205	0.0000	1	0.611	15	0.0007
			0.5	2	95	190			0.1					1		15	
	3-2	2	0.5	1	87	174	185	366	0.1	140575	0.25	205	0.0000	1	0.611	15	0.0007
			0.5	2	96	192			0.1					1		15	
	3-3	2	0.5	1	87	174	178	356	0.1	140575	0.24	199	0.0000	1	0.611	15	0.0680
			0.5	2	91	182			0.1					1		15	
	4-1	2	0.5	1	153	306	306	606	0.1	140575	0.43	409	0.0001	1	0.611	15	0.0014
			0.5	2	153	306			0.1					1		15	
	4-2	2	0.5	1	139	278	276	556	0.1	140575	0.40	399	0.0000	1	0.611	15	0.0013
			0.5	2	138	276			0.1					1		15	
	4-3	2	0.5	1	136	272	266	532	0.1	140575	0.38	393	0.0000	1	0.611	15	0.0013
			0.5	2	140	280			0.1					1		15	
	5-1	2	0.5	1	240	480	484	968	0.1	140575	0.69	811	0.0002	1	0.611	15	0.0027
			0.5	2	244	488			0.1					1		15	
	5-2	2	0.5	1	234	468	423	846	0.1	140575	0.61	709	0.0002	1	0.611	15	0.0024
			0.5	2	211	422			0.1					1		15	
	5-3	2	0.5	1	237	474	473	946	0.1	140575	0.67	789	0.0002	1	0.611	15	0.0026
			0.5	2	236	472			0.1					1		15	
	6-1	2	0.5	1	537	1074	1065	2130	0.1	140575	1.52	1973	0.0005	1	0.611	15	0.0061
			0.5	2	528	1056			0.1					1		15	
	6-2	2	0.5	1	432	864	888	1776	0.1	140575	1.22	1585	0.0004	1	0.611	15	0.0049
			0.5	2	456	912			0.1					1		15	
	6-3	2	0.5	1	528	1056	1066	2132	0.1	140575	1.51	1965	0.0005	1	0.611	15	0.0060
			0.5	2	532	1064			0.1					1		15	
	7-1	2	0.5	1	1364	2728	2915	5814	0.1	140575	4.01	5387	0.0014	1	0.611	15	0.0222
			0.5	2	1429	2858			0.1					1		15	
	7-2	2	0.5	1	1445	2890	2820	5640	0.1	140575	4.15	5653	0.0015	1	0.611	15	0.0231
			0.5	2	1373	2746			0.1					1		15	
	7-3	2	0.5	1	1452	2904	2978	5956	0.1	140575	4.14	5969	0.0015	1	0.611	15	0.0250
			0.5	2	1505	3010			0.1					1		15	
	8-1	2	0.5	1	2302	4604	4551	9102	0.1	140575	6.22	9029	0.0017	1	0.611	15	0.0419
			0.5	2	2299	4598			0.1					1		15	
	8-2	2	0.5	1	2183	4366	4343	8686	0.1	140575	5.98	8529	0.0130	1	0.611	15	0.0392
			0.5	2	2180	4360			0.1					1		15	
	8-3	2	0.5	1	2159	4318	4368	8736	0.1	140575	6.21	8579	0.0134	1	0.611	15	0.0394
			0.5	2	2177	4354			0.1					1		15	

Assay Date	2/23/2005	Test Chemical ID	1/C	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0626	0.0013
Full activity	End	0.0511	0.0034
Full activity	Overall	0.0569	0.0069
Background	Beginning	0.0001	0.000324849
Background	End	-0.0001	0
Background	Overall	0.0000	0.000235987
Positive	Beginning	0.0318	0.0005
Positive	End	0.0313	0.0005
Positive	Overall	0.0315	0.0005
Negative	Beginning	0.0592	0.0001
Negative	End	0.0556	0.0014
Negative	Overall	0.0574	0.0022

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/C	1	1	1.00E-06	-6.00	0.0001
1/C	1	2	1.00E-06	-6.00	-0.0001
1/C	1	3	1.00E-06	-6.00	0.0001
1/C	2	1	1.00E-06	-6.00	0.0009
1/C	2	2	1.00E-06	-6.00	0.0002
1/C	2	3	1.00E-06	-6.00	0.0001
1/C	3	1	1.00E-07	-7.00	0.0009
1/C	3	2	1.00E-07	-7.00	0.0010
1/C	3	3	1.00E-07	-7.00	0.0009
1/C	4	1	5.00E-08	-7.30	0.0021
1/C	4	2	5.00E-08	-7.30	0.0019
1/C	4	3	5.00E-08	-7.30	0.0017
1/C	5	1	2.50E-08	-7.60	0.0037
1/C	5	2	2.50E-08	-7.60	0.0036
1/C	5	3	2.50E-08	-7.60	0.0036
1/C	6	1	1.00E-08	-8.00	0.0091
1/C	6	2	1.00E-08	-8.00	0.0072
1/C	6	3	1.00E-08	-8.00	0.0090
1/C	7	1	2.50E-09	-8.60	0.0252
1/C	7	2	2.50E-09	-8.60	0.0261
1/C	7	3	2.50E-09	-8.60	0.0260
1/C	8	1	1.00E-09	-9.00	0.0414
1/C	8	2	1.00E-09	-9.00	0.0392
1/C	8	3	1.00E-09	-9.00	0.0394

Percent of control values				
	Log[test	Replicate		
Level	substance]	1	2	3
1	-6.00	0.09	-0.11	0.09
2	-6.00	0.59	0.38	0.23
3	-7.00	1.64	1.69	1.61
4	-7.30	3.63	3.22	3.03
5	-7.60	6.55	6.37	6.37
6	-8.00	15.94	12.59	15.87
7	-8.60	44.32	45.90	45.71
8	-9.00	72.74	68.69	69.30

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0193	21231	1100052	
2	0.0194	23485	1210587	
3	0.0198	24202	1222323	
4	0.0196	24313	1240459	
5	0.0196	24485	1249235	
			Average DPM/g soln	1204527
			SD	60329
			CV	5.01
			µCi/g soln	0.543

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.8	10		1080.00
Dilution A			100	10.80
Dilution B			10	1.08

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.03 g
Mass of dilution B used in substrate prep	4.5227 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.608283 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00614 µg/g soln.
µg/g soln.

a. µCi/g soln 0.543
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.608283 + 0.00614
= 0.614426 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.883 µCi/µg ASDN

561462 dpm/nmol

Assay Date 2/24/2005 | Test Chemical ID 1/C | # Concentrations tested 8

Technician ID TD | Replicate # 3 | Microsome type placental | Microsome ID 11343-7

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210900238
	0.269	0.247	0.203	0.139	0.075	0.054	0.000			
	0.299	0.246	0.203	0.143	0.061	0.042	0.000			
	0.303	0.254	0.192	0.146	0.052	0.047	0.000			

Samples:	11343-7	QC1	QC2	QC3
	0.075	0.041	0.157	0.294
	0.076	0.039	0.166	0.298
	0.086			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.300	0.300	0.0243	m, b	0.085	-0.001
0.8	14.3	25	0.00080	25	0.0200	0.249	0.249	0.0200	se_m, se_b	0.004	0.001
0.6	10.7	25	0.00060	25	0.0150	0.199	0.199	0.0157	r^2, se_y	0.992	0.001
0.4	7.1	25	0.00040	25	0.0099	0.143	0.143	0.0109	F, df	505	4
0.2	3.6	25	0.00020	25	0.0050	0.063	0.063	0.0041	ss_{reg}, ss_{resid}	0.000	0.000
0.11	2	25	0.00011	25	0.0028	0.048	0.048	0.0028			

Regression results are calculated using the function LINEST

Blank 0.000

r^2 = 0.992
m = 0.085
b = -0.001

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.075	0.075	0.005	25	100	5000	0.010	0.011	10.941
11343-7	0.076	0.076	0.005	25	100	5000	0.010		
11343-7	0.086	0.086	0.006	25	100	5000	0.012		
QC1	0.041	0.041	0.002	25	1	1	0.000	0.000	0.086
QC1	0.039	0.039	0.002	25	1	1	0.000		
QC1									
QC2	0.157	0.157	0.012	25	1	1	0.000	0.001	0.500
QC2	0.166	0.166	0.013	25	1	1	0.001		
QC2									
QC3	0.294	0.294	0.024	25	1	1	0.001	0.001	0.958
QC3	0.298	0.298	0.024	25	1	1	0.001		
QC3									

Assay Date	2/24/2005	Test Chemical ID	1/C	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	10.941
Protein Concentration (dilution added to assay, mg/mL):	0.021882

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	1.00E-08
4	5.00E-09
5	2.50E-09
6	1.00E-09
7	2.50E-10
8	1.00E-10

Assay Date	20040805	Test Chemical ID	12	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample tube	Replicate # or mI	Incubation total volume (mL)	Abs Volume (mL)	Adj. F	DPM/mL	DPM/mL	Ave DPM/mL	Total (DPM)	Volume of substrate solution used to prep tube (mL)	Total DPM in assay tube (dpm)	% conversion to product	Total DPM corrected for background (Blank ground Tubes)	nmol ^3H_2O formed	Volume diluted microsome used in assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	3533	6994	5967	11934	0.1	120453	9.94	11862	0.6211	1	0.011	15	0.0643
			0.5	2	8955	8955			0.1					1		15	
	2	2	0.5	1	2981	5965	6265	12530	0.1	120453	9.87	11885	0.6222	1	0.011	15	0.0643
			0.5	2	3029	6040			0.1					1		15	
	3	2	0.5	1	2673	5346	5395	10790	0.1	120453	9.85	10950	0.6166	1	0.011	15	0.0572
			0.5	2	2968	5764			0.1					1		15	
	4	2	0.5	1	2699	5398	5894	10788	0.1	120453	9.84	10950	0.6166	1	0.011	15	0.0572
			0.5	2	2790	5412			0.1					1		15	
Blank/solvent control	1	2	0.5	1	38	65	65	130	0.1	120451	0.11	12	0.0000	1	0.011	15	0.0000
			0.5	2	38	65			0.1					1		15	
	2	2	0.5	1	31	52	54	105	0.1	120453	0.11	9	0.0000	1	0.011	15	0.0000
			0.5	1	33	56			0.1					1		15	
	3	2	0.5	1	28	47	52	104	0.1	120453	0.09	-10	0.0000	1	0.011	15	-0.0008
			0.5	2	29	50			0.1					1		15	
	4	2	0.5	1	29	50	51	102	0.1	120453	0.09	0	0.0000	1	0.011	15	0.0000
			0.5	2	30	51			0.1					1		15	
Positive control	1	2	0.5	1	1750	3460	3472	6943	0.1	120453	5.77	5888	0.6122	1	0.011	15	0.0371
			0.5	2	1740	3439			0.1					1		15	
	2	2	0.5	1	1569	3176	3312	6624	0.1	120453	5.53	5592	0.6143	1	0.011	15	0.0342
			0.5	2	1624	3249			0.1					1		15	
	3	2	0.5	1	1619	3200	3282	6564	0.1	120453	5.57	5682	0.6152	1	0.011	15	0.0351
			0.5	2	1680	3364			0.1					1		15	
	4	2	0.5	1	1638	3270	3235	6472	0.1	120453	5.37	5550	0.6115	1	0.011	15	0.0345
			0.5	2	1599	3199			0.1					1		15	
Negative Control	1	2	0.5	1	3251	6504	5995	11990	0.1	120453	9.95	11748	0.6199	1	0.011	15	0.0652
			0.5	2	2823	5651			0.1					1		15	
	2	2	0.5	1	2882	5765	5582	11164	0.1	120453	9.80	11442	0.6206	1	0.011	15	0.0621
			0.5	2	2901	5801			0.1					1		15	
	3	2	0.5	1	2436	4873	5246	10493	0.1	120453	9.11	10760	0.6183	1	0.011	15	0.0569
			0.5	2	2713	5427			0.1					1		15	
	4	2	0.5	1	2625	5250	5293	10586	0.1	120453	9.19	10868	0.6196	1	0.011	15	0.0580
			0.5	2	2670	5340			0.1					1		15	
0.5	1-1	2	0.5	1	41	82	84	168	0.1	120453	0.14	46	0.0001	1	0.011	15	0.0005
			0.5	1	43	86			0.1					1		15	
	1-2	2	0.5	1	38	80	84	168	0.1	120453	0.11	9	0.0000	1	0.011	15	0.0000
			0.5	2	39	80			0.1					1		15	
	1-3	2	0.5	1	43	86	92	184	0.1	120453	0.14	42	0.0001	1	0.011	15	0.0002
			0.5	2	49	97			0.1					1		15	
	2-1	2	0.5	1	77	152	156	312	0.1	120453	0.26	136	0.0007	1	0.011	15	0.0031
			0.5	1	80	164			0.1					1		15	
	2-2	2	0.5	1	82	164	156	312	0.1	120453	0.26	314	0.0004	1	0.011	15	0.0012
			0.5	1	78	156			0.1					1		15	
	2-3	2	0.5	1	94	188	181	362	0.1	120453	0.30	348	0.0004	1	0.011	15	0.0013
			0.5	1	87	174			0.1					1		15	
	3-1	2	0.5	1	458	916	909	1818	0.1	120453	1.61	1686	0.6036	1	0.011	15	0.0059
			0.5	1	453	906			0.1					1		15	
	3-2	2	0.5	1	472	944	937	1874	0.1	120453	1.69	1752	0.6021	1	0.011	15	0.0006
			0.5	1	468	936			0.1					1		15	
	3-3	2	0.5	1	461	922	910	1820	0.1	120453	1.31	1599	0.6030	1	0.011	15	0.0042
			0.5	2	443	886			0.1					1		15	
	4-1	2	0.5	1	763	1526	1527	3054	0.1	120453	2.74	2933	0.6027	1	0.011	15	0.0139
			0.5	1	774	1548			0.1					1		15	
	4-2	2	0.5	1	1597	3193	1595	3191	0.1	120453	2.59	2880	0.6051	1	0.011	15	0.0135
			0.5	2	798	1597			0.1					1		15	
	4-3	2	0.5	1	793	1587	1495	2989	0.1	120453	2.40	2598	0.6051	1	0.011	15	0.0158
			0.5	1	704	1408			0.1					1		15	
	5-1	2	0.5	1	1356	2713	2581	5163	0.1	120453	4.26	5096	0.6089	1	0.011	15	0.0272
			0.5	2	1279	2558			0.1					1		15	
	5-2	2	0.5	1	1231	2462	2454	4908	0.1	120453	4.12	4940	0.6086	1	0.011	15	0.0263
			0.5	2	1263	2526			0.1					1		15	
	5-3	2	0.5	1	1247	2493	2493	4986	0.1	120453	4.12	4974	0.6087	1	0.011	15	0.0264
			0.5	2	1250	2501			0.1					1		15	
	6-1	2	0.5	1	1921	3841	3688	7376	0.1	120453	6.25	7419	0.6132	1	0.011	15	0.0400
			0.5	2	1844	3689			0.1					1		15	
	6-2	2	0.5	1	1824	3648	3863	7726	0.1	120453	6.49	7644	0.6106	1	0.011	15	0.0415
			0.5	1	1954	3908			0.1					1		15	
	6-3	2	0.5	1	1904	3808	3968	7936	0.1	120453	6.59	7814	0.6129	1	0.011	15	0.0424
			0.5	2	1908	3816			0.1					1		15	
	7-1	2	0.5	1	2653	5306	5029	10058	0.1	120453	8.75	10404	0.6186	1	0.011	15	0.0556
			0.5	1	2615	5229			0.1					1		15	
	7-2	2	0.5	1	2583	5166	4934	10069	0.1	120453	8.36	9918	0.6123	1	0.011	15	0.0529
			0.5	2	2017	4034			0.1					1		15	
	7-3	2	0.5	1	2489	4978	4969	9938	0.1	120453	8.22	9778	0.6174	1	0.011	15	0.0523
			0.5	2	2481	4962			0.1					1		15	
	8-1	2	0.5	1	2556	5112	5152	10304	0.1	120453	8.55	10182	0.6191	1	0.011	15	0.0553
			0.5	2	2595	5191			0.1					1		15	
	8-2	2	0.5	1	2457	5914	5893	10918	0.1	120453	8.47	10026	0.6139	1	0.011	15	0.0547
			0.5	2	2921	5842			0.1					1		15	
	8-3	2	0.5	1	2641	5345	5784	11529	0.1	120453	8.94	10696	0.6195	1	0.011	15	0.0575
			0.5	2	3038	6076			0.1					1		15	

Assay Date	2/24/2005	Test Chemical ID	1/C	# Concentrations tested		Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0644	0.0001
Full activity	End	0.0576	0.0005
Full activity	Overall	0.0610	0.0039
Background	Beginning	0.0000	2.30217E-05
Background	End	0.0000	6.90651E-05
Background	Overall	0.0000	7.03325E-05
Positive	Beginning	0.0356	0.0020
Positive	End	0.0343	0.0003
Positive	Overall	0.0349	0.0014
Negative	Beginning	0.0629	0.0012
Negative	End	0.0578	0.0015
Negative	Overall	0.0604	0.0031

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
1/C	1	1	1.00E-06	-6.00	0.0002
1/C	1	2	1.00E-06	-6.00	0.0000
1/C	1	3	1.00E-06	-6.00	0.0002
1/C	2	1	1.00E-07	-7.00	0.0011
1/C	2	2	1.00E-07	-7.00	0.0012
1/C	2	3	1.00E-07	-7.00	0.0013
1/C	3	1	1.00E-08	-8.00	0.0092
1/C	3	2	1.00E-08	-8.00	0.0095
1/C	3	3	1.00E-08	-8.00	0.0092
1/C	4	1	5.00E-09	-8.30	0.0159
1/C	4	2	5.00E-09	-8.30	0.0157
1/C	4	3	5.00E-09	-8.30	0.0156
1/C	5	1	2.50E-09	-8.60	0.0272
1/C	5	2	2.50E-09	-8.60	0.0263
1/C	5	3	2.50E-09	-8.60	0.0264
1/C	6	1	1.00E-09	-9.00	0.0402
1/C	6	2	1.00E-09	-9.00	0.0415
1/C	6	3	1.00E-09	-9.00	0.0424
1/C	7	1	2.50E-10	-9.60	0.0566
1/C	7	2	2.50E-10	-9.60	0.0540
1/C	7	3	2.50E-10	-9.60	0.0531
1/C	8	1	1.00E-10	-10.00	0.0553
1/C	8	2	1.00E-10	-10.00	0.0547
1/C	8	3	1.00E-10	-10.00	0.0575

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-6.00	0.41	0.05	0.37
2	-7.00	1.74	1.90	2.13
3	-8.00	15.09	15.59	15.10
4	-8.30	26.08	25.71	25.51
5	-8.60	44.53	43.11	43.36
6	-9.00	65.92	68.00	69.51
7	-9.60	92.82	88.49	86.98
8	-10.00	90.58	89.63	94.26

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	26802	1360508	
2	0.0198	26520	1339394	
3	0.0198	27217	1374596	
4	0.0195	26725	1370513	
5	0.0197	26883	1364619	
			Average DPM/g soln	1361926
			SD	13704
			CV	1.01
			µCi/g soln	0.613

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.8	10		1080.00
Dilution A			100	10.80
Dilution B			10	1.08

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0454 g
Mass of dilution B used in substrate prep	4.5186 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.606569 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00694 µg/g soln.
µg/g soln.

a. µCi/g soln 0.613
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.606569 + 0.00694
= 0.613513 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.000 µCi/µg ASDN

635774 dpm/nmol

Assay Date	2/28/2005	Test Chemical ID	2/D	# Concentrations tested	8		
Technician ID	TD	Replicate #	1	Microsome type	placental	Microsome ID	11343-7

Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank
	0.287	0.239	0.197	0.140	0.077	0.047	0.000
	0.301	0.238	0.192	0.126	0.051	0.026	0.000
	0.284	0.243	0.196	0.149	0.059	0.045	0.000

Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
28	20	210000288

Samples:	11343-7	QC1	QC2	QC3
	0.089	0.037	0.149	0.273
	0.085	0.042	0.157	0.286
	0.106			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.291	0.291	0.0243
0.8	14.3	25	0.00080	25	0.0200	0.240	0.240	0.0198
0.6	10.7	25	0.00060	25	0.0150	0.195	0.195	0.0159
0.4	7.1	25	0.00040	25	0.0099	0.138	0.138	0.0110
0.2	3.6	25	0.00020	25	0.0050	0.062	0.062	0.0044
0.11	2	25	0.00011	25	0.0028	0.039	0.039	0.0024
			Blank	0.000				

Variables	Regression results	
m, b	0.087	-0.001
se_m, se_b	0.004	0.001
r^2, se_y	0.991	0.001
F, df	445	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2= 0.991
m= 0.087
b= -0.001

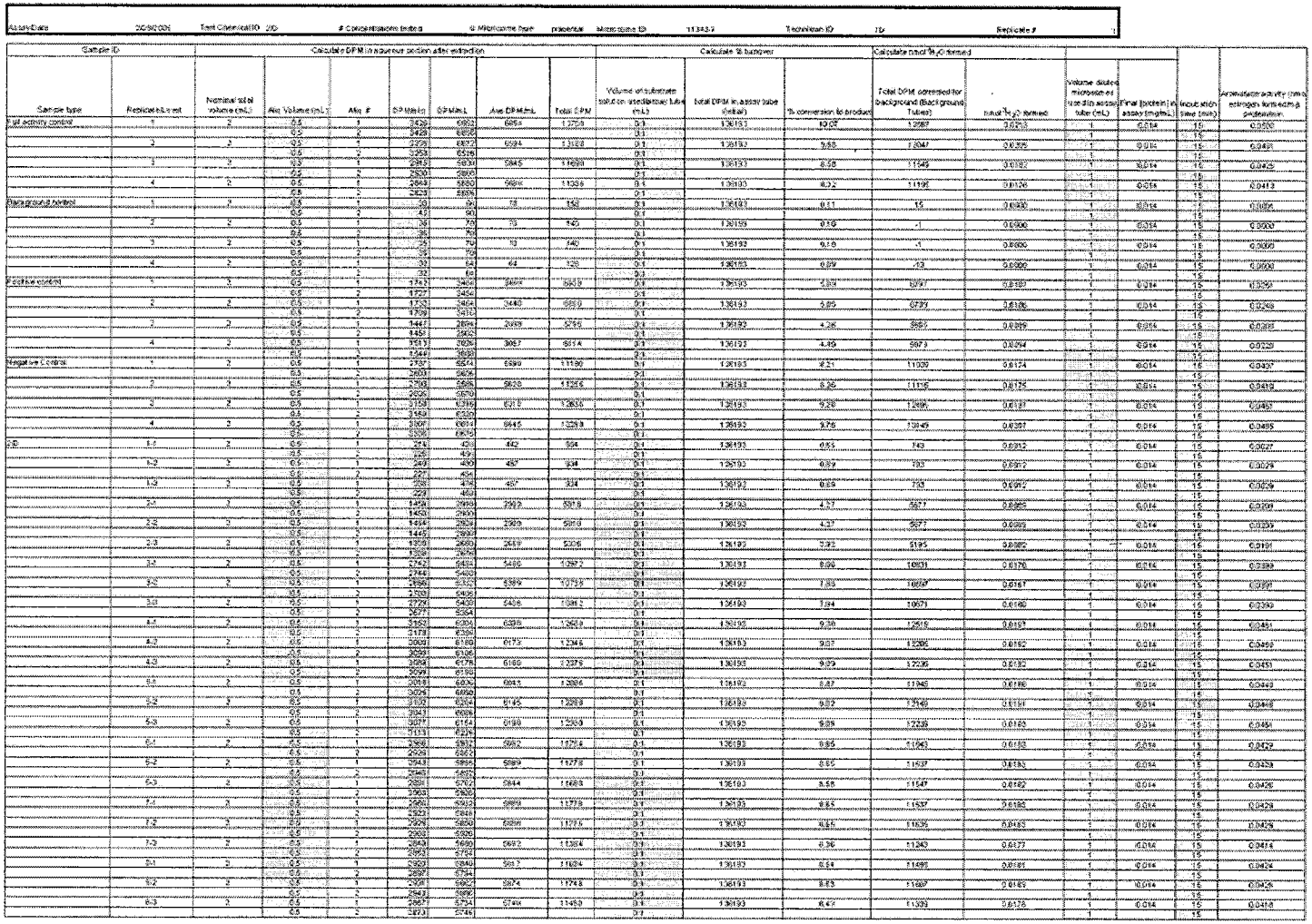
	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.089	0.089	0.007	25	100	5000	0.013	0.014	14.225
11343-7	0.085	0.085	0.006	25	100	5000	0.013		
11343-7	0.106	0.106	0.008	25	100	5000	0.016		
QC1	0.037	0.037	0.002	25	1	1	0.000	0.000	0.096
QC1	0.042	0.042	0.003	25	1	1	0.000		
QC1									
QC2	0.149	0.149	0.012	25	1	1	0.000	0.000	0.492
QC2	0.157	0.157	0.013	25	1	1	0.001		
QC2									
QC3	0.273	0.273	0.025	25	1	1	0.001	0.001	0.930
QC3	0.286	0.286	0.024	25	1	1	0.001		
QC3									

Assay Date	2/26/2005	Test Chemical ID	2/D	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	14.225
Protein Concentration (dilution added to assay, mg/mL):	0.02845

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	1.00E-06
4	2.50E-07
5	1.00E-07
6	1.00E-08
7	1.00E-09
8	1.00E-10



Assay Date	06/30/2006	Test Chemical ID	0/0	# Concentrations tested		# Microtome type	proprietary	Mass tube ID	11343-2	Thawaway ID	10	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % transfer			Calculate nmol ^3H_2O formed					
Sample type	Replicate/Unit	Nominal total volume (mL)	Ave. Volume (mL)	Ave. #	DPM/mL	DPM/mL	Ave. DPM/mL	Total DPM	Volume of substrate added to test/blank tube (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	total ^3H_2O formed	Volume diluted microliters of test/blank assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Aromatase activity (nmol estrogen formed/h/mg protein)
Full activity control	1	2	0.5	1	3439	6883	6854	13708	0.1	1.36193	10.07	13687	0.0218	1	0.014	15	0.0500
			0.5	1	3428	6857			0.1					1		15	
	2	2	0.5	1	3339	6677	6684	13368	0.1	1.36193	9.85	13347	0.0205	1	0.014	15	0.0481
			0.5	1	3353	6706			0.1					1		15	
	3	2	0.5	1	2931	5863	5845	11690	0.1	1.36193	8.58	11649	0.0182	1	0.014	15	0.0425
			0.5	1	2930	5860			0.1					1		15	
	4	2	0.5	1	2648	5296	5299	10599	0.1	1.36193	7.79	10558	0.0162	1	0.014	15	0.0378
			0.5	1	2649	5302			0.1					1		15	
Background, Aminase	1	1	0.5	1	78	156	78	156	0.1	1.36193	0.11	15	0.0030	1	0.014	15	0.0070
			0.5	1	78	156			0.1					1		15	
	2	2	0.5	1	36	72	73	145	0.1	1.36193	0.09	-1	0.0000	1	0.014	15	0.0000
			0.5	1	36	72			0.1					1		15	
	3	2	0.5	1	75	150	73	146	0.1	1.36193	0.10	-1	0.0000	1	0.014	15	0.0000
			0.5	1	37	74			0.1					1		15	
	4	2	0.5	1	32	64	64	128	0.1	1.36193	0.09	-13	0.0000	1	0.014	15	0.0000
			0.5	1	32	64			0.1					1		15	
Positive control	1	1	0.5	1	17421	34842	34842	69683	0.1	1.36193	4.84	69671	0.0987	1	0.014	15	0.2350
			0.5	2	17421	34842			0.1					1		15	
	2	2	0.5	1	1720	3440	3440	6880	0.1	1.36193	0.85	6739	0.0186	1	0.014	15	0.0248
			0.5	1	1720	3440			0.1					1		15	
	3	2	0.5	1	1447	2894	2939	5758	0.1	1.36193	4.26	5683	0.0809	1	0.014	15	0.0286
			0.5	2	1483	2966			0.1					1		15	
	4	2	0.5	1	3513	7026	3967	7914	0.1	1.36193	4.49	5873	0.0094	1	0.014	15	0.0229
			0.5	1	1544	3088			0.1					1		15	
Negative Control	1	1	0.5	1	2737	5474	5599	11190	0.1	1.36193	8.21	11039	0.0124	1	0.014	15	0.0437
			0.5	1	2803	5606			0.1					1		15	
	2	2	0.5	1	2798	5596	5620	11236	0.1	1.36193	8.26	11135	0.0125	1	0.014	15	0.0435
			0.5	1	2825	5650			0.1					1		15	
	3	2	0.5	1	3139	6278	6315	12630	0.1	1.36193	9.28	12490	0.0187	1	0.014	15	0.0485
			0.5	1	3158	6317			0.1					1		15	
	4	2	0.5	1	3307	6614	6643	13286	0.1	1.36193	9.76	13149	0.0357	1	0.014	15	0.0485
			0.5	1	3336	6670			0.1					1		15	
2,6	1-1	3	0.5	1	214	428	402	804	0.1	1.36193	0.55	743	0.0352	1	0.014	15	0.0027
			0.5	1	203	406			0.1					1		15	
	1-2	3	0.5	1	223	446	467	934	0.1	1.36193	0.69	793	0.0912	1	0.014	15	0.0029
			0.5	2	227	454			0.1					1		15	
	1-3	3	0.5	1	208	416	467	934	0.1	1.36193	0.69	793	0.0912	1	0.014	15	0.0029
			0.5	2	227	454			0.1					1		15	
	2-1	3	0.5	1	1474	2948	2959	5918	0.1	1.36193	4.37	5677	0.0995	1	0.014	15	0.0208
			0.5	1	1480	2960			0.1					1		15	
	2-2	3	0.5	1	1464	2928	2959	5918	0.1	1.36193	4.37	5677	0.0099	1	0.014	15	0.0208
			0.5	1	1445	2890			0.1					1		15	
	2-3	3	0.5	1	1676	3352	3419	6838	0.1	1.36193	5.02	6195	0.0092	1	0.014	15	0.0191
			0.5	1	1708	3416			0.1					1		15	
	3-1	3	0.5	1	2742	5483	5499	10997	0.1	1.36193	8.09	10857	0.0376	1	0.014	15	0.0399
			0.5	1	2744	5488			0.1					1		15	
	3-2	3	0.5	1	2696	5392	5399	10798	0.1	1.36193	7.93	10657	0.0167	1	0.014	15	0.0391
			0.5	1	2703	5406			0.1					1		15	
	3-3	3	0.5	1	2729	5458	5426	10851	0.1	1.36193	7.94	10671	0.0180	1	0.014	15	0.0393
			0.5	2	2677	5354			0.1					1		15	
	4-1	3	0.5	1	2552	5104	5265	10529	0.1	1.36193	9.36	12618	0.0157	1	0.014	15	0.0451
			0.5	2	2626	5252			0.1					1		15	
	4-2	3	0.5	1	3083	6166	6175	12349	0.1	1.36193	9.07	12208	0.0152	1	0.014	15	0.0440
			0.5	2	3089	6178			0.1					1		15	
	4-3	3	0.5	1	3094	6188	6188	12375	0.1	1.36193	9.09	12236	0.0152	1	0.014	15	0.0441
			0.5	2	3099	6198			0.1					1		15	
	5-1	3	0.5	1	3081	6163	6063	12366	0.1	1.36193	8.87	11945	0.0186	1	0.014	15	0.0440
			0.5	2	3045	6090			0.1					1		15	
	5-2	3	0.5	1	3107	6215	6145	12289	0.1	1.36193	9.02	12148	0.0191	1	0.014	15	0.0446
			0.5	1	3043	6086			0.1					1		15	
	5-3	3	0.5	1	3077	6154	6199	12399	0.1	1.36193	9.06	12239	0.0183	1	0.014	15	0.0451
			0.5	2	3123	6246			0.1					1		15	
	6-1	2	0.5	1	2536	5071	5662	11324	0.1	1.36193	8.85	11043	0.0183	1	0.014	15	0.0429
			0.5	2	2829	5657			0.1					1		15	
	6-2	2	0.5	1	2943	5886	5889	11778	0.1	1.36193	8.65	11537	0.0183	1	0.014	15	0.0429
			0.5	2	2945	5891			0.1					1		15	
	6-3	2	0.5	1	2901	5802	5844	11689	0.1	1.36193	8.58	11547	0.0182	1	0.014	15	0.0426
			0.5	1	2903	5806			0.1					1		15	
	7-1	2	0.5	1	2890	5780	5889	11778	0.1	1.36193	8.65	11537	0.0183	1	0.014	15	0.0429
			0.5	2	2923	5846			0.1					1		15	
	7-2	2	0.5	1	2923	5846	5699	11398	0.1	1.36193	8.37	11636	0.0183	1	0.014	15	0.0415
			0.5	2	2853	5706			0.1					1		15	
	7-3	2	0.5	1	2843	5686	5699	11398	0.1	1.36193	8.36	11340	0.0177	1	0.014	15	0.0414
			0.5	1	2843	5686			0.1					1		15	
	8-1	2	0.5	1	2803	5840	5617	11234	0.1	1.36193	8.24	11495	0.0181	1	0.014	15	0.0424
			0.5	2	2807	5614			0.1					1		15	
	8-2	2	0.5	1	2908	5982	5874	11748	0.1	1.36193	8.63	11607	0.0183	1	0.014	15	0.0426
			0.5	1	2843	5686			0.1					1		15	
	8-3	2	0.5	1	2867	5734	5744	11489	0.1	1.36193	8.47	11309	0.0176	1	0.014	15	0.0418
			0.5	1	2873	5746			0.1								

Assay Date	2/28/2005	Test Chemical ID	2/D	# Concentrations tested		Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0490	0.0014
Full activity	End	0.0419	0.0009
Full activity	Overall	0.0455	0.0042
Background	Beginning	0.0000	4.16993E-05
Background	End	0.0000	3.12745E-05
Background	Overall	0.0000	4.23459E-05
Positive	Beginning	0.0249	0.0002
Positive	End	0.0214	0.0006
Positive	Overall	0.0232	0.0021
Negative	Beginning	0.0406	0.0002
Negative	End	0.0473	0.0017
Negative	Overall	0.0440	0.0038

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/D	1	1	1.00E-04	-4.00	0.0027
2/D	1	2	1.00E-04	-4.00	0.0029
2/D	1	3	1.00E-04	-4.00	0.0029
2/D	2	1	1.00E-05	-5.00	0.0209
2/D	2	2	1.00E-05	-5.00	0.0209
2/D	2	3	1.00E-05	-5.00	0.0191
2/D	3	1	1.00E-06	-6.00	0.0399
2/D	3	2	1.00E-06	-6.00	0.0391
2/D	3	3	1.00E-06	-6.00	0.0393
2/D	4	1	2.50E-07	-6.60	0.0461
2/D	4	2	2.50E-07	-6.60	0.0450
2/D	4	3	2.50E-07	-6.60	0.0451
2/D	5	1	1.00E-07	-7.00	0.0440
2/D	5	2	1.00E-07	-7.00	0.0446
2/D	5	3	1.00E-07	-7.00	0.0451
2/D	6	1	1.00E-08	-8.00	0.0429
2/D	6	2	1.00E-08	-8.00	0.0429
2/D	6	3	1.00E-08	-8.00	0.0426
2/D	7	1	1.00E-09	-9.00	0.0429
2/D	7	2	1.00E-09	-9.00	0.0429
2/D	7	3	1.00E-09	-9.00	0.0414
2/D	8	1	1.00E-10	-10.00	0.0424
2/D	8	2	1.00E-10	-10.00	0.0428
2/D	8	3	1.00E-10	-10.00	0.0416

		Percent of control values		
	Log[test	Replicate		
Level	substance]	1	2	3
1	-4.00	6.02	6.43	6.43
2	-5.00	46.01	46.01	42.10
3	-6.00	87.78	85.86	86.48
4	-6.60	101.45	98.91	99.15
5	-7.00	96.80	98.46	99.19
6	-8.00	94.36	94.31	93.58
7	-9.00	94.31	94.29	91.11
8	-10.00	93.14	94.06	91.89

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0198	23543	1189040	
2	0.0198	25414	1283535	
3	0.0196	26021	1327602	
4	0.0198	26098	1318081	
5	0.0196	25533	1302704	
			Average DPM/g soln	1284193
			SD	55745
			CV	4.34
			µCi/g soln	0.578

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.4	10		1040.00
Dilution A			100	10.40
Dilution B			10	1.04

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0801 g
Mass of dilution B used in substrate prep	4.5431 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.584748 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00655 µg/g soln.
µg/g soln.

a. µCi/g soln 0.578
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.584748 + 0.00655
= 0.591297 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 0.978 µCi/µg ASDN

622011 dpm/nmol

Assay Date 3/1/2005 | Test Chemical ID 2/D | # Concentrations tested 8

Technician ID TD | Replicate # 2 | Microsome type placental | Microsome ID 11343-7

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	28	20	210000238
	0.307	0.240	0.184	0.136	0.089	0.025	0.000			
	0.299	0.245	0.185	0.131	0.079	0.061	0.000			
	0.298	0.235	0.185	0.123	0.080	0.048	0.000			

Samples:	11343-7	QC1	QC2	QC3
	0.091	0.043	0.153	0.283
	0.063	0.044	0.161	0.299
	0.073			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
1	17.9	25	0.00100	25	0.0251	0.301	0.301	0.0252	m, b	0.088	-0.001
0.8	14.3	25	0.00080	25	0.0200	0.240	0.240	0.0198	se_m, se_b	0.001	0.000
0.6	10.7	25	0.00060	25	0.0150	0.185	0.185	0.0149	r^2, se_y	0.999	0.000
0.4	7.1	25	0.00040	25	0.0099	0.130	0.130	0.0101	F, df	6724	4
0.2	3.6	25	0.00020	25	0.0050	0.076	0.076	0.0053	ss_{reg}, ss_{resid}	0.000	0.000
0.11	2	25	0.00011	25	0.0023	0.045	0.045	0.0026			

Regression results are calculated using the function LINEST

Blank 0.000

r^2= 0.999
m= 0.088
b= -0.001

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usome prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
11343-7	0.091	0.091	0.007	25	100	5000	0.013	0.011	10.584
11343-7	0.063	0.063	0.004	25	100	5000	0.008		
11343-7	0.073	0.073	0.005	25	100	5000	0.010		
QC1	0.043	0.043	0.002	25	1	1	0.000	0.000	0.099
QC1	0.044	0.044	0.003	25	1	1	0.000		
QC1									
QC2	0.153	0.153	0.012	25	1	1	0.000	0.000	0.499
QC2	0.161	0.161	0.013	25	1	1	0.001		
QC2									
QC3	0.283	0.283	0.024	25	1	1	0.001	0.001	0.970
QC3	0.299	0.299	0.025	25	1	1	0.001		
QC3									

Assay Date	3/1/2005	Test Chemical ID	2/D	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	2

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	10.584
Protein Concentration (dilution added to assay, mg/mL):	0.021168

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	2.50E-05
3	1.00E-05
4	5.00E-06
5	2.50E-06
6	1.00E-06
7	2.50E-07
8	1.00E-07

AssayDate	3/8/2006	Test Chemical ID	2D	# Concentrations tested	8	Microbiocide type	paracetic	Inoculome ID	113457	Technician ID	TD	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate mmol H_2O_2 Cleaved					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq. Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	total DPM in assay tube (mDa)	% conversion to product	Total DPM corresponding to background (Background Tubes)	mmol H_2O_2 /assay	Volume diluted microbiocide use into assay tube (mL)	Final [protein] in assay (mg/mL)	Incubation time (min)	Antimicrobial activity (rate entrapped formaldehyde preformed)
Full activity control	1	2	0.5	1	2497	4994	4951	9902	0.1	1.28419	7.72	9274	0.0157	1	0.011	15	0.0496
			0.5	1	2408	4816			0.1					1		15	
	2	2	0.5	1	2254	4508	4918	9836	0.1	1.28419	7.92	9992	0.0143	1	0.011	15	0.0450
			0.5	1	2660	5321			0.1					1		15	
	3	2	0.5	1	2094	4188	4119	8238	0.1	1.28419	8.41	9100	0.0135	1	0.011	15	0.0411
			0.5	2	2050	4100			0.1					1		15	
	4	2	0.5	1	2001	4002	4072	8144	0.1	1.28419	8.04	8956	0.0129	1	0.011	15	0.0406
			0.5	2	2047	4094			0.1					1		15	
Blocking agent control	1	2	0.5	1	54	108	68	136	0.1	1.28419	0.11	6	0.0000	1	0.011	15	0.0000
			0.5	2	34	68			0.1					1		15	
	2	2	0.5	1	26	52	62	125	0.1	1.28419	0.09	-8	0.0000	1	0.011	15	0.0000
			0.5	2	32	64			0.1					1		15	
	3	2	0.5	1	27	54	58	116	0.1	1.28419	0.09	-12	0.0000	1	0.011	15	0.0000
			0.5	2	29	58			0.1					1		15	
	4	2	0.5	1	36	72	70	140	0.1	1.28419	0.11	12	0.0000	1	0.011	15	0.0000
			0.5	2	33	66			0.1					1		15	
Positive control	1	2	0.5	1	1279	2558	2393	5785	0.1	1.28419	4.04	3962	0.0081	1	0.011	15	0.0259
			0.5	2	1207	2414			0.1					1		15	
	2	2	0.5	1	1297	2594	2591	5182	0.1	1.28419	4.04	3964	0.0081	1	0.011	15	0.0259
			0.5	1	1294	2588			0.1					1		15	
	3	2	0.5	1	1243	2487	2397	4794	0.1	1.28419	3.73	4660	0.0095	1	0.011	15	0.0238
			0.5	2	1173	2346			0.1					1		15	
	4	2	0.5	1	1185	2370	2382	4764	0.1	1.28419	3.69	4576	0.0094	1	0.011	15	0.0232
			0.5	1	1197	2394			0.1					1		15	
Negative Control	1	2	0.5	1	2347	4694	4649	9298	0.1	1.28419	7.24	9170	0.0147	1	0.011	15	0.0424
			0.5	2	2303	4606			0.1					1		15	
	2	2	0.5	1	2196	4393	4431	8862	0.1	1.28419	6.90	8734	0.0140	1	0.011	15	0.0403
			0.5	1	2237	4474			0.1					1		15	
	3	2	0.5	1	2113	4226	4269	8538	0.1	1.28419	6.65	8392	0.0135	1	0.011	15	0.0389
			0.5	1	2168	4336			0.1					1		15	
	4	2	0.5	1	2018	4036	4025	8049	0.1	1.28419	6.32	7968	0.0128	1	0.011	15	0.0369
			0.5	1	2065	4130			0.1					1		15	
2D	1-1	2	0.5	1	147	294	256	706	0.1	1.28419	0.66	468	0.0008	1	0.011	15	0.0024
			0.5	2	150	301			0.1					1		15	
	1-2	2	0.5	1	151	302	308	616	0.1	1.28419	0.48	430	0.0006	1	0.011	15	0.0026
			0.5	2	156	312			0.1					1		15	
	1-3	2	0.5	1	156	312	321	642	0.1	1.28419	0.50	314	0.0006	1	0.011	15	0.0026
			0.5	1	155	310			0.1					1		15	
	2-1	2	0.5	1	849	1698	1064	2128	0.1	1.28419	1.66	2080	0.0032	1	0.011	15	0.0101
			0.5	1	524	1048			0.1					1		15	
	2-2	2	0.5	1	463	926	921	1842	0.1	1.28419	1.45	1734	0.0028	1	0.011	15	0.0088
			0.5	1	460	920			0.1					1		15	
	2-3	2	0.5	1	505	1010	1064	2128	0.1	1.28419	1.66	2080	0.0032	1	0.011	15	0.0102
			0.5	1	534	1068			0.1					1		15	
	3-1	2	0.5	1	473	946	1709	3862	0.1	1.28419	2.69	3224	0.0066	1	0.011	15	0.0165
			0.5	1	953	1906			0.1					1		15	
	3-2	2	0.5	1	881	1762	1884	3862	0.1	1.28419	2.97	3564	0.0073	1	0.011	15	0.0177
			0.5	1	906	1810			0.1					1		15	
	3-3	2	0.5	1	969	1938	1998	3862	0.1	1.28419	2.84	3524	0.0087	1	0.011	15	0.0175
			0.5	1	909	1806			0.1					1		15	
	4-1	2	0.5	1	1054	2108	2021	4145	0.1	1.28419	4.51	5618	0.0091	1	0.011	15	0.0254
			0.5	1	1099	2198			0.1					1		15	
	4-2	2	0.5	1	1202	2404	2489	4979	0.1	1.28419	3.35	4570	0.0095	1	0.011	15	0.0247
			0.5	1	1287	2574			0.1					1		15	
	4-3	2	0.5	1	1238	2475	2519	5038	0.1	1.28419	3.52	4393	0.0073	1	0.011	15	0.0248
			0.5	1	1278	2556			0.1					1		15	
	5-1	2	0.5	1	1625	3249	3225	6450	0.1	1.28419	5.02	6522	0.0102	1	0.011	15	0.0325
			0.5	2	1609	3218			0.1					1		15	
	5-2	2	0.5	1	1540	3080	3164	6328	0.1	1.28419	4.93	6080	0.0096	1	0.011	15	0.0306
			0.5	1	1584	3168			0.1					1		15	
	5-3	2	0.5	1	1590	3180	3141	6282	0.1	1.28419	4.89	6154	0.0099	1	0.011	15	0.0312
			0.5	2	1561	3122			0.1					1		15	
	6-1	2	0.5	1	1868	3736	3735	7470	0.1	1.28419	5.82	7342	0.0118	1	0.011	15	0.0373
			0.5	1	1864	3728			0.1					1		15	
	6-2	2	0.5	1	1871	3742	3721	7442	0.1	1.28419	5.80	7314	0.0118	1	0.011	15	0.0372
			0.5	1	1853	3706			0.1					1		15	
	6-3	2	0.5	1	1477	2954	2912	5824	0.1	1.28419	4.63	5814	0.0094	1	0.011	15	0.0294
			0.5	1	1459	2918			0.1					1		15	
	7-1	2	0.5	1	1885	3770	3813	7554	0.1	1.28419	5.94	7496	0.0121	1	0.011	15	0.0380
			0.5	1	1927	3854			0.1					1		15	
	7-2	2	0.5	1	1917	3834	3887	7774	0.1	1.28419	6.06	7654	0.0123	1	0.011	15	0.0388
			0.5	1	1953	3906			0.1					1		15	
	7-3	2	0.5	1	1963	3926	3962	7924	0.1	1.28419	6.17	7796	0.0125	1	0.011	15	0.0396
			0.5	1	1963	3926			0.1					1		15	
	8-1	2	0.5	1	1997	3994	4005	8010	0.1	1.28419	6.27	7922	0.0127	1	0.011	15	0.0400
			0.5	2	2008	4016			0.1					1		15	
	8-2	2	0.5	1	1969	3938	3970	7940	0.1	1.28419	6.18	7810	0.0126	1	0.011	15	0.0396
			0.5	1	2002	4004			0.1					1		15	
	8-3		0.5	1	1969	3938	3926	7852	0.1	1.28419	6.13	7744	0.0124	1	0.011	15	0.0392
			0.5	1	1955	3910			0.1					1		15	

Assay Date	3/1/2005	Test Chemical ID	2/D	# Concentrations tested		Microsome S type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0473	0.0032
Full activity	End	0.0406	0.0003
Full activity	Overall	0.0440	0.0041
Background	Beginning	0.0000	5.72844E-05
Background	End	0.0000	8.59265E-05
Background	Overall	0.0000	5.96235E-05
Positive	Beginning	0.0256	0.0000
Positive	End	0.0234	0.0003
Positive	Overall	0.0245	0.0013
Negative	Beginning	0.0453	0.0016
Negative	End	0.0413	0.0017
Negative	Overall	0.0433	0.0027

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/D	1	1	1.00E-04	-4.00	0.0024
2/D	1	2	1.00E-04	-4.00	0.0025
2/D	1	3	1.00E-04	-4.00	0.0026
2/D	2	1	2.50E-05	-4.60	0.0101
2/D	2	2	2.50E-05	-4.60	0.0088
2/D	2	3	2.50E-05	-4.60	0.0102
2/D	3	1	1.00E-05	-5.00	0.0189
2/D	3	2	1.00E-05	-5.00	0.0177
2/D	3	3	1.00E-05	-5.00	0.0176
2/D	4	1	5.00E-06	-5.30	0.0254
2/D	4	2	5.00E-06	-5.30	0.0247
2/D	4	3	5.00E-06	-5.30	0.0249
2/D	5	1	2.50E-06	-5.60	0.0320
2/D	5	2	2.50E-06	-5.60	0.0308
2/D	5	3	2.50E-06	-5.60	0.0312
2/D	6	1	1.00E-06	-6.00	0.0372
2/D	6	2	1.00E-06	-6.00	0.0370
2/D	6	3	1.00E-06	-6.00	0.0294
2/D	7	1	2.50E-07	-6.60	0.0380
2/D	7	2	2.50E-07	-6.60	0.0388
2/D	7	3	2.50E-07	-6.60	0.0395
2/D	8	1	1.00E-07	-7.00	0.0401
2/D	8	2	1.00E-07	-7.00	0.0396
2/D	8	3	1.00E-07	-7.00	0.0392

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-4.00	5.38	5.63	5.91
2	-4.60	22.99	19.94	23.11
3	-5.00	42.81	40.29	40.52
4	-5.30	57.69	56.99	56.43
5	-5.60	72.68	69.90	70.75
6	-6.00	84.41	84.09	66.87
7	-6.60	86.18	88.02	89.63
8	-7.00	91.08	89.81	89.03

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0198	25391	1282374	
2	0.0198	26724	1349697	
3	0.0197	26996	1370355	
4	0.0197	26716	1356142	
5	0.0197	28058	1424264	
			Average DPM/g soln	1356566
			SD	50806
			CV	3.75
			µCi/g soln	0.611

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	total volume (mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.0902 g
Mass of dilution B used in substrate prep	4.5457 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.561877 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00692 µg/g soln.
µg/g soln.

a. µCi/g soln 0.611
b. Specific activity of [^{3}H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.561877 + 0.00692
= 0.568795 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.074 µCi/µg ASDN

683060 dpm/nmol

Assay Date	3/2/2005	Test Chemical ID	2/D	# Concentrations tested	8
Technician ID	TD	Replicate #	3	Microsome type	placental

Microsome ID 11343-7

								Protein stock (mg BSA)	Total volume of stock (mL)	Protein stock ID
Standards:	1	0.8	0.6	0.4	0.2	0.11	Blank	26	20	210000238
	0.287	0.253	0.193	0.115	0.050	0.043	0.000			
	0.285	0.244	0.186	0.107	0.048	0.051	0.000			
	0.287	0.243	0.186	0.116	0.064	0.049	0.000			

Samples:	11343-7	QC1	QC2	QC3
	0.080	0.041	0.149	0.276
	0.082	0.040	0.160	0.295
	0.083			

Standard concentration (mg/mL)	Volume of stock used	Final volume of Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
1	17.9	25	0.00100	25	0.0251	0.286	0.286	0.0242
0.8	14.3	25	0.00080	25	0.0200	0.247	0.247	0.0203
0.6	10.7	25	0.00060	25	0.0150	0.189	0.189	0.0158
0.4	7.1	25	0.00040	25	0.0099	0.113	0.113	0.0091
0.2	3.6	25	0.00020	25	0.0050	0.059	0.059	0.0044
0.11	2	25	0.00011	25	0.0028	0.049	0.049	0.0026

Variables	Regression results	
m, b	0.087	-0.001
se_m, se_b	0.004	0.001
r^2, se_y	0.990	0.001
F, df	409	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

Blank	0.000	
	r^2=	0.990
	m=	0.087
	b=	-0.001

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Vol usomic prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep	average mg/µL	mg/mL
11343-7	0.080	0.080	0.006	25	100	5000	0.012	0.013	12.767
11343-7	0.082	0.082	0.006	25	100	5000	0.013		
11343-7	0.083	0.083	0.006	25	100	5000	0.013		
QC1	0.041	0.041	0.003	25	1	1	0.000	0.000	0.113
QC1	0.040	0.040	0.003	25	1	1	0.000		
QC1									
QC2	0.149	0.149	0.012	25	1	1	0.000	0.001	0.511
QC2	0.160	0.160	0.013	25	1	1	0.001		
QC2									
QC3	0.276	0.276	0.023	25	1	1	0.001	0.001	0.957
QC3	0.295	0.295	0.025	25	1	1	0.001		
QC3									

Assay Date	3/2/2005	Test Chemical ID	2/D	# Concentrations tested	8	Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	4.5	mL microsome Dilution A used
	45	mL total volume
	10	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.767
Protein Concentration (dilution added to assay, mg/mL):	0.025534

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	2.50E-05
3	1.00E-05
4	5.00E-06
5	2.50E-06
6	1.00E-06
7	2.50E-07
8	1.00E-07

Assay Date:	10/4/05	Test Chemical ID:	2D	# Concentrations tested:	6	Microsome type:	placental	Microsome ID:	113401	Technician ID:	TD	Replicate #:	3

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate value	Nominal total volume (mL)	Abs Volume (mL)	Abs. #	DPM/Abs	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (dilution)	% conversion to product	Total DPM corrected for background (Blank ground Tubes)	nmol ^3H_2O formed	Volume diluted microsomes used in assay tube (mL)	Final [protein] in assay (mg prot/mL)	Incubation time (min)	Aromatase activity (nmol estrogen form edthg prot/min)
Full activity control	1	2	0.5	1	2376	4752	4757	9514	0.1	139557	7.01	9386	0.0137	1	0.013	15	0.0169
			0.5	2	2379	4758			0.1					1		15	
	2	2	0.5	1	2478	4956	4988	9972	0.1	139557	7.15	9844	0.0144	1	0.013	15	0.0256
			0.5	2	2511	5022			0.1					1		15	
	3	2	0.5	1	2271	4542	4505	9010	0.1	139557	6.64	9882	0.0130	1	0.013	15	0.0240
			0.5	2	2234	4468			0.1					1		15	
	4	3	0.5	1	2311	4623	4629	9258	0.1	139557	6.82	9136	0.0134	1	0.013	15	0.0349
			0.5	2	2315	4630			0.1					1		15	
Background control	1	2	0.5	1	90	180	80	160	0.1	139557	0.03	-4	0.0000	1	0.013	15	0.0000
			0.5	2	90	180			0.1					1		15	
	2	3	0.5	1	41	82	76	152	0.1	139557	0.01	74	0.0000	1	0.013	15	0.0000
			0.5	2	38	76			0.1					1		15	
	3	2	0.5	1	36	72	68	136	0.1	139557	0.05	20	0.0000	1	0.013	15	0.0000
			0.5	2	30	64			0.1					1		15	
	4	2	0.5	1	30	60	66	132	0.1	139557	0.02	4	0.0000	1	0.013	15	0.0000
			0.5	2	34	72			0.1					1		15	
Positive control	1	3	0.5	1	1359	2718	2815	5636	0.1	139557	0.61	5156	0.0086	1	0.013	15	0.0150
			0.5	2	1340	2681			0.1					1		15	
	2	2	0.5	1	1242	2484	2482	4964	0.1	139557	0.67	4246	0.0071	1	0.013	15	0.0165
			0.5	2	1241	2482			0.1					1		15	
	3	3	0.5	1	1103	2206	2241	4482	0.1	139557	0.50	3364	0.0056	1	0.013	15	0.0156
			0.5	2	1150	2300			0.1					1		15	
	4	2	0.5	1	1192	2384	2412	4824	0.1	139557	0.58	4206	0.0069	1	0.013	15	0.0160
			0.5	2	1220	2440			0.1					1		15	
Negative Control	1	3	0.5	1	2342	4684	4602	9204	0.1	139557	0.00	9136	0.0134	1	0.013	15	0.0351
			0.5	2	2310	4620			0.1					1		15	
	2	2	0.5	1	2451	4902	4849	9698	0.1	139557	7.27	9756	0.0143	1	0.013	15	0.0367
			0.5	2	2427	4854			0.1					1		15	
	3	2	0.5	1	2388	4776	4741	9494	0.1	139557	7.00	9586	0.0137	1	0.013	15	0.0358
			0.5	2	2353	4706			0.1					1		15	
	4	2	0.5	1	2378	4756	4521	9042	0.1	139557	6.67	9944	0.0131	1	0.013	15	0.0341
			0.5	2	2243	4486			0.1					1		15	
4Z	1-1	3	0.5	1	185	370	347	694	0.1	139557	0.51	996	0.0009	1	0.013	15	0.0022
			0.5	2	128	256			0.1					1		15	
	1-2	2	0.5	1	176	352	353	706	0.1	139557	0.52	973	0.0009	1	0.013	15	0.0022
			0.5	2	177	354			0.1					1		15	
	1-3	3	0.5	1	140	280	357	714	0.1	139557	0.53	496	0.0009	1	0.013	15	0.0022
			0.5	2	174	348			0.1					1		15	
	2-1	2	0.5	1	531	1062	1071	2142	0.1	139557	1.58	2284	0.0029	1	0.013	15	0.0077
			0.5	2	534	1068			0.1					1		15	
	2-2	3	0.5	1	345	1035	1020	2076	0.1	139557	1.50	2082	0.0030	1	0.013	15	0.0078
			0.5	2	342	1026			0.1					1		15	
	2-3	2	0.5	1	551	1102	1084	2168	0.1	139557	1.60	2080	0.0030	1	0.013	15	0.0078
			0.5	2	532	1064			0.1					1		15	
	3-1	2	0.5	1	1967	3934	4020	8040	0.1	139557	2.92	3942	0.0057	1	0.013	15	0.0150
			0.5	2	2073	4146			0.1					1		15	
	3-2	2	0.5	1	1919	3838	3919	7838	0.1	139557	2.83	3716	0.0054	1	0.013	15	0.0141
			0.5	2	964	1928			0.1					1		15	
	3-3	2	0.5	1	971	1942	1935	3870	0.1	139557	2.81	3742	0.0055	1	0.013	15	0.0143
			0.5	2	964	1928			0.1					1		15	
	4-1	2	0.5	1	1351	2702	2745	5490	0.1	139557	4.05	5362	0.0078	1	0.013	15	0.0200
			0.5	2	1388	2776			0.1					1		15	
	4-2	2	0.5	1	1331	2662	2655	5310	0.1	139557	3.91	5182	0.0076	1	0.013	15	0.0193
			0.5	2	1344	2688			0.1					1		15	
	4-3	2	0.5	1	511	1022	1238	2476	0.1	139557	1.85	2246	0.0034	1	0.013	15	0.0090
			0.5	2	627	1254			0.1					1		15	
	5-1	2	0.5	1	1760	3520	3402	6804	0.1	139557	5.06	6726	0.0099	1	0.013	15	0.0257
			0.5	2	1719	3438			0.1					1		15	
	5-2	2	0.5	1	1611	3222	3323	6646	0.1	139557	4.77	6538	0.0093	1	0.013	15	0.0242
			0.5	2	1822	3644			0.1					1		15	
	5-3	2	0.5	1	1607	3214	3172	6344	0.1	139557	4.66	6216	0.0091	1	0.013	15	0.0236
			0.5	2	1565	3130			0.1					1		15	
	6-1	2	0.5	1	1309	2618	2814	5628	0.1	139557	5.07	7140	0.0105	1	0.013	15	0.0274
			0.5	2	1465	2930			0.1					1		15	
	6-2	2	0.5	1	1734	3468	3487	6974	0.1	139557	5.13	6948	0.0103	1	0.013	15	0.0262
			0.5	2	1753	3506			0.1					1		15	
	6-3	2	0.5	1	1720	3440	3481	6963	0.1	139557	5.15	6954	0.0100	1	0.013	15	0.0262
			0.5	2	1771	3542			0.1					1		15	
	7-1	2	0.5	1	2214	4428	4405	8811	0.1	139557	6.56	8664	0.0127	1	0.013	15	0.0332
			0.5	2	2192	4384			0.1					1		15	
	7-2	2	0.5	1	2023	4046	4041	8082	0.1	139557	5.99	7954	0.0116	1	0.013	15	0.0306
			0.5	2	2036	4072			0.1					1		15	
	7-3	2	0.5	1	2116	4232	4569	9138	0.1	139557	6.87	9206	0.0121	1	0.013	15	0.0315
			0.5	2	2357	4714			0.1					1		15	
	8-1	2	0.5	1	2459	4918	4682	9363	0.1	139557	6.97	9536	0.0125	1	0.013	15	0.0329
			0.5	2	2194	4388			0.1					1		15	
	8-2	2	0.5	1	2147	4294	4267	8534	0.1	139557	6.23	8408	0.0123	1	0.013	15	0.0321
			0.5	2	2120	4240			0.1					1		15	
	8-3	3	0.5	1	2360	4720	4355	8710	0.1	139557	6.42	8582	0.0126	1	0.013	15	0.0329
			0.5	2	2145	4290			0.1					1		15	

Assay Date	3/2/2005	Test Chemical ID	2/D	# Concentrations tested		Microsome type	placental	Microsome ID	11343-7	Technician ID	TD	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0368	0.0012
Full activity	End	0.0344	0.0007
Full activity	Overall	0.0356	0.0016
Background	Beginning	0.0000	8.64901E-05
Background	End	0.0000	6.48676E-05
Background	Overall	0.0000	7.17138E-05
Positive	Beginning	0.0192	0.0009
Positive	End	0.0173	0.0010
Positive	Overall	0.0182	0.0013
Negative	Beginning	0.0361	0.0015
Negative	End	0.0349	0.0012
Negative	Overall	0.0355	0.0013

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
2/D	1	1	1.00E-04	-4.00	0.0022
2/D	1	2	1.00E-04	-4.00	0.0022
2/D	1	3	1.00E-04	-4.00	0.0022
2/D	2	1	2.50E-05	-4.60	0.0077
2/D	2	2	2.50E-05	-4.60	0.0078
2/D	2	3	2.50E-05	-4.60	0.0078
2/D	3	1	1.00E-05	-5.00	0.0150
2/D	3	2	1.00E-05	-5.00	0.0142
2/D	3	3	1.00E-05	-5.00	0.0143
2/D	4	1	5.00E-06	-5.30	0.0205
2/D	4	2	5.00E-06	-5.30	0.0198
2/D	4	3	5.00E-06	-5.30	0.0090
2/D	5	1	2.50E-06	-5.60	0.0257
2/D	5	2	2.50E-06	-5.60	0.0242
2/D	5	3	2.50E-06	-5.60	0.0238
2/D	6	1	1.00E-06	-6.00	0.0274
2/D	6	2	1.00E-06	-6.00	0.0262
2/D	6	3	1.00E-06	-6.00	0.0262
2/D	7	1	2.50E-07	-6.60	0.0332
2/D	7	2	2.50E-07	-6.60	0.0304
2/D	7	3	2.50E-07	-6.60	0.0315
2/D	8	1	1.00E-07	-7.00	0.0328
2/D	8	2	1.00E-07	-7.00	0.0321
2/D	8	3	1.00E-07	-7.00	0.0328

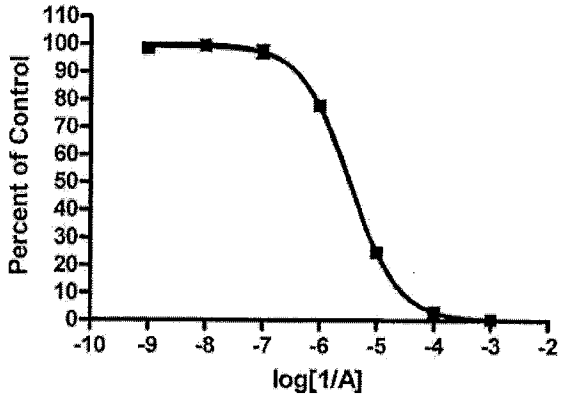
Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-4.00	6.08	6.21	6.29
2	-4.60	21.63	22.00	21.91
3	-5.00	42.02	39.85	40.19
4	-5.30	57.59	55.66	25.22
5	-5.60	72.24	68.07	66.76
6	-6.00	76.90	73.53	73.62
7	-6.60	93.27	85.43	88.48
8	-7.00	92.13	90.29	92.18

APPENDIX F

Prism Output F-1

Placental Assay 2-2-2005

Replicate 1 (1/A)

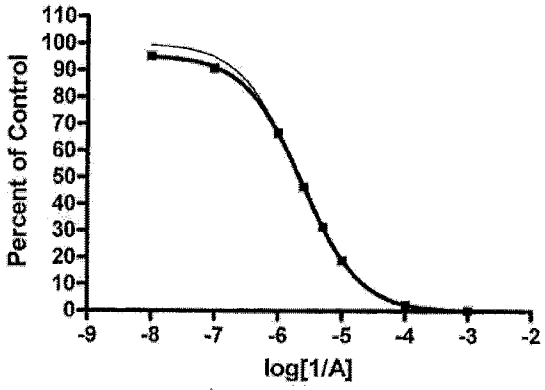


-3.00	0.22	0.56	0.23
-4.00	3.10	3.36	3.14
-5.00	25.61	24.72	23.92
-6.00	75.98	78.06	79.71
-7.00	99.50	99.50	92.77
-8.00	99.50	99.50	99.50
-9.00	99.50	99.50	96.44

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.470
HILLSLOPE	-1.018
EC50	3.385e-006
Std. Error	
LOGEC50	0.01351
HILLSLOPE	0.01797
95% Confidence Intervals	
LOGEC50	-5.499 to -5.442
HILLSLOPE	-1.056 to -0.9807
EC50	3.172e-006 to 3.613e-006
Goodness of Fit	
Degrees of Freedom	19
R^2 (unweighted)	0.9986
Weighted Sum of Squares (1/Y)	0.8064
Absolute Sum of Squares	52.30
Sy.x	1.659
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	7
Number of Y replicates	3
Total number of values	21
Number of missing values	0

Placental Microsomes Assay 02-04-2005

Replicate 2 (1/A)

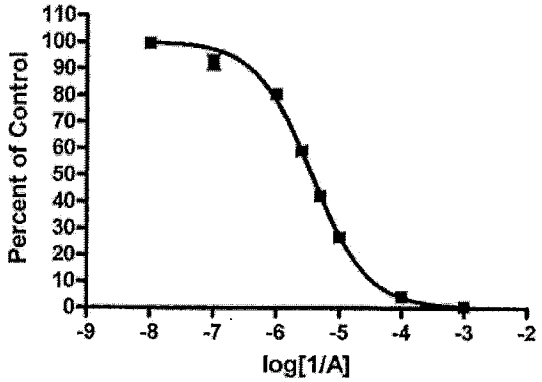


-3.00	0.21	0.11	0.25
-4.00	2.39	2.33	2.38
-5.00	19.31	19.09	18.31
-5.30	31.67	31.42	31.24
-5.60	46.96	46.43	46.02
-6.00	66.32	67.33	66.22
-7.00	89.46	90.51	92.17
-8.00	94.74	94.53	96.48

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.670
HILLSLOPE	-0.9532
EC50	2.137e-006
Std. Error	
LOGEC50	0.01218
HILLSLOPE	0.01882
95% Confidence Intervals	
LOGEC50	-5.695 to -5.645
HILLSLOPE	-0.9923 to -0.9142
EC50	2.016e-006 to 2.265e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9961
Weighted Sum of Squares (1/Y)	1.654
Absolute Sum of Squares	115.2
Sy.x	2.288
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay 02-07-2005

Replicate 3 (1/A)

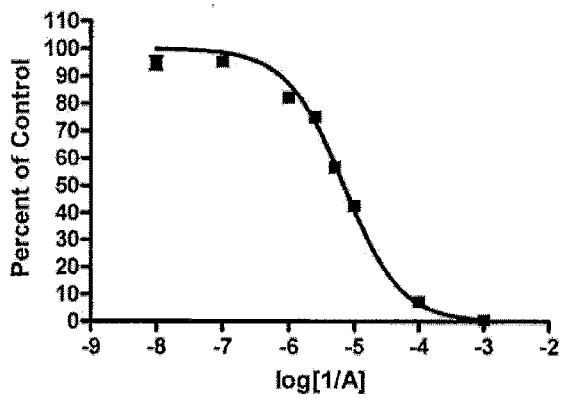


-3.00	0.29	0.62	0.33
-4.00	3.83	3.85	4.60
-5.00	25.89	26.89	26.99
-5.30	42.69	41.08	42.46
-5.60	60.94	57.26	59.10
-6.00	81.14	81.43	78.67
-7.00		94.89	89.58
-8.00	99.50	99.50	99.50

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.436
HILLSLOPE	-0.9816
EC50	3.660e-006
Std. Error	
LOGEC50	0.01089
HILLSLOPE	0.01953
95% Confidence Intervals	
LOGEC50	-5.459 to -5.414
HILLSLOPE	-1.022 to -0.9410
EC50	3.474e-006 to 3.856e-006
Goodness of Fit	
Degrees of Freedom	21
R^2 (unweighted)	0.9968
Weighted Sum of Squares (1/Y)	1.469
Absolute Sum of Squares	93.99
Sy.x	2.116
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	23
Number of missing values	1

Placental Aromatase Assay (03-14-05)

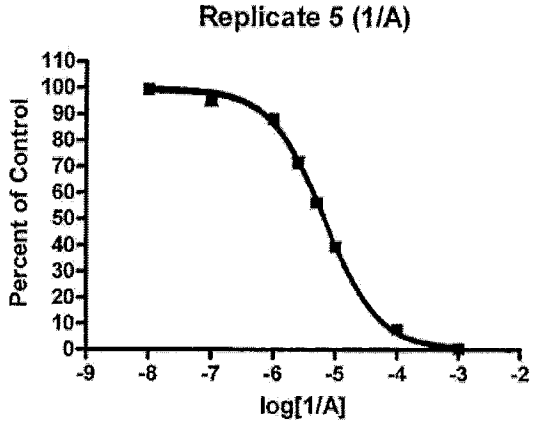
Replicate 4(1/A)



-3.00	0.66	0.22	0.54
-4.00	6.75	7.14	7.61
-5.00	40.48	42.80	44.21
-5.30	55.55	55.06	59.36
-5.60	75.61	76.33	73.49
-6.00	80.38	84.05	82.38
-7.00	93.15	96.64	96.10
-8.00	92.79	99.50	91.81

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.161
HILLSLOPE	-0.9950
EC50	6.906e-006
Std. Error	
LOGEC50	0.01985
HILLSLOPE	0.03047
95% Confidence Intervals	
LOGEC50	-5.202 to -5.120
HILLSLOPE	-1.058 to -0.9318
EC50	6.281e-006 to 7.592e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9904
Weighted Sum of Squares (1/Y)	4.791
Absolute Sum of Squares	282.0
Sy.x	3.580
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Aromatase Assay (03-16-05)

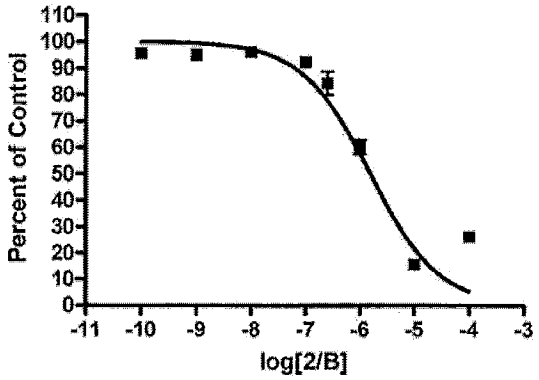


-3.00	0.50	0.52	0.59
-4.00	8.29	7.93	7.59
-5.00	40.24	38.60	39.83
-5.30	54.53	57.14	56.71
-5.60	74.47	72.17	67.19
-6.00	90.74	88.20	85.51
-7.00	99.50	91.27	96.19
-8.00	99.26	99.50	99.24

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.181
HILLSLOPE	-0.9592
EC50	6.596e-006
Std. Error	
LOGEC50	0.01405
HILLSLOPE	0.02130
95% Confidence Intervals	
LOGEC50	-5.210 to -5.152
HILLSLOPE	-1.003 to -0.9151
EC50	6.168e-006 to 7.054e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9961
Weighted Sum of Squares (1/Y)	2.338
Absolute Sum of Squares	122.4
Sy.x	2.359
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay (2-15-05)

Replicate 1 (2/B)

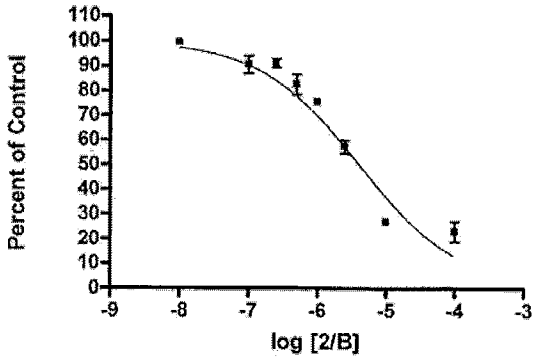


-4.00	24.83	24.70	29.04
-5.00	14.69	16.48	15.62
-6.00	64.44	60.88	54.95
-6.60	78.95	80.92	92.95
-7.00	92.61	94.25	90.06
-8.00	95.45	96.44	96.53
-9.00	94.25	95.90	95.14
-10.00	95.10	94.10	97.71

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.807
HILLSLOPE	-0.6870
EC50	1.558e-006
Std. Error	
LOGEC50	0.1134
HILLSLOPE	0.1015
95% Confidence Intervals	
LOGEC50	-6.043 to -5.572
HILLSLOPE	-0.8976 to -0.4765
EC50	9.064e-007 to 2.678e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9158
Weighted Sum of Squares (1/Y)	63.00
Absolute Sum of Squares	1947
Sy.x	9.409
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay (2-16-05)

Replicate 2 (2/B)

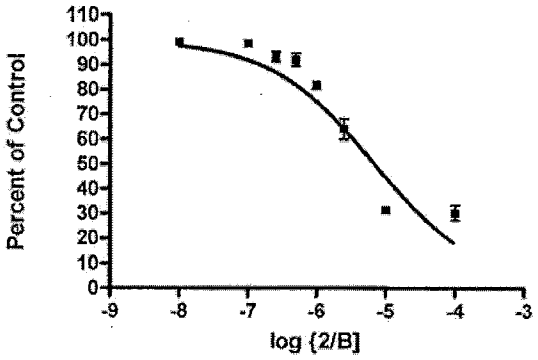


-4.00	22.70	27.38	19.03
-5.00	26.46	26.14	28.16
-5.60	55.26	56.23	60.14
-6.00	74.75	75.61	76.02
-6.30	82.56	86.64	78.23
-6.60	89.69	93.13	90.70
-7.00	94.59	88.08	88.62
-8.00	99.50	99.50	99.50

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.393
HILLSLOPE	-0.5970
EC50	4.042e-006
Std. Error	
LOGEC50	0.07050
HILLSLOPE	0.05858
95% Confidence Intervals	
LOGEC50	-5.540 to -5.247
HILLSLOPE	-0.7185 to -0.4755
EC50	2.887e-006 to 5.660e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9426
Weighted Sum of Squares (1/Y)	30.02
Absolute Sum of Squares	1062
Sy.x	6.947
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay (02-17-05)

Replicate 3 (2/B)

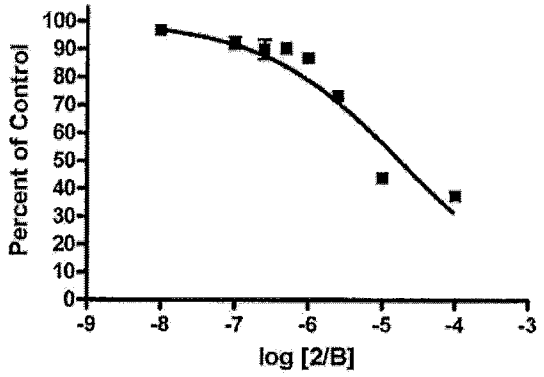


-4.00	31.60	32.54	26.84
-5.00	31.67	31.14	31.75
-5.60	68.14	64.12	60.02
-6.00	80.03	82.82	82.73
-6.30	89.50	94.62	91.73
-6.60	92.82	91.49	95.49
-7.00	99.12	99.22	97.80
-8.00	99.50	99.50	98.79

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.160
HILLSLOPE	-0.5723
EC50	6.921e-006
Std. Error	
LOGEC50	0.09077
HILLSLOPE	0.07143
95% Confidence Intervals	
LOGEC50	-5.348 to -4.972
HILLSLOPE	-0.7205 to -0.4242
EC50	4.487e-006 to 1.068e-005
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.8981
Weighted Sum of Squares (1/Y)	40.93
Absolute Sum of Squares	1786
Sy.x	9.009
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay 3-15-05

Replicate 4 (2/B)

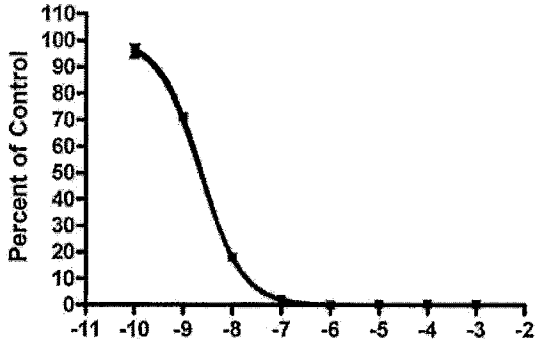


-4.00	35.63	40.09	37.01
-5.00	46.07	43.74	42.04
-5.60	73.60	73.14	73.48
-6.00	89.77	87.35	83.38
-6.30	87.93	91.20	91.69
-6.60	83.61	90.92	95.35
-7.00	95.23	88.04	93.36
-8.00	95.17	95.92	99.23

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-4.752
HILLSLOPE	-0.4597
EC50	1.772e-005
Std. Error	
LOGEC50	0.08748
HILLSLOPE	0.04547
95% Confidence Intervals	
LOGEC50	-4.933 to -4.570
HILLSLOPE	-0.5540 to -0.3654
EC50	1.167e-005 to 2.691e-005
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9023
Weighted Sum of Squares (1/Y)	20.09
Absolute Sum of Squares	1106
Sy.x	7.090
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay 02-22-05

Replicate 1 (1/C)

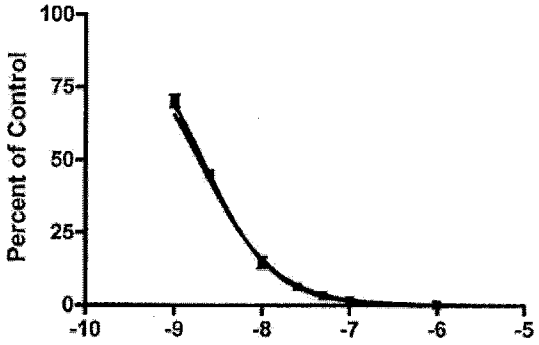


log[1/C]			
-3.00	0.50	0.50	0.50
-4.00	0.50	0.50	0.50
-5.00	0.50	0.50	0.50
-6.00	0.50	0.09	0.18
-7.00	1.75	2.00	2.22
-8.00	18.38	17.85	17.97
-9.00	70.80	71.62	71.07
-10.00	93.30	96.43	98.13

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-8.628
HILLSLOPE	-1.043
EC50	2.355e-009
Std. Error	
LOGEC50	0.03035
HILLSLOPE	0.04083
95% Confidence Intervals	
LOGEC50	-8.691 to -8.565
HILLSLOPE	-1.128 to -0.9588
EC50	2.038e-009 to 2.723e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9995
Weighted Sum of Squares (1/Y)	4.895
Absolute Sum of Squares	15.73
Sy.x	0.8456
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay 02-23-05

Replicate 2 (1/C)

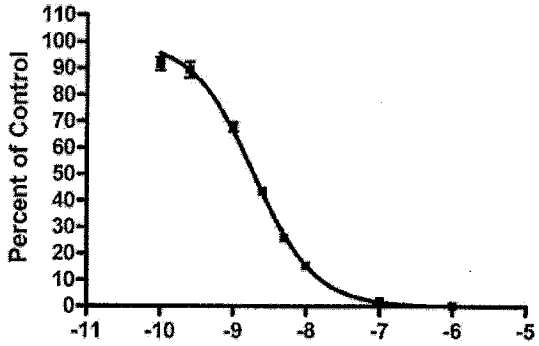


log[1/C]			
-6.00	0.09	0.50	0.09
-6.00	0.59	0.38	0.23
-7.00	1.64	1.69	1.61
-7.30	3.63	3.22	3.03
-7.60	6.55	6.37	6.37
-8.00	15.94	12.59	15.87
-8.60	44.32	45.90	45.71
-9.00	72.74	68.89	69.30

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-8.678
HILLSLOPE	-1.083
EC50	2.099e-009
Std. Error	
LOGEC50	0.01378
HILLSLOPE	0.02014
95% Confidence Intervals	
LOGEC50	-8.706 to -8.649
HILLSLOPE	-1.125 to -1.041
EC50	1.966e-009 to 2.242e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9982
Weighted Sum of Squares (1/Y)	1.956
Absolute Sum of Squares	25.05
Sy.x	1.067
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay 02-24-05

Replicate 3 (1/C)

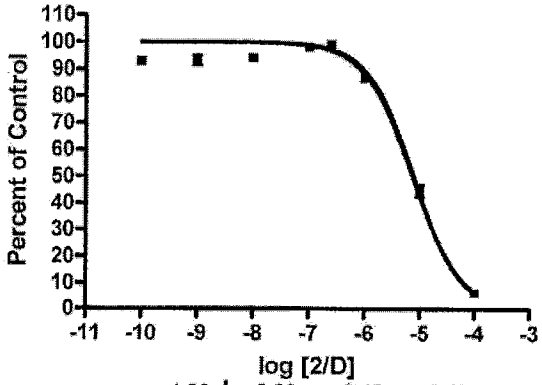


log[1/C]			
-6.00	0.41	0.05	0.37
-7.00	1.74	1.90	2.13
-8.00	15.09	15.59	15.10
-8.30	26.08	25.71	25.51
-8.60	44.53	43.11	43.36
-9.00	65.92	68.00	69.51
-9.60	92.82	88.49	86.98
-10.00	90.58	89.63	94.28

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-8.718
HILLSLOPE	-1.035
EC50	1.913e-009
Std. Error	
LOGEC50	0.01230
HILLSLOPE	0.02272
95% Confidence Intervals	
LOGEC50	-8.744 to -8.693
HILLSLOPE	-1.082 to -0.9882
EC50	1.804e-009 to 2.028e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9965
Weighted Sum of Squares (1/Y)	1.976
Absolute Sum of Squares	100.8
Sy.x	2.140
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay 02-28-05

Replicate 1(2/D)

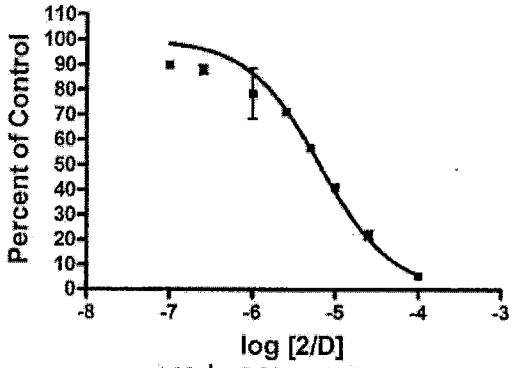


log [2/D]			
-4.00	6.02	6.43	6.43
-5.00	46.01	46.01	42.10
-6.00	87.78	85.88	86.48
-6.60	99.50	98.91	99.15
-7.00	96.80	98.46	99.19
-8.00	94.36	94.31	93.58
-9.00	94.31	94.29	91.11
-10.00	93.14	94.06	91.89

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.106
HILLSLOPE	-1.043
EC50	7.830e-006
Std. Error	
LOGEC50	0.02991
HILLSLOPE	0.04869
95% Confidence Intervals	
LOGEC50	-5.168 to -5.044
HILLSLOPE	-1.144 to -0.9420
EC50	6.788e-006 to 9.033e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9810
Weighted Sum of Squares (1/Y)	5.043
Absolute Sum of Squares	449.0
Sy.x	4.518
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay (03-01-05)

Replicate 2 (2/D)

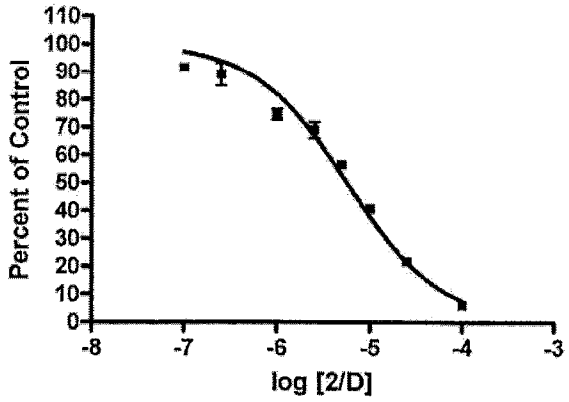


-4.00	5.38	5.63	5.91
-4.60	22.99	19.94	23.11
-5.00	42.81	40.29	40.52
-5.30	57.69	55.99	56.43
-5.60	72.68	69.90	70.75
-6.00	84.41	84.09	66.87
-6.60	86.18	88.02	89.63
-7.00	91.08	89.81	89.03

	Data Set-A
Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.216
HILLSLOPE	-0.9201
EC50	6.081e-006
Std. Error	
LOGEC50	0.02884
HILLSLOPE	0.04367
95% Confidence Intervals	
LOGEC50	-5.276 to -5.156
HILLSLOPE	-1.011 to -0.8295
EC50	5.298e-006 to 6.978e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9662
Weighted Sum of Squares (1/Y)	10.70
Absolute Sum of Squares	699.2
Sy.x	5.638
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Placental Microsomes Assay (03-02-05)

Replicate 3 (2/D)



-4.00	6.08	6.21	6.29
-4.60	21.63	22.00	21.91
-5.00	42.02	39.85	40.19
-5.30	57.59	55.66	
-5.60	72.24	68.07	66.76
-6.00	76.90	73.53	73.62
-6.60	93.27	85.43	88.48
-7.00	92.13	90.29	92.18

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-5.246
HILLSLOPE	-0.8685
EC50	5.675e-006
Std. Error	
LOGEC50	0.02597
HILLSLOPE	0.03461
95% Confidence Intervals	
LOGEC50	-5.300 to -5.192
HILLSLOPE	-0.9404 to -0.7965
EC50	5.011e-006 to 6.426e-006
Goodness of Fit	
Degrees of Freedom	21
R^2 (unweighted)	0.9780
Weighted Sum of Squares (1/Y)	6.985
Absolute Sum of Squares	448.0
Sy.x	4.619
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	23
Number of missing values	1

APPENDIX G

Statistician's Report G-1

DRAFT REPORT

On

PLACENTAL AROMATASE VALIDATION STUDY
INTRALABORATORY STATISTICAL ANALYSIS OF BATTELLE DATA

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 5

January 9, 2006

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

BATTELLE
505 King Avenue
Columbus, Ohio 43201

Placental Aromatase Validation Study

Conduct Studies with Centrally Prepared Microsomes
Intralaboratory Statistical Analysis of Battelle Data

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 5

_____ _____

Ying-Liang Chou, Author Date

_____ _____

Paul I. Feder, Reviewer Date

Quality Assurance Statement

Study Number:

This study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Battelle Task Leader/ Battelle Management
Audit study file	12/19/2005	12/19/2005
Audit draft report	12/19/2005	12/19/2005

Quality Assurance Unit **Date**

Hillary Flory 1-9-06

This report discusses the methods and results of the intralaboratory statistical analysis on the data collected at Battelle with the placental aromatase assay for Task 5: Conduct Studies with Centrally Prepared Microsomes.

Summary and Conclusions

There were four reference chemicals analyzed in this task: aminoglutethimide, chrysin, econazole, and ketoconazole (coded Chemicals 1/A, 2/B, 1/C, and 2/D, respectively). Five replicates were carried out for Chemical 1/A, four replicates for Chemical 2/B, and three replicates for both Chemicals 1/C and 2/D. For each chemical the statistical analyses were based on the data from all the replicates.

Two types of data were obtained: inhibition curve fit data and control activity data. Statistical analyses were carried out separately for these two types of data. The response variable was the "percent of control". Percent of control is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the average background adjusted aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. Control activity data consists of four types of controls: full enzyme activity control, background activity control, positive control (4-OH ASDN), and negative control (lindane). Statistical analyses were performed separately for these four types of controls.

For the inhibition curve fit data, separate analyses were carried out for each of the four reference chemicals. For each reference chemical, concentration response curves were fitted within each replicate to describe the relation between reference chemical concentrations and extent of inhibition. The concentration response curves were summarized by the IC_{50} (concentration corresponding to 50 percent inhibition) and slope. Results were compared across replicates within reference chemical. For the control activity data, statistical analyses were carried out for the controls combined across reference chemicals. Full enzyme activity, background activity, positive, and negative control tube responses were compared between the beginning and the end of each replicate to identify differences within replicates, differences among replicates within reference chemicals, and differences among reference chemicals.

The following results were observed:

1. The estimated $\log_{10}IC_{50}$ ranged from -5.671 to -5.159 for the five replicates of Chemical 1/A, ranged from -5.808 to -4.752 for the four replicates of Chemical 2/B, ranged from -8.719 to -8.628 for the three replicates of Chemical 1/C, and ranged from -5.350 to -5.105 for the three replicates of Chemical 2/D. Chemical 1/C had orders of magnitude lower IC_{50} than the other three chemicals.

2. For Chemicals 1/A, 1/C, and 2/D the estimated slopes were close to -1. The estimated slopes ranged from -1.018 to -0.916 for Chemical 1/A, ranged from -1.083 to -1.035 for

Chemical 1/C, and ranged from -1.045 to -0.803 for Chemical 2/D. Chemical 2/B had a flatter slope, averaging -0.56 across the four replicates.

3. Figures 2-1 and 2-2 show that for Chemical 2/B the bottom threshold lies between 20% and 40%, depending on replicate. The two-parameter concentration response model used in this report assumes a bottom threshold of 0% and so does not fit the Chemical 2/B response data. This implies that four parameter, variable top and bottom threshold models will need to be used in future response curve fits for this assay.

4. The majority of variation for $\log_{10}IC_{50}$ was from replicate-to-replicate variation. It ranged from 0.0127 to 0.1832 for the four chemicals. The within-replicate variations were of lower order of magnitude for all four chemicals. The replicate-to-replicate and within-replicate variations for slope were all small and or the most part of the same order of magnitude for all four chemicals.

5. For all four control types the differences between the beginning and the end portions, when averaged across replicates, were significant. The end portion was significantly lower than the beginning portion. This implies a reduction in aromatase activity between the beginning and the end of a replicate.

6. For all the control types chemical by portion interaction was not significant and was not included in the tables.

7. For Chemical 1/A the variation in Replicate 1 for the full enzyme activity controls and for the negative controls was substantially larger and out of line with than that for any replicate for all the chemicals (Figures 5, 8).

8. For positive controls the majority of variation was from replicate-to-replicate.

Introduction and Background

In Task 5 of the Placental Aromatase Validation Study, each laboratory carried out multiple independent replicates of the placental aromatase assay with four reference chemicals according to a common test protocol. For each reference chemical concentration, response curves were fitted to the results from each replicate. Graphical displays and analysis of variance summary comparisons of the concentration response curves and the full enzyme activity control, background activity control, positive, and negative controls were prepared for each inhibitor compound.

This report discusses the methods and results of the statistical analyses of the data collected at Battelle for the four reference chemicals: aminoglutethimide, chrysin, econazole, and

ketoconazole coded Chemicals 1/A, 2/B, 1/C, and 2/D, respectively.

Data Used in the Analyses

Aromatase activity levels were determined for the full enzyme activity control, background activity control, positive control, and negative control, and for eight graded concentrations of each of the four reference chemicals. The specific concentration levels varied for each of the reference chemicals.

Five replicates were carried out with Chemical 1/A, four replicates with Chemical 2/B, and three replicates each with Chemicals 1/C and 2/D. Within each replicate of each reference chemical three repetitions were run at each of the eight concentrations. In addition, two repeat tubes of the full enzyme activity, background activity, positive, and negative controls were run prior to the inhibition concentration runs and two repeat tubes of each the four control types were run following the inhibition concentration runs.

Statistical analyses were carried out on the "percent of control" responses. Percent of control is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the background adjusted average aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background activity control tubes is necessarily 0 percent within each replicate.

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibition concentrations, but this may vary with the inhibitor. Chemical 2/B departs from this behavior with respect to the bottom threshold.

The percent of control activity responses at varying inhibitor concentrations within each replicate are displayed in Tables A-1a, A-1b, A-1c, and A-1d for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively. The full enzyme activity, background activity, positive, and negative controls background adjusted aromatase activity and the percent of control data by replicate and portion (beginning or end) are displayed in Tables A-2a, A-2b, A-2c, and A-2d for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively.

Several potentially outlying responses were identified in the data, in that they differed from surrounding data values more than typically:

- Chemical 1/A – Replicate 1 (assay date 2-2-05), level 8, Percent of Control values 80.75, 77.04, 75.64
- Chemical 1/A – Replicate 3 (assay date 2-7-05), level 7, Percent of Control value 49.35

- Chemical 2/D – Replicate 3 (assay date 3-2-05), level 4, Percent of Control value 25.22

There were no indications for any of these values of departures from protocol test procedures or of clerical errors. It was thus decided to include all these values in the statistical analyses.

Objectives

The primary objectives of the statistical analysis are:

1. Fit concentration curves within each replicate to describe the trend in the percent of control activity across varying inhibitor concentrations for each of the four reference chemicals.
2. Estimate the IC_{50} concentration, slope, and associated standard errors within each replicate of each reference chemical.
3. Combine results across replicates within reference chemicals to determine the average IC_{50} concentration, average slope, and associated standard errors among replicates.
4. Determine whether there are differences between the full enzyme activity, background activity, positive, and negative controls obtained at the beginning and those obtained at the end of each replicate within each reference chemical.
5. Assess the consistency of test conditions within replicates, across replicates within reference chemicals, and across reference chemicals based on the full enzyme activity, background activity, positive, and negative control values.

Statistical Analysis Methods

Concentration Response Trend Curves

For each reference chemical and replicate within reference chemical a concentration response curve was fitted to the percent of control activity values at the three repetitions at each of the eight graded reference chemical inhibitor concentrations.

For purposes of response curve fitting, concentration was expressed on the log scale. In agreement with past convention, common logarithms (i.e. base 10) were used. Let X denote the logarithm of the concentration of inhibitor compound (e.g. if concentration = 10^{-5} then X = -5). Let

Y ≡ (background corrected) percent of control activity in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average (not corrected for background) DPMs across the repeat tubes with the same inhibitor concentration

$\beta \equiv$ slope of the concentration response curve (β is negative)
$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%)

The following two parameter concentration response curve was fitted to relate percent of control activity to logarithm of concentration within each replicate

$$Y = 100/[1 + 10^{(\mu - X)\beta}] + \epsilon$$

where ϵ is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts) and also approximately proportional to the response Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. This weighting system gives greater weight to the lower end of the concentration response curve, where greater inhibition occurs.

Model fits were carried out using PRISM software (Version 4). Observed percent of control values above 100% were set to 99.5%. Observed percent of control values below 0% were set to 0.5%. This adjustment tacitly assumes an upper bound of 100% on the concentration response curve and a lower bound of 0%.

For each replicate the estimated $\log_{10}IC_{50}$ (μ) and its associated standard error, the IC_{50} and its associated geometric standard error, the slope (β) and its associated standard error, and the "Status" of each response curve are reported. The "Status" of each response curve is indicated as "C", complete, if the concentration response curve inhibition ranges from essentially 0 percent to at least 80 percent of control. It is indicated as "I", incomplete, if the concentration response curve inhibition ranges from essentially 100% inhibition to less than 50% inhibition. It is also indicated as "I", incomplete, if the concentration response curve decreases to between 80% and 50% of control. It is indicated as "N", no inhibition, if the concentration response curve inhibition does not go below 80% of control. If the concentration response curve does not extend beyond 50% of control, an IC_{50} estimate is not calculated.

It should be noted that the above two parameter model assumes that the top and bottom thresholds of the concentration response models are at 100% and 0% respectively. This is often so, but not always. The two parameter model will be used in the analyses in this task for all the chemicals, to demonstrate its lack-of-fit when the assumptions concerning the thresholds are not satisfied. This is the case for the bottom threshold for chrysin (Chemical 2/B). The two parameter model will be expanded to a four parameter model, with variable top and bottom thresholds, in Task 7.

For each of the reference chemicals a one-way random effects analysis of variance model

with heterogeneous variances among the replicates was fitted to the parameter estimates, $\log_{10}IC_{50}$ (μ) and slope (β), from the concentration response curve fits within each replicate, using weights incorporating within replicate variances. The random effect was replicate within reference chemical. The within replicate variances were estimated as the squares of the standard errors for each replicate. The analysis of variance fits provide estimated weighted averages effects (mean) across the replicates within reference chemicals and their associated standard errors. These standard errors include both within replicate and between replicate components of variation. Degrees of freedom associated with the mean effects were calculated based on Satterthwaite's approximation.

The estimated IC_{50} for the reference chemical was estimated as 10 to the power mean $\log_{10}IC_{50}$. The geometric standard error associated with the estimated IC_{50} was calculated as 10 to the power standard error associated with mean $\log_{10}IC_{50}$.

Slope (β) and $\log_{10}IC_{50}$ (μ) were each compared across replicates within reference chemicals based on the one-way random effects analysis of variance model fits. For each of β and μ, plots were prepared that display the parameters within each replicate with associated 95% confidence intervals based on the within replicate standard error and the average across replicates with associated 95% confidence interval incorporating replicate-to-replicate variation.

Concentration response curves were fitted to the averages of the three repetitions within each replicate. Estimates and associated standard errors (or geometric standard error) for $\log_{10}IC_{50}$ (μ), IC_{50}, and slope (β) were displayed. The averages of the three repetitions for each replicate were plotted in the same plot with plotting symbols distinguishing among replicates. The concentration response curves for each replicate, fitted to the average data, were superimposed on the same plot to compare the percent of control activity values across replicates.

On a separate plot the average percent of control values for each replicate were plotted versus logarithm of inhibitor concentrations. The average concentration response curve across replicates was superimposed on the same plot. The average response curve was calculated as

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}]$$

where β_{avg} and μ_{avg} were estimated across the three replicates, based on the random effects one-way analysis of variance model discussed above.

All concentration response curves were fitted to the data using the nonlinear regression analysis features in the PRISM statistical analysis package, Version 4. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons were carried out using PRISM and the SAS statistical analysis system-Version 9.

Analysis of Full Enzyme Activity, Background Activity, Positive, and Negative Controls Across Reference Chemicals and Replicates Within Reference Chemicals

Within each reference chemical and replicate within reference chemical, quadruplicate repetitions were made of the full enzyme activity, background activity, positive, and negative control responses. Half the repetitions were carried out at the beginning of the replicate and half at the end. If the test conditions were consistent throughout the replicate, the control tube responses at the beginning should be equivalent to those at the end.

The control response analyses were carried out with the control data combined across 15 replicates within the four reference chemicals. The control responses were expressed as percent of control. The full enzyme activity, background activity, positive, and negative controls percent of control responses were plotted across replicates, with plotting symbol distinguishing between beginning and end, and with reference line at 0% (background activity controls), at 100% (full enzyme activity controls), or at 100% (negative controls),. These plots indicate the extent of consistency across replicates with respect to average value and variability, and provide comparisons of beginning versus end of each replicate. Additional plots were prepared displaying the difference of the average of the first two percent of control values (i.e. those based on the "beginning" tubes) and the average of the last two percent of control values (i.e. those based on the "end" tubes) across replicates. Each plot has a reference line at 0.

Mixed effects analysis of variance models were fitted to the full enzyme activity, background activity, positive, and negative controls percent of control responses. The fixed effect factors in the analysis of variance were reference chemical, portion (beginning or end), and portion by reference chemical interaction. The random effects were replicate nested within reference chemical and portion by replicate within reference chemical interaction. The residual error variation was based on the variation among repetitions within replicate and portion. The response was percent of control. For the background activity and full enzyme activity controls the average of the repetitions within a replicate are constrained to be 0 and 100 respectively, which implies that the variation associated with the replication effect is necessarily constrained to be 0.

Roundoff

Some derived numbers in the results tables may differ from those in the computer printouts or from those obtained using hand calculations by several units in the least significant digit due to round off in intermediate numbers and in intermediate calculations.

Statistical Analysis Results

Concentration Response Trend Curves

Concentration response curves were fitted separately to the repeat tubes data within each replicate and to the averages of the repetitions within each replicate. Percent of control, the response variable for the statistical analyses, is displayed in Tables A-1a, A-1b, A-1c, and A-1d for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively. The estimated parameters of the fitted concentration response curves are displayed in Tables 1-1, 2-1, 3-1, and 4-1 for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively. The concentration response curves fitted to the averages of the three repetitions within each replicate for Chemicals 1/A, 2/B, 1/C, and 2/D are displayed in Figures 1-1, 2-1, 3-1, and 4-1.

The parameters of the average concentration response curves, based on random effects analysis of variance model fits with replicate as a random effect are displayed in Tables 1-1, 2-1, 3-1, and 4-1 for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively ("Mean" under "Replicate" column). The parameters within each replicate are also displayed. The average concentration response curves, along with the averages of three repetitions within each replicate are plotted together in Figures 1-2, 2-2, 3-2, and 4-2 for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively.

The parameter estimates for each replicate and the average parameter estimates across replicates and their associated 95% confidence intervals are displayed in Tables 1-2, 2-2, 3-2, and 4-2 for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively. The corresponding graphs for $\log_{10}IC_{50}$ are presented in Figures 1-3, 2-3, 3-3, and 4-3; and for slope, Figures 1-4, 2-4, 3-4, 4-4.

The results of analyses of variance for these estimates are presented in Tables 1-3, 2-3, 3-3, and 4-3 for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively. For each replicate, the squares of the standard errors associated with each parameter are given. These estimates include only within replicate variation. Across replicates, the replicate-to-replicate variation and the square of the standard error of the overall average are displayed. These estimates include both within replicate variation and replicate-to-replicate variation.

Chemical 1/A: Response Curve Analysis

In Figure 1-3, replicates 4 and 5 are seen to have higher IC_{50} than the average. In Figure 1-4, the slopes are seen to be relatively consistent among replicates. The parameter estimates are displayed in Table 1-2. The variability in replicates 1 and 3 is somewhat greater than that in replicates 2, 4, and 5. Replicates 1 and 3 were identified as having potentially outlying observations that were retained in the analyses.

Table 1-3 shows the estimated variance components for the parameter estimates. For $\log_{10}IC_{50}$ the replicate-to-replicate variation was more than ten times the individual replicate within-replicate variances. For slope the replicate-to-replicate variation was essentially zero and the individual replicate within-replicate variances were close to zero, with those for replicates 1 and 3 substantially larger than those for replicates 2, 4, and 5.

Chemical 2/B: Response Curve Analysis

Figures 2-1 and 2-2 show substantial lack-of-fit of the concentration response model fits to the data. The two parameter model forces the lower estimated threshold to 0% whereas the data suggest lower thresholds in the range from 20% to 30%. This inflates the estimates of both the within replicate variation and the replicate-to-replicate variation and results in the relatively large standard errors of the overall means, as seen in Figures 2-3 and 2-4. Both the $\log_{10}IC_{50}$ and the slope estimates display upward trends with replicate number. This is due to the upward trend in the lower threshold, as is seen in Figures 2-1 and 2-2.

Table 2-3 shows that the replicate-to-replicate variation accounted for the majority of the overall variation for $\log_{10}IC_{50}$. The replicate-to-replicate variation was more than 15 times the individual replicate within-replicate variances. For slope, the replicate-to-replicate variation was about the same size as the within replicate variation.

Chemical 1/C: Response Curve Analysis

Figures 3-1 and 3-2 show that all three replicates are in line with the fitted response curve. As shown in Table 3-2 and in Figure 3-3, parameter estimates for $\log_{10}IC_{50}$ are consistent across three replicates. Table 3-3 shows that the replicate-to-replicate variation accounted for the majority of the overall variation for $\log_{10}IC_{50}$. The replicate-to-replicate variation was more than 10 times the individual replicate within-replicate variances. The slopes are consistent across replicates, as shown in Tables 3-2, 3-3 and in Figure 3-4.

Chemical 2/D: Response Curve Analysis

Figure 4-1 shows that the two parameter model, with upper threshold of 100%, does not fit the upper threshold of the data from replicate 1. Figure 4-2 shows that an outlying value in replicate 3 ($\log_{10}$ concentration = -5.3) increased the within replicate variability of the $\log_{10}IC_{50}$ and the slope estimates as compared to the other two replicates.

Figures 4-3 and 4-4 show that for replicate 2 both the estimated $\log_{10}IC_{50}$ and slope were about average. For replicate 1, the estimated $\log_{10}IC_{50}$ was relatively high and the estimated slope was relatively low. For replicate 3, the estimated $\log_{10}IC_{50}$ was relatively low and the estimated slope was relatively high.

Table 4-3 shows that for $\log_{10}IC_{50}$, the replicate-to-replicate variation was more than ten times the individual replicate within-replicate variances for replicates 1 and 2 and more than four 4 times the individual replicate within-replicate variances for replicate 3. For slope, the replicate-to-replicate variation was more than twice the within replicate variation for replicate 3 and more than four times the within replicate variation for replicates 1 and 2.

Analysis of Full Enzyme Activity, Background Activity, Positive, and Negative Controls Across Reference Chemicals and Replicates Within Reference Chemicals

The control data were combined across the 15 replicates among the four reference chemicals. The control responses were expressed as percent of control. The full enzyme activity, background activity, positive, and negative control responses for each replicate are displayed in Tables A-2a, A-2b, A-2c, and A-2d for Chemicals 1/A, 2/B, 1/C, and 2/D, respectively. The percent of control data are plotted by replicate in Figures 5 to 8, with plotting symbol distinguishing between beginning and end of the replicate. Figures 9 to 12 show the differences between the averages at the beginning and at the end within each replicate (end minus beginning). In the horizontal axis of these figures, "1/A-1" stands for replicate 1 of Chemical 1/A, "2/B-2" stands for replicate 2 of Chemical 2/B, and so on.

Mixed effects analysis of variance models were fitted to the full enzyme activity, background activity, positive, and negative controls percent of control data. The fixed effect factors in the analysis of variance were reference chemical, portion (beginning or end), and portion by reference chemical interaction. The random effects were replicate nested within reference chemical and portion by replicate within reference chemical interaction. The residual error variation corresponds to variation among repetitions within reference chemical, replicate, and portion. For the background and full enzyme activity control responses the component of variation due to replicate is constrained to be 0 by the definitions of these control responses. The analysis results for the four types of controls are displayed in Table 5. The left panel of the table displays the results of the tests for the differences between the responses collected at the beginning and at the end of a replicate. The right panel displays the estimated variance components across replicate within reference chemicals and across portion by replicate interaction within reference chemicals. The chemical by portion interaction was not significant for any of the four chemicals and is not included in Table 5.

Figures 5 to 8 show that the control responses were nearly always lower at the end of a replicate than at the beginning. This was true for the full enzyme activity, background activity, positive, and negative controls. This is reflected in the curves in Figures 9 to 12 nearly always falling below zero and in the significant portion effects in Table 5. Figures 5, 7, and 8 show that except for the first replicate for reference chemical 1/A the average levels and the variability about the average levels were consistent across all the replicates. The test conditions appear to have been consistent among the reference chemicals and the replicates within reference chemicals. Figures 5 and 8 show that for the full enzyme activity controls and for the negative controls, replicate 1 of reference chemical 1/A had greater variability between the beginning and the end of the replicate than did the other replicates. Figure 7 shows that the positive controls both at the beginning and the end of the replicates tended to have greater activity for reference chemical 1/A and 2/B than for reference chemicals 1/C and 2/D. However Figure 7 shows that

the differences between the beginning and the end were about the same for nearly all the replicates and all the reference chemicals. Replicate 1 for reference chemical 1/A looked different than the other replicates. The positive control values at the beginning of the replicate were smaller than those at the end. This is also reflected in the relatively large portion by replicate interaction for the positive controls for reference chemical 1/A shown in Table 5.

In general the test system appears to be consistent across all the replicates within all the chemicals. However there was consistent decrease in control activity between the beginning and the end of nearly all the test replicates, which was significant for each of the four control responses. This can result in potential bias or potential loss of precision due to decrease of aromatase activity within replicates. If the inhibition concentration tubes are tested in order of inhibitor concentration, the decrease in aromatase activity across the replicate can accentuate the apparent inhibition if the reference chemical concentrations are varied from low to high or can lessen the apparent inhibition if the reference chemical concentrations are varied from high to low. If the 24 tubes (8 inhibitor concentrations × 3 repetitions per concentration) were tested in randomized order then the decrease in aromatase activity within the replicate would increase the residual variation about the fitted concentration response curves for each replicate but would avoid resulting in biased response curves.

Table 1-1. Chemical 1/A: Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Placental Aromatase Assay.

Replicate	$Log_{10}IC_{50}$ (SE)	IC_{50} (GSE)[d]	Slope (SE)	Status
Individual Values[a]				
1	-5.470 (0.063)	$3.385x10^{-6}$ (1.155)	-1.018 (0.083)	C
2	-5.671 (0.012)	$2.132x10^{-6}$ (1.028)	-0.953 (0.019)	C
3	-5.466 (0.064)	$3.422x10^{-6}$ (1.159)	-0.916 (0.092)	C
4	-5.159 (0.020)	$6.927x10^{-6}$ (1.046)	-0.996 (0.030)	C
5	-5.181 (0.014)	$6.586x10^{-6}$ (1.033)	-0.959 (0.021)	C
Mean[c]	-5.387 (0.099)	$4.099x10^{-6}$ (1.257)	-0.963 (0.012)	--
Average Values[b]				
1	-5.467 (0.117)	$3.409x10^{-6}$ (1.310)	-1.019 (0.158)	C
2	-5.672 (0.021)	$2.127x10^{-6}$ (1.050)	-0.948 (0.033)	C
3	-5.455 (0.066)	$3.511x10^{-6}$ (1.164)	-0.934 (0.102)	C
4	-5.158 (0.029)	$6.948x10^{-6}$ (1.069)	-0.981 (0.044)	C
5	-5.180 (0.019)	$6.603x10^{-6}$ (1.046)	-0.959 (0.029)	C

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each Chemical 1/A concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each Chemical 1/A concentration level within each replicate.
c. Weighted averages of the parameter estimates across the five replicates.
d. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

Table 1-2. Chemical 1/A: Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Estimate (95% CI)					
	Replicate 1[a]	Replicate 2[a]	Replicate 3[a]	Replicate 4[a]	Replicate 5[a]	Mean[b]
$Log_{10}IC_{50}$	-5.470 (-5.600, -5.340)	-5.671 (-5.696, -5.646)	-5.466 (-5.599, -5.333)	-5.159 (-5.200, -5.118)	-5.181 (-5.210, -5.152)	-5.387 (-5.658, -5.117)
Slope	-1.018 (-1.191, -0.845)	-0.953 (-0.992, -0.914)	-0.916 (-1.108, -0.724)	-0.996 (-1.058, -0.933)	-0.959 (-1.003, -0.914)	-0.963 (-0.988, -0.938)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition values within replicates.
b. Mean and its associated 95% confidence interval, based on a one-way analysis of variance model with replicate treated as a random effect.

Table 1-3. Chemical 1/A: Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Variance/Degree of Freedom[a,b,c]						
						Overall	
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Random Replicate (p-value)[d]	Variance of Mean
$Log_{10}IC_{50}$	0.00392 /df=22	0.00015 /df=22	0.00408 /df=22	0.00039 /df=22	0.00020 /df=22	0.04778 /df=4 (p=0.081)	0.00989 /df=4.212
Slope	0.00694 /df=22	0.00035 /df=22	0.00855 /df=22	0.00091 /df=22	0.00045 /df=22	6.28×10^{-22} /df=4 (p=NA)	0.00016 /df=53.209

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition results within each concentration level.
b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.
c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(Si^4/df_i))$, where S_r^2 is random replicate variance, S_i^2 and df_i are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of S_r^2 and K is the number of replicates (Hartung and Makambi, 2001).

d. p-value is based on the Wald Z-test result.

Table 2-1. Chemical 2/B: Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Placental Aromatase Assay.

Replicate	$Log_{10}IC_{50}$ (SE)	IC_{50} (GSE)[d]	Slope (SE)	Status
Individual Values[a]				
1	-5.808 (0.114)	$1.557x10^{-6}$ (1.299)	-0.687 (0.101)	C
2	-5.394 (0.071)	$4.037x10^{-6}$ (1.176)	-0.597 (0.059)	C
3	-5.160 (0.091)	$6.914x10^{-6}$ (1.233)	-0.572 (0.071)	C
4	-4.752 (0.087)	$1.771x10^{-5}$ (1.223)	-0.460 (0.045)	C
Mean[c]	-5.276 (0.219)	$5.303x10^{-6}$ (1.655)	-0.558 (0.047)	--
Average Values[b]				
1	-5.803 (0.213)	$1.575 x10^{-6}$ (1.633)	-0.690 (0.192)	C
2	-5.390 (0.132)	$4.075 x10^{-6}$ (1.355)	-0.593 (0.109)	C
3	-5.157 (0.172)	$6.961 x10^{-6}$ (1.486)	-0.571 (0.135)	C
4	-4.749 (0.159)	$1.783 x10^{-5}$ (1.443)	-0.460 (0.083)	C

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each chemical 2/B concentration level.
b. Concentration response curve fitted to the averages of the four repetitions at each chemical 2/B concentration level within each replicate.
c. Weighted averages of the parameter estimates across the four replicates.
d. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

Table 2-2. Chemical 2/B: Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Estimate (95% CI)				
	Replicate 1[a]	Replicate 2[a]	Replicate 3[a]	Replicate 4[a]	Mean[b]
$Log_{10}IC_{50}$	-5.808 (-6.043, -5.572)	-5.394 (-5.540, -5.248)	-5.160 (-5.349, -4.972)	-4.752 (-4.933, -4.571)	-5.276 (-5.979, -4.572)
Slope	-0.687 (-0.897, -0.476)	-0.597 (-0.718, -0.475)	-0.572 (-0.720, -0.424)	-0.460 (-0.554, -0.365)	-0.558 (-0.687, -0.429)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition values within replicates.
b. Mean and its associated 95% confidence interval, based on a one-way analysis of variance model with replicate treated as a random effect.

Table 2-3. Chemical 2/B: Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Variance/Degree of Freedom[a,b,c]					
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Overall	
					Random Replicate (p-value)[d]	Variance of Mean
$Log_{10}IC_{50}$	0.01288 /df=22	0.00500 /df=22	0.00824 /df=22	0.00765 /df=22	0.1832 /df=3 (p=0.123)	0.04792 /df=2.950
Slope	0.01028 /df=22	0.00342 /df=22	0.00509 /df=22	0.00206 /df=22	0.00445 /df=3 (p=0.254)	0.00221 /df=4.078

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition results within each concentration level.
b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.
c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(Si^4/df_i))$, where S_r^2 is random replicate variance, S_i^2 and df_i are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of S_r^2 and K is the number of replicates (Hartung and Makambi, 2001).
d. p-value is based on the Wald Z-test result.

Table 3-1. Chemical 1/C: Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Placental Aromatase Assay.

Replicate	$Log_{10}IC_{50}$ (SE)	IC_{50} (GSE)[d]	Slope (SE)	Status
Individual Values[a]				
1	-8.628 (0.030)	2.355×10^{-9} (1.072)	-1.043 (0.041)	C
2	-8.679 (0.014)	2.094×10^{-9} (1.032)	-1.083 (0.020)	C
3	-8.719 (0.012)	1.908×10^{-9} (1.029)	-1.035 (0.023)	C
Mean[c]	-8.681 (0.024)	2.084×10^{-9} (1.057)	-1.058 (0.018)	--
Average Values[b]				
1	-8.617 (0.060)	2.416×10^{-9} (1.148)	-1.091 (0.081)	C
2	-8.668 (0.027)	2.148×10^{-9} (1.063)	-1.117 (0.039)	C
3	-8.722 (0.018)	1.897×10^{-9} (1.042)	-1.022 (0.033)	C

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each chemical 1/C concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each chemical 1/C concentration level within each replicate.
c. Weighted averages of the parameter estimates across the three replicates.
d. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

Table 3-2. Chemical 1/C: Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Estimate (95% CI)			
	Replicate 1[a]	Replicate 2[a]	Replicate 3[a]	Mean[b]
$Log_{10}IC_{50}$	-8.628 (-8.691, -8.565)	-8.679 (-8.708, -8.650)	-8.719 (-8.745, -8.693)	-8.681 (-8.796, -8.567)
Slope	-1.043 (-1.128, -0.958)	-1.083 (-1.125, -1.041)	-1.035 (-1.082, -0.988)	-1.058 (-1.111, -1.005)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition values within replicates.
b. Mean and its associated 95% confidence interval, based on a one-way analysis of variance model with replicate treated as a random effect.

Table 3-3. Chemical 1/C: Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Variance/Degree of Freedom[a,b,c]				
	Replicate 1	Replicate 2	Replicate 3	Overall	
				Random Replicate (p-value)[d]	Variance of Mean
$Log_{10}IC_{50}$	0.00092 /df=22	0.00019 /df=22	0.00015 /df=22	0.00140 /df=2 (p=0.2310)	0.00059 /df=1.824
Slope	0.00167 /df=22	0.00040 /df=22	0.00052 /df=22	0.00035 /df=2 (p=0.344)	0.00034 /df=3.675

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition results within each concentration level.
b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.
c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(Si^4/df_i))$, where S_r^2 is random replicate variance, S_i^2 and df_i are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of S_r^2 and K is the number of replicates (Hartung and Makambi, 2001).
d. p-value is based on the Wald Z-test result.

Table 4-1. Chemical 2/D: Estimated Parameters of the Concentration Response Curve Fits by Replicate and Averaged Across Replicates. Percent of Control Activity. Placental Aromatase Assay.

Replicate	$Log_{10}IC_{50}$ (SE)	IC_{50} (GSE)[d]	Slope (SE)	Status
Individual Values[a]				
1	-5.105 (0.030)	7.850×10^{-6} (1.072)	-1.045 (0.049)	C
2	-5.217 (0.029)	6.066×10^{-6} (1.069)	-0.920 (0.044)	C
3	-5.350 (0.054)	4.469×10^{-6} (1.132)	-0.803 (0.065)	C
Mean[c]	-5.218 (0.069)	6.048×10^{-6} (1.172)	-0.927 (0.068)	--
Average Values[b]				
1	-5.104 (0.055)	7.869×10^{-6} (1.136)	-1.046 (0.090)	C
2	-5.213 (0.044)	6.124×10^{-6} (1.107)	-0.925 (0.067)	C
3	-5.302 (0.047)	4.988×10^{-6} (1.114)	-0.830 (0.060)	C

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each chemical 2/D concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each chemical 2/D concentration level within each replicate.
c. Weighted averages of the parameter estimates across the three replicates.
d. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

Table 4-2. Chemical 2/D: Parameter Estimates of the Concentration Response Curves and Associated 95% Confidence Intervals. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Estimate (95% CI)			
	Replicate 1[a]	Replicate 2[a]	Replicate 3[a]	Mean[b]
$Log_{10}IC_{50}$	-5.105 (-5.168, -5.042)	-5.217 (-5.277, -5.157)	-5.350 (-5.461, -5.239)	-5.218 (-5.530, -4.907)
Slope	-1.045 (-1.148, -0.943)	-0.920 (-1.010, -0.829)	-0.803 (-0.938, -0.667)	-0.927 (-1.237, -0.618)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual repetition values within replicates.
b. Mean and its associated 95% confidence interval, based on a one-way analysis of variance model with replicate treated as a random effect.

Table 4-3. Chemical 2/D: Variances Associated with Estimated Parameters of Concentration Response Curves. Percent of Control Activity. Placental Aromatase Assay.

Parameter	Variance/Degree of Freedom[a,b,c]				
	Replicate 1	Replicate 2	Replicate 3	Overall	
				Random Replicate (p-value)[d]	Variance of Mean
$Log_{10}IC_{50}$	0.00091 /df=22	0.00083 /df=22	0.00289 /df=22	0.01270 /df=2 (p=0.192)	0.00473 /df=1.899
Slope	0.00244 /df=22	0.00190 /df=22	0.00425 /df=22	0.0109 /df=2 (p=0.221)	0.0046 /df=1.881

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual repetition results within each concentration level.
b. Variance estimates for the random replicate were estimated based on a one-way random effects analysis of variance. The variances for each replicate were fixed at their reported values.
c. Degrees of freedom for the variance of mean were estimated by $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(Si^4 /df_i))$, where S_r^2 is random replicate variance, S_i^2 and df_i are estimated variance and degree of freedom for a given replicate, $var(S_r^2)$ is the variance associated with the estimation of S_r^2 and K is the number of replicates (Hartung and Makambi, 2001).
d. p-value is based on the Wald Z-test result.

Table 5. **All Chemicals All Replicates: Variance Components of the Percent of Control Values for Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control. Portion Effects and Variation Across Replicates of Portion Effects Within Replicates.**

Parameter	Difference Between Beginning and End Portions (End-Beginning)		Variance Components								
	Estimate (%) (Std. Error)	p-Value/ Degree of Freedom	Replicate				Replicate* Portion				Residual (Repetition)
			Chem 1/A	Chem 2/B	Chem 1/C	Chem 2/D	Chem 1/A	Chem 2/B	Chem 1/C	Chem 2/D	
Full Enzyme Activity Control	-12.955 (2.051)	<0.0001/ df=21.8	0	0	0	0	80.98	2.49	0	0	30.12
Background Activity Control	-0.165 (0.041)	0.0002/ df=51	0	0	0	0	0	0	0	0	0.02
Positive Control	-2.299 (0.989)	0.0427/ df=9.84	36.48	49.28	0	5.24	20.98	0.49	0	0	6.27
Negative Control	-6.391 (2.249)	0.0111/ df=17.3	0	51.20	0	0	87.95	0	0	26.51	25.15

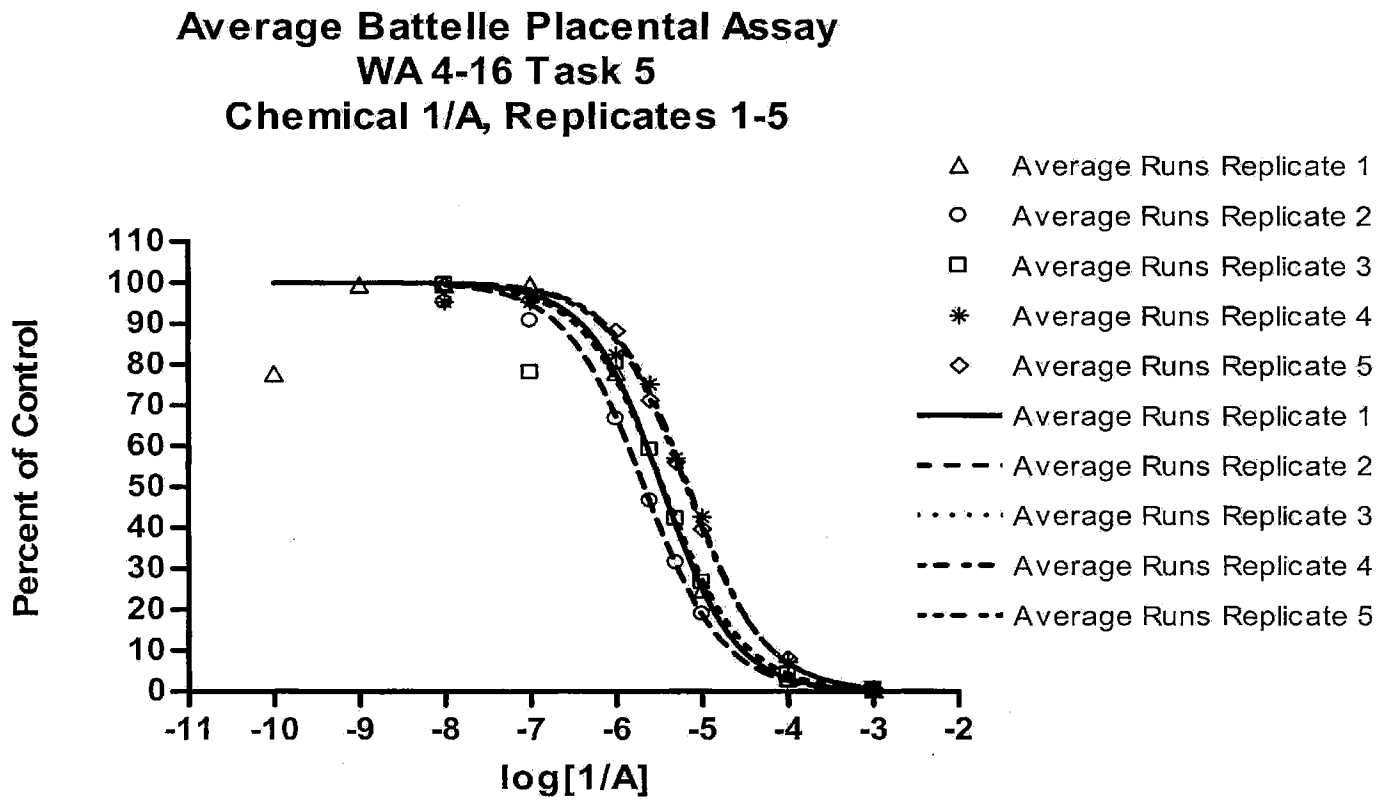


Figure 1-1. Chemical 1/A: Concentration Response Curves and Averages of Repetitions Within Chemical 1/A Concentrations. Placental Aromatase Assay. By Replicate.

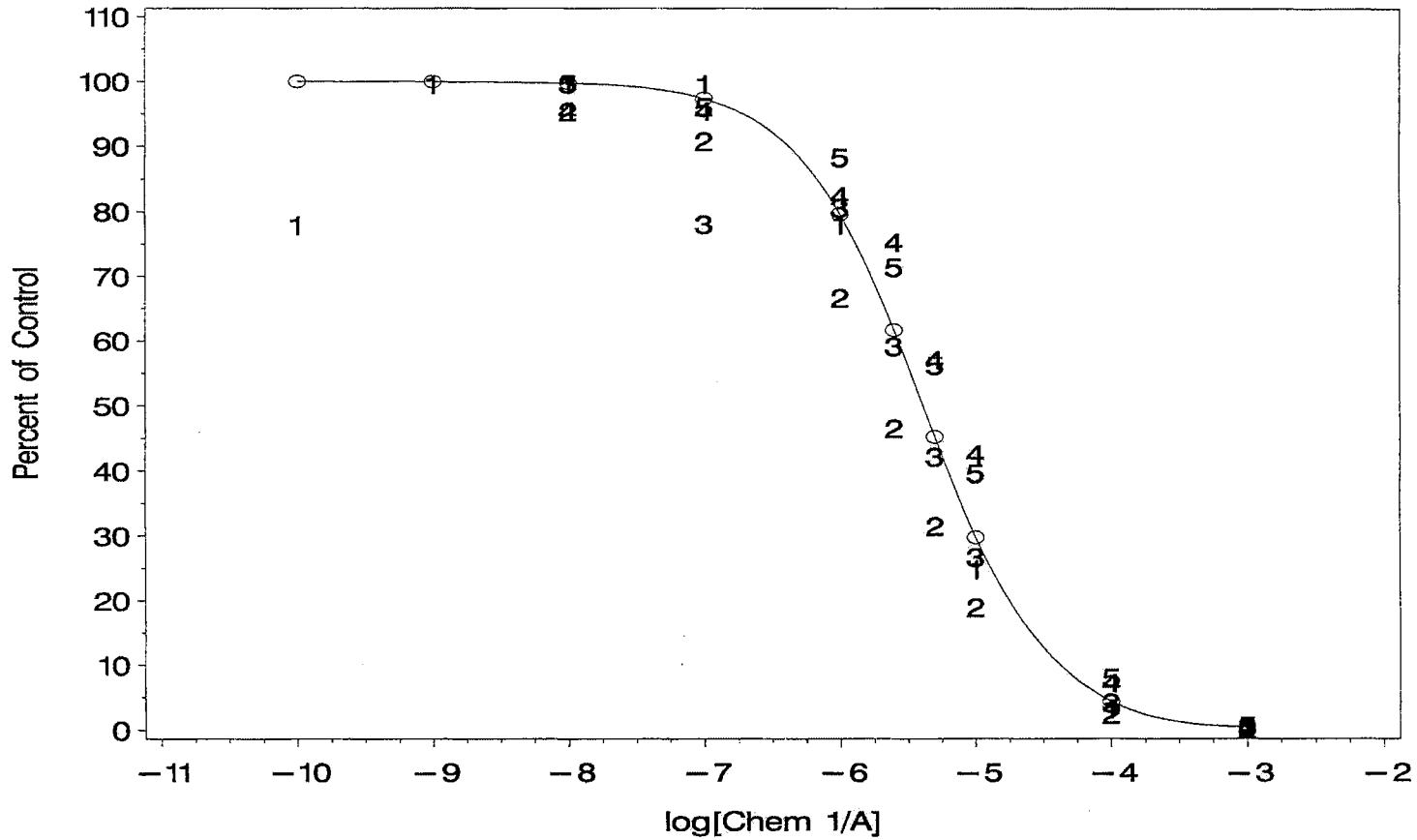


Figure 1-2. **Chemical 1/A: Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within Chemical 1/A Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values.**

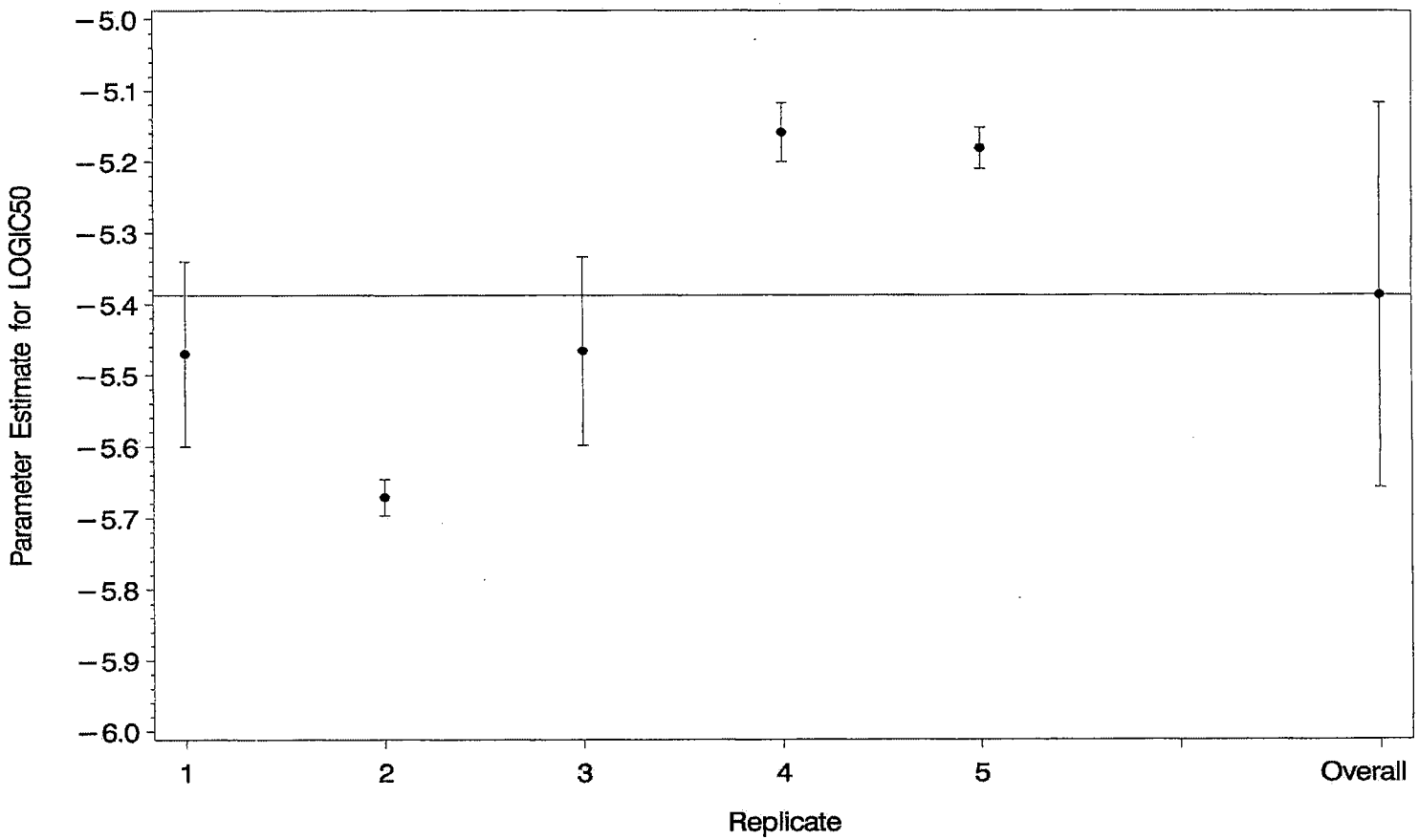


Figure 1-3. Chemical 1/A: $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.

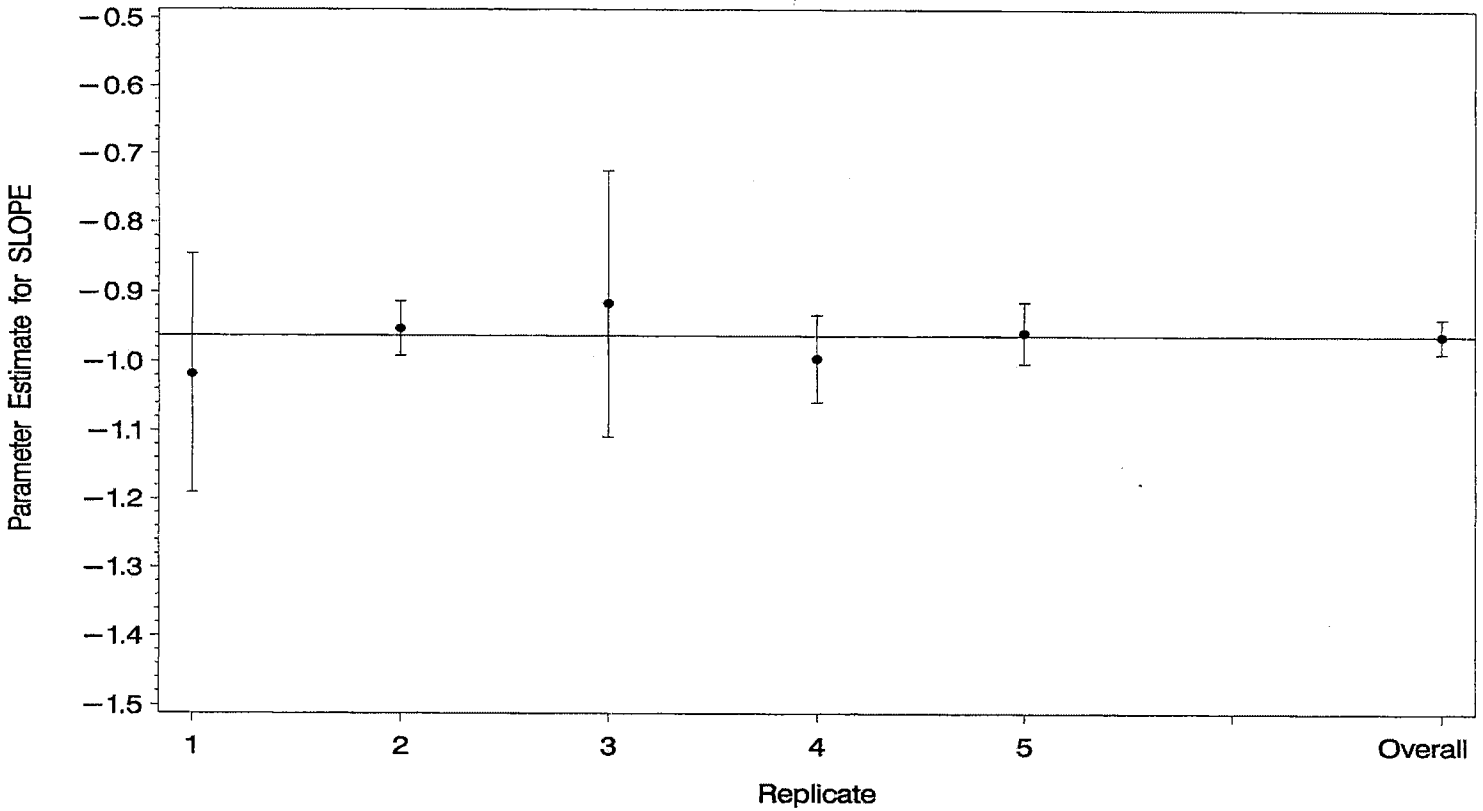


Figure 1-4. Chemical 1/A: Slope Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.

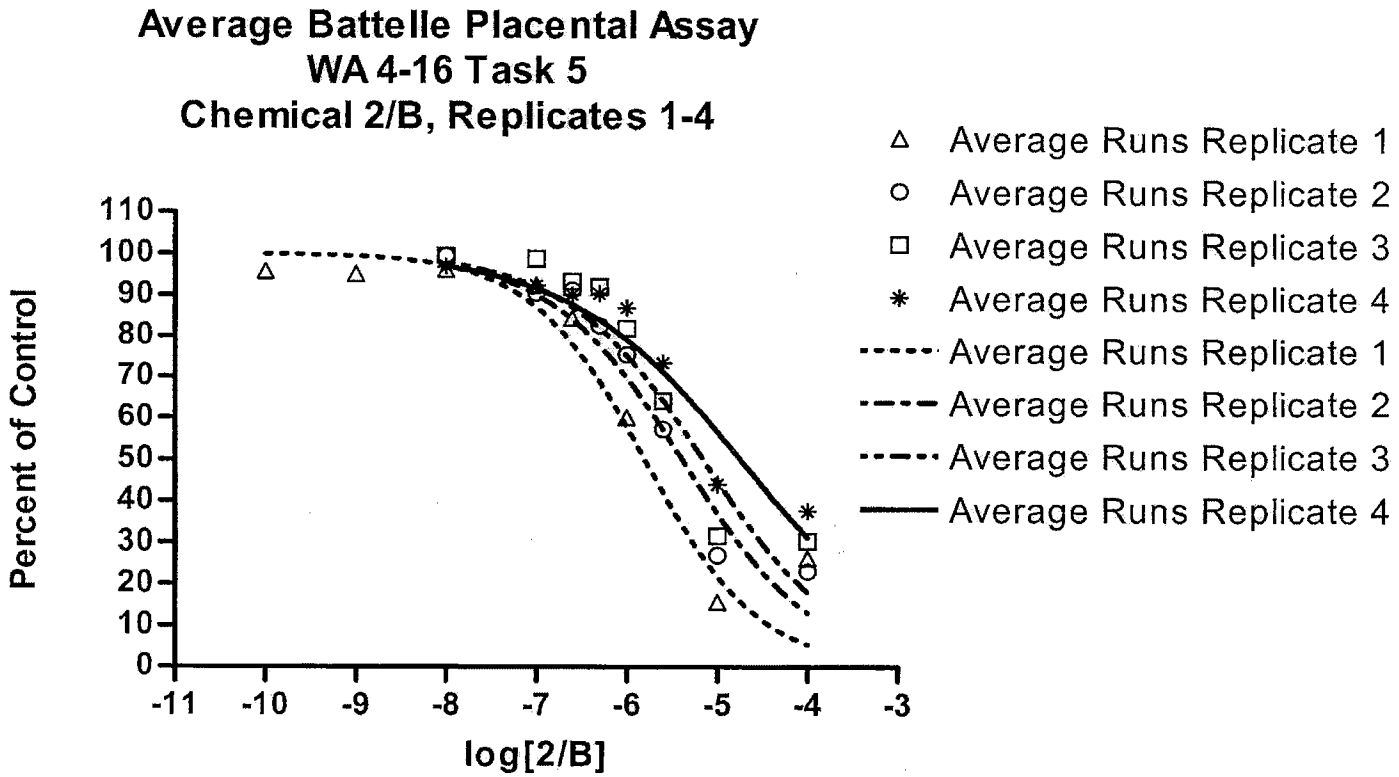


Figure 2-1. **Chemical 2/B: Concentration Response Curves and Averages of Repetitions Within Chemical 2/B Concentrations. Placental Aromatase Assay. By Replicate.**

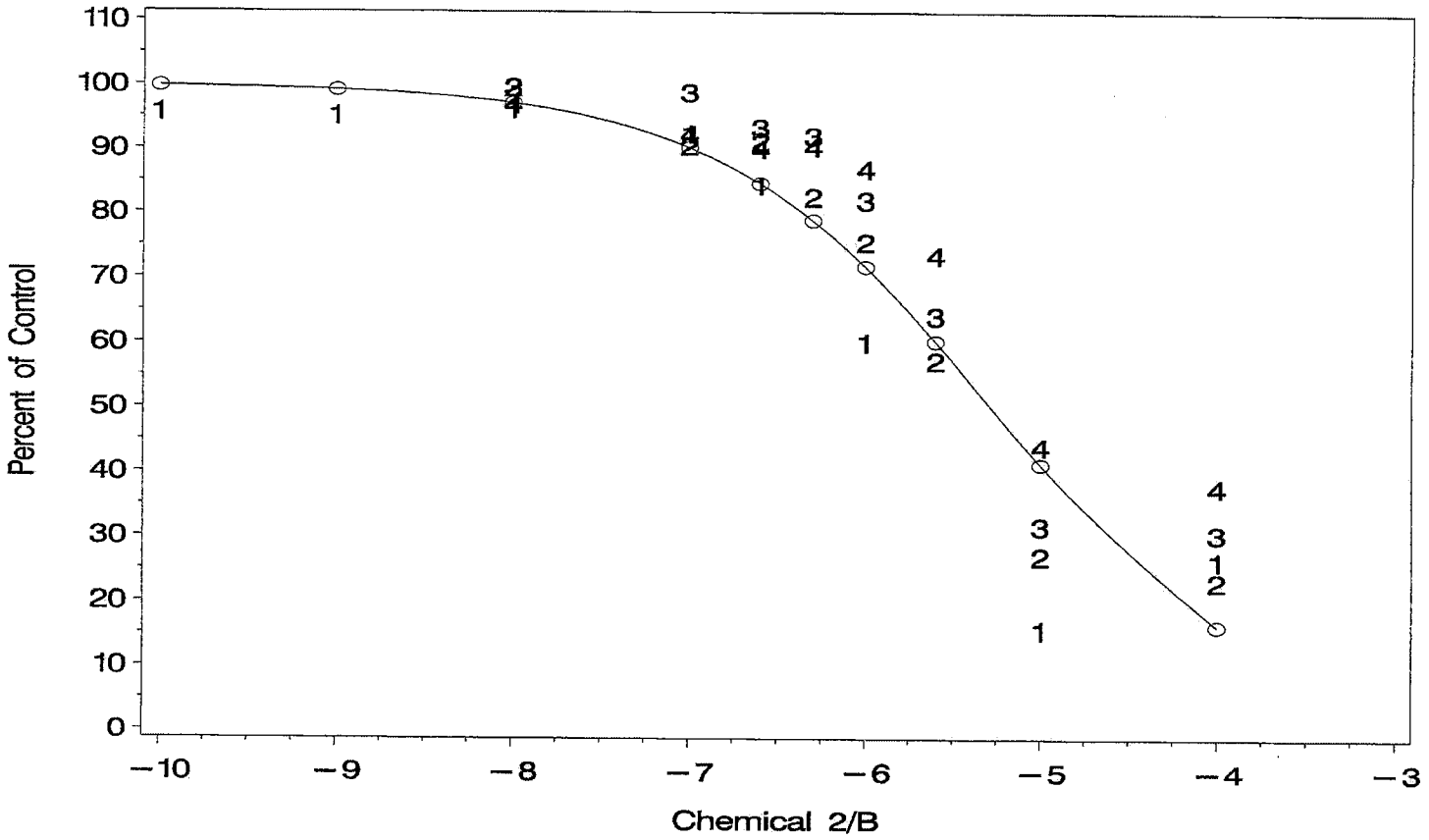


Figure 2-2. **Chemical 2/B: Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within Chemical 2/B Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values.**

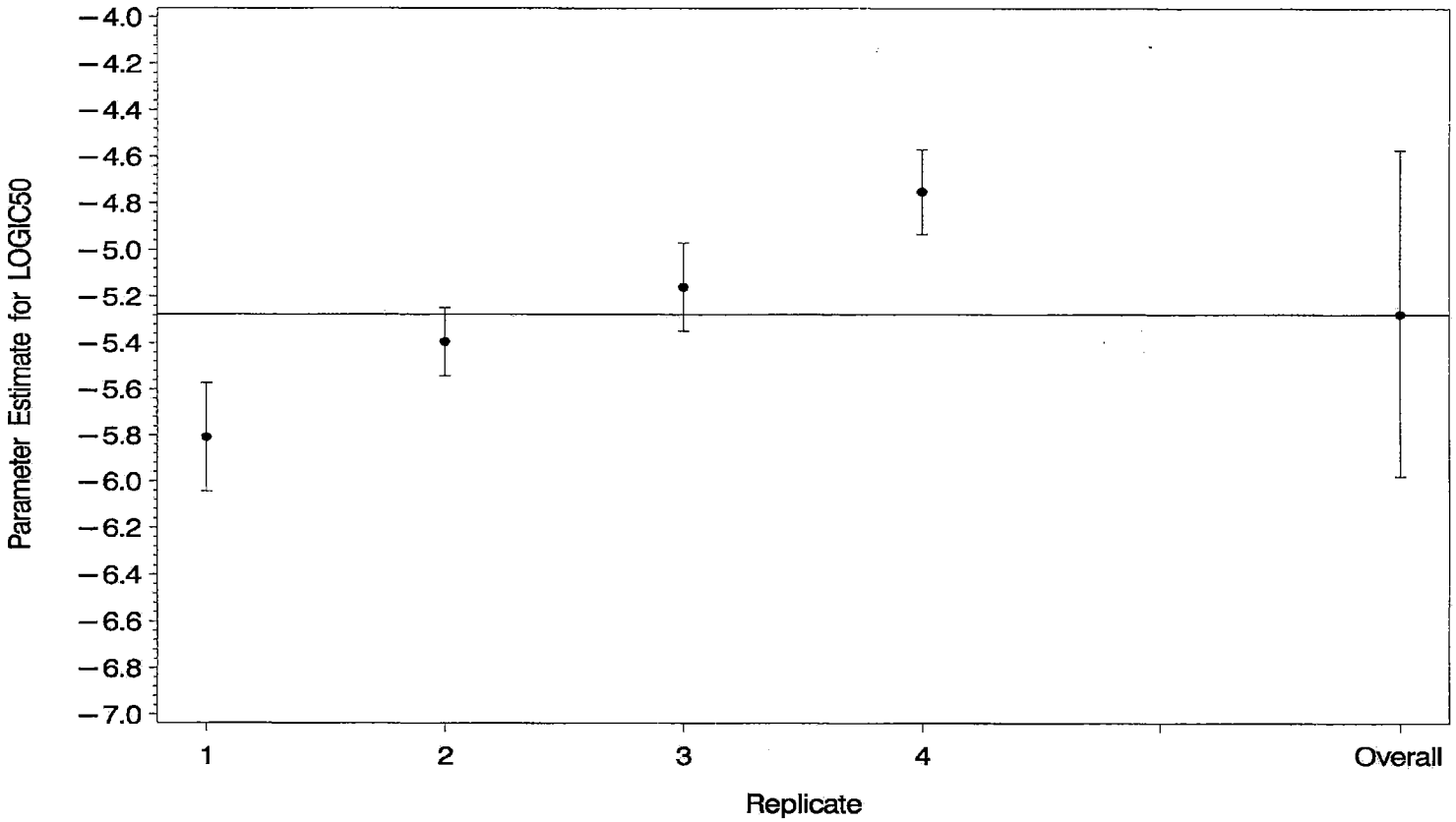


Figure 2-3. Chemical 2/B: $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.

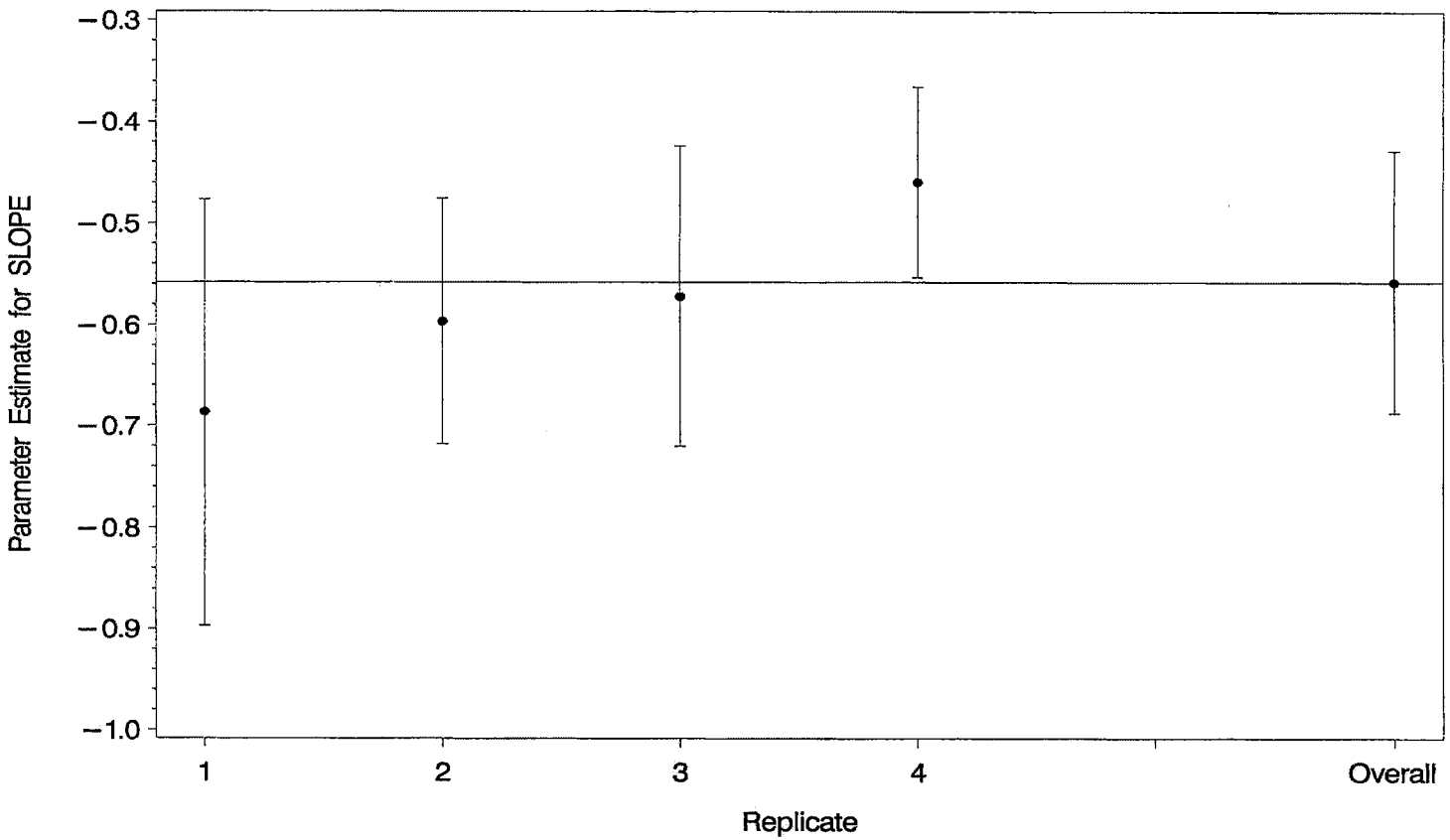


Figure 2-4. **Chemical 2/B: Slope Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.**

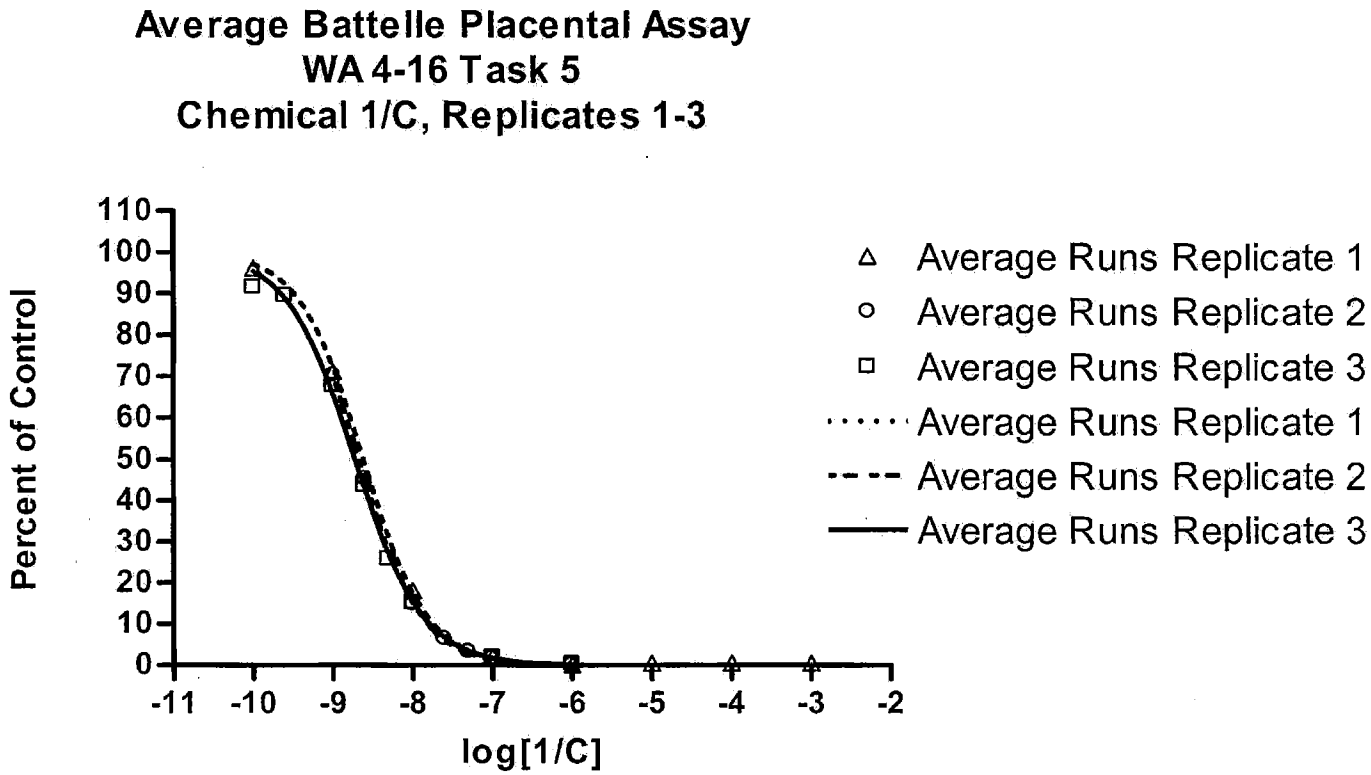


Figure 3-1. Chemical 1/C: Concentration Response Curves and Averages of Repetitions Within Chemical 1/C Concentrations. Placental Aromatase Assay. By Replicate.

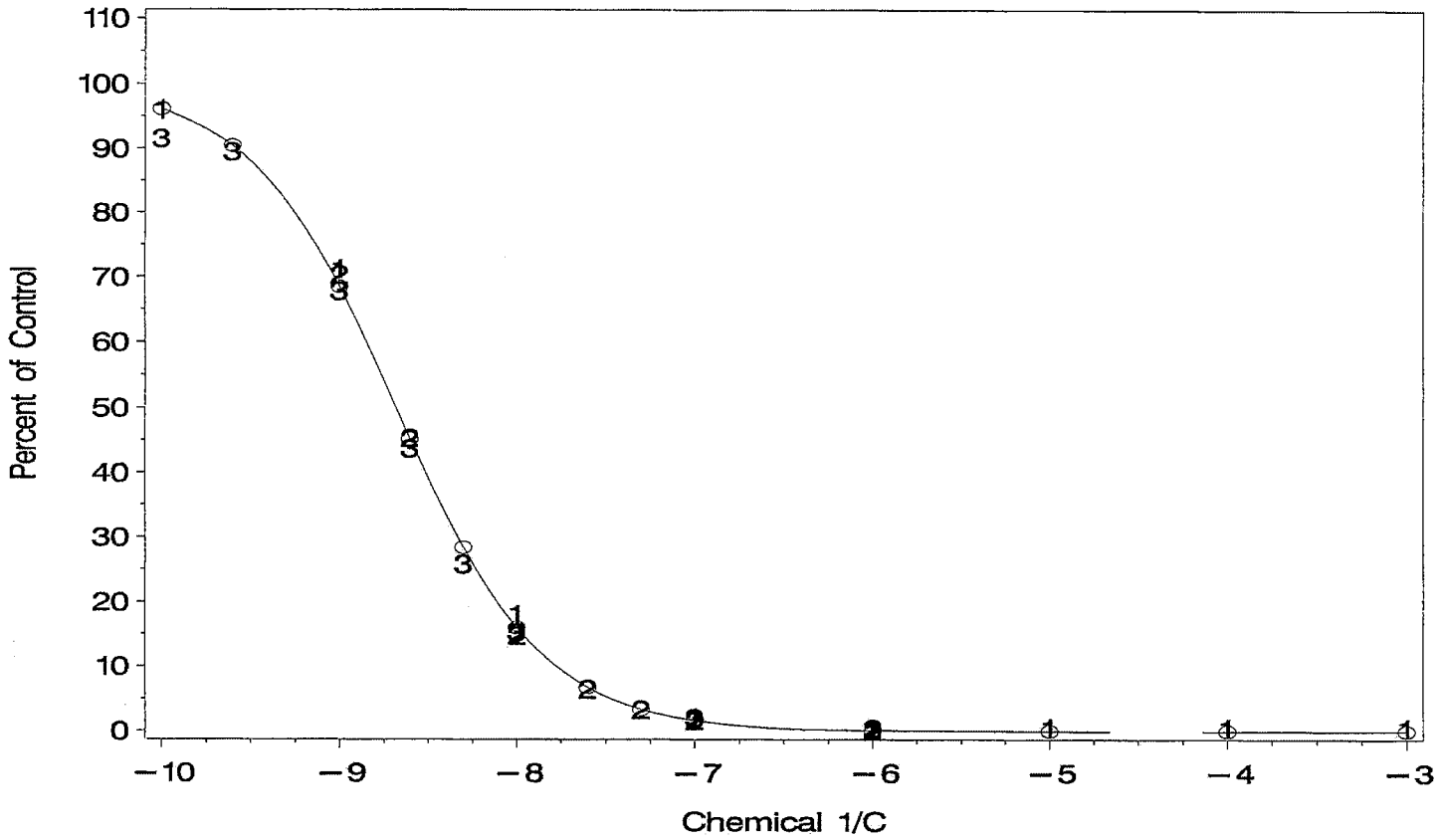


Figure 3-2. **Chemical 1/C: Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within Chemical 1/C Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values.**

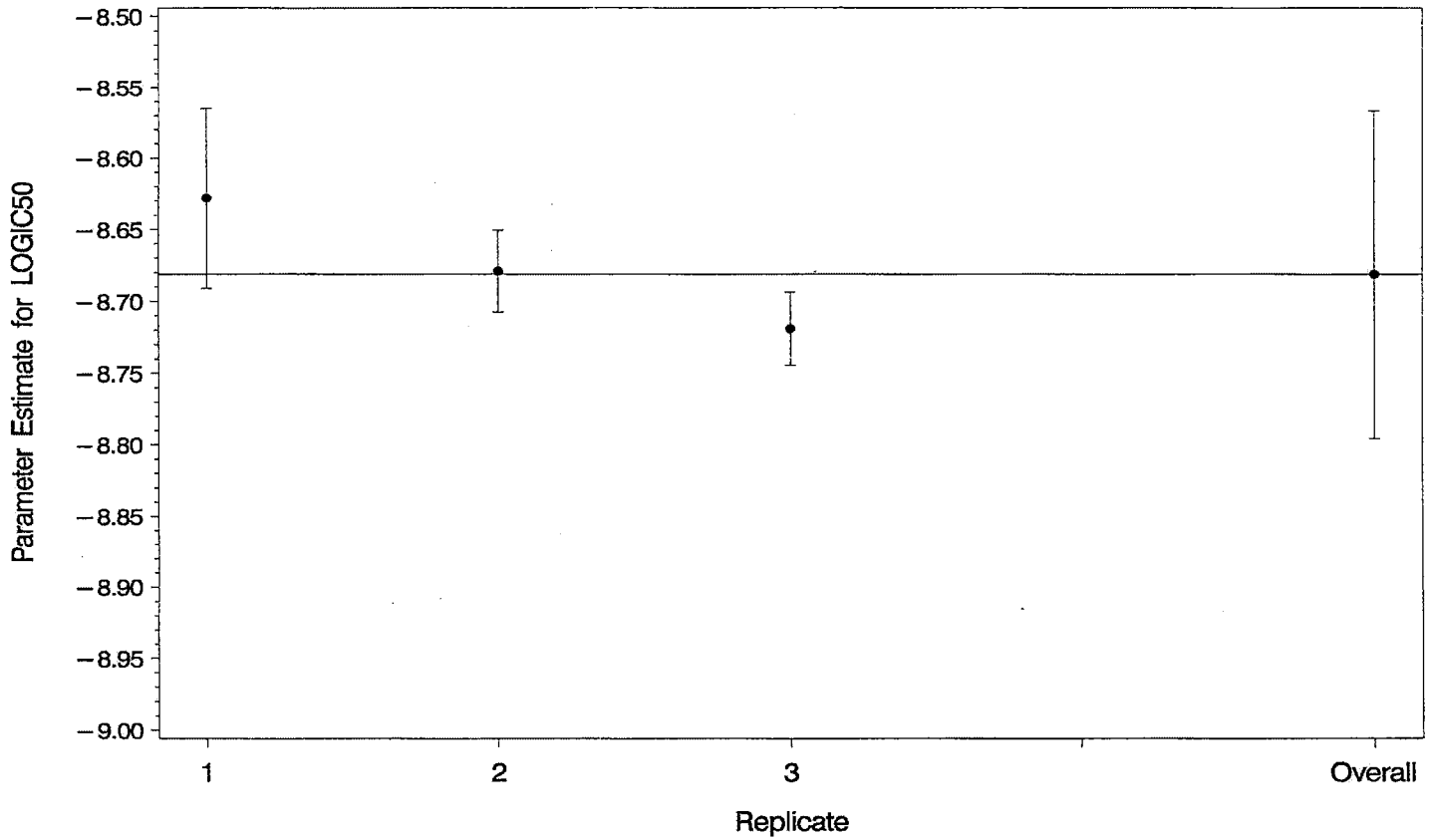


Figure 3-3. Chemical 1/C: $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.

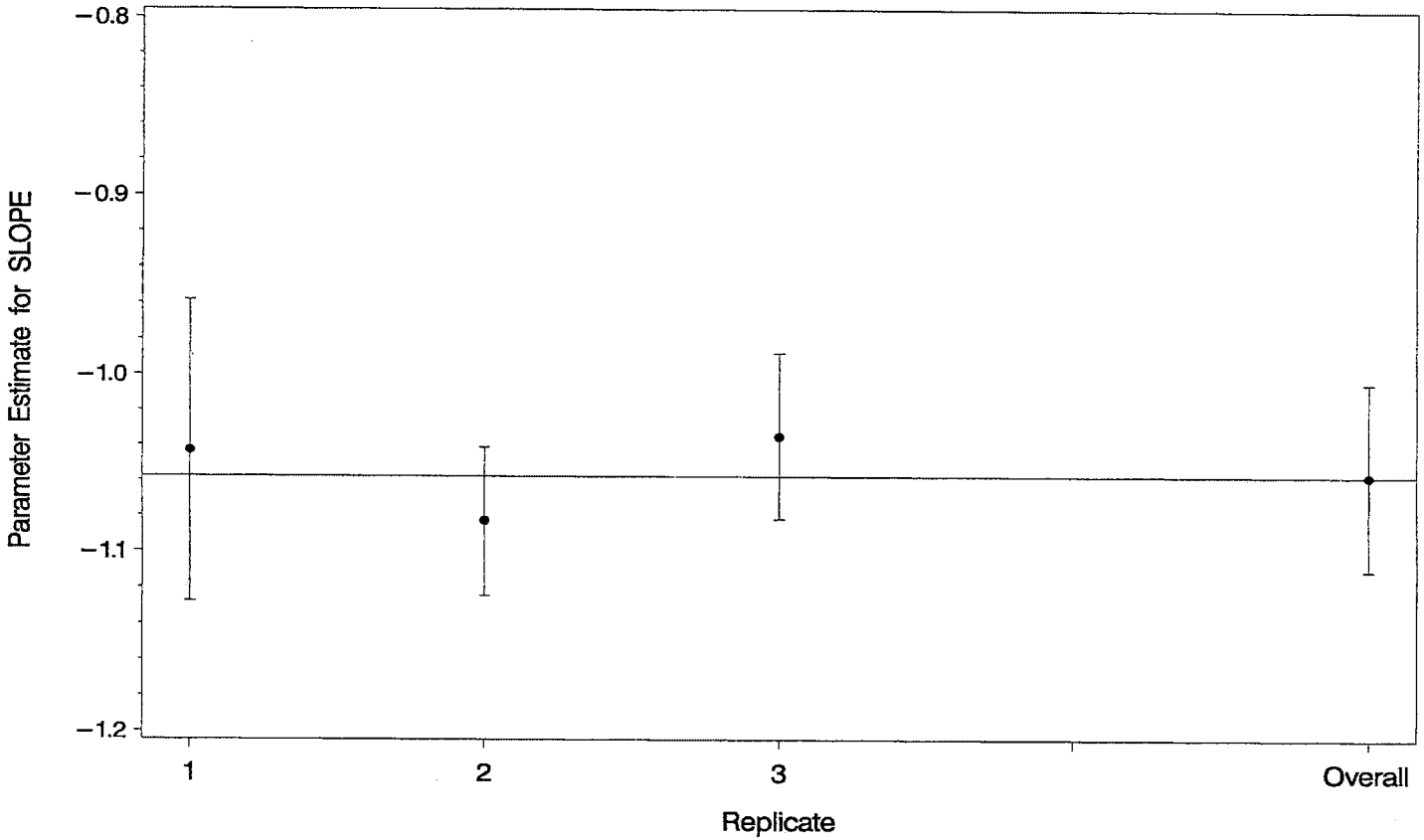


Figure 3-4. **Chemical 1/C: Slope Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds t the Average Across Replicates.**

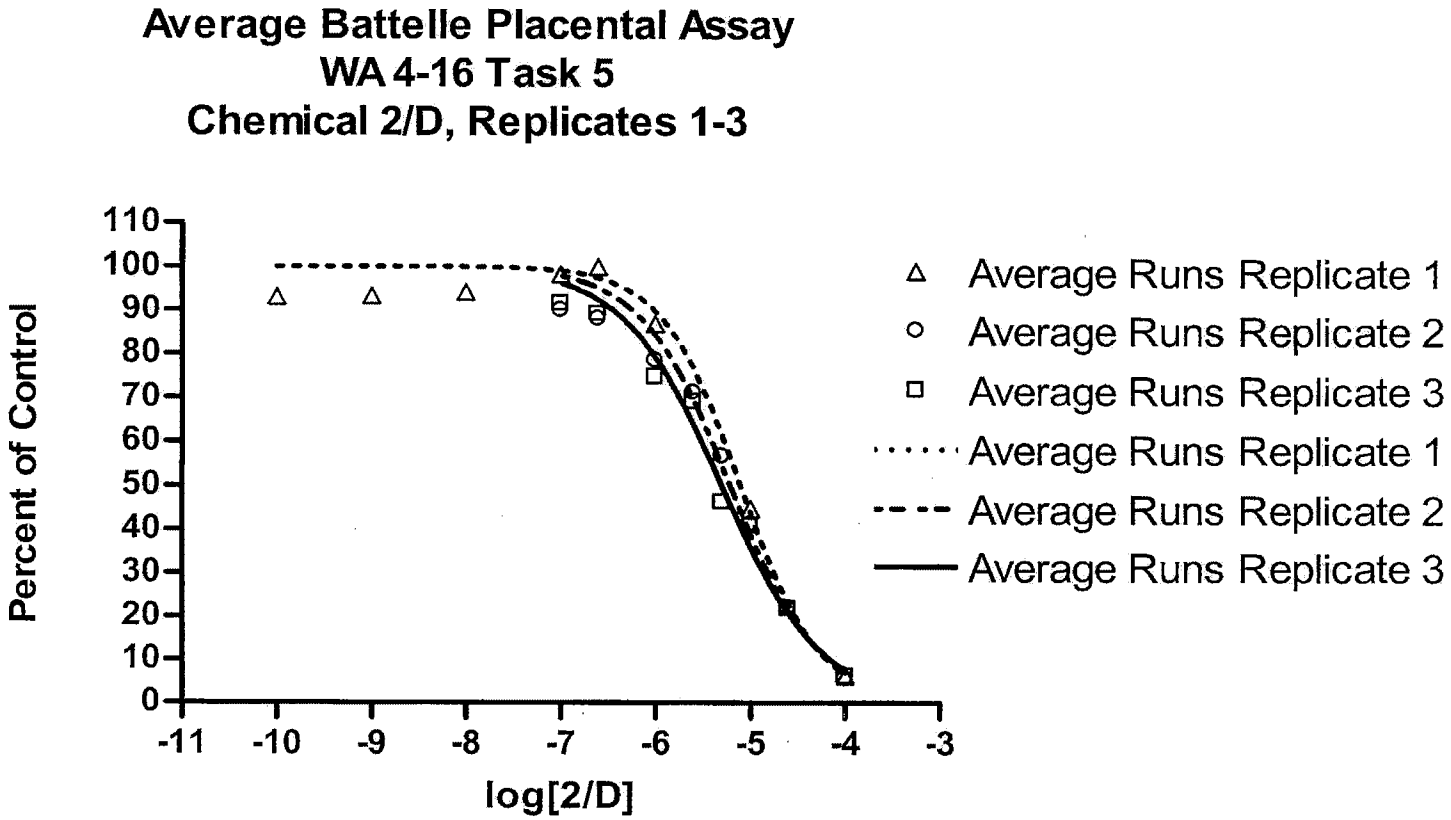


Figure 4-1. **Chemical 2/D: Concentration Response Curves and Averages of Repetitions Within Chemical 2/D Concentrations. Placental Aromatase Assay. By Replicate.**

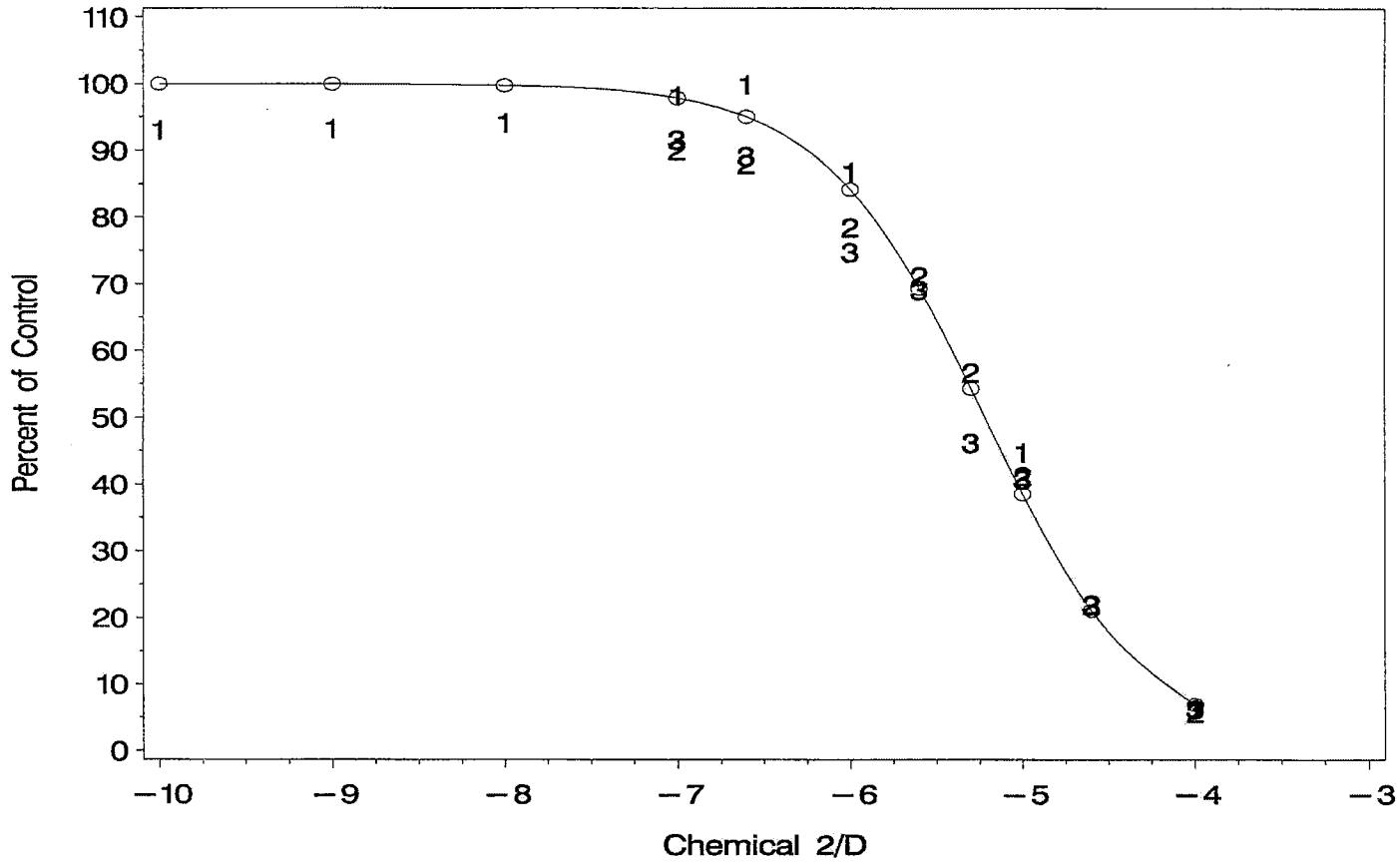


Figure 4-2. **Chemical 2/D: Overall Average Concentration Response Curve Across Replicates and Average Responses Across Repetitions Within Chemical 2/D Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate Parameter Values.**

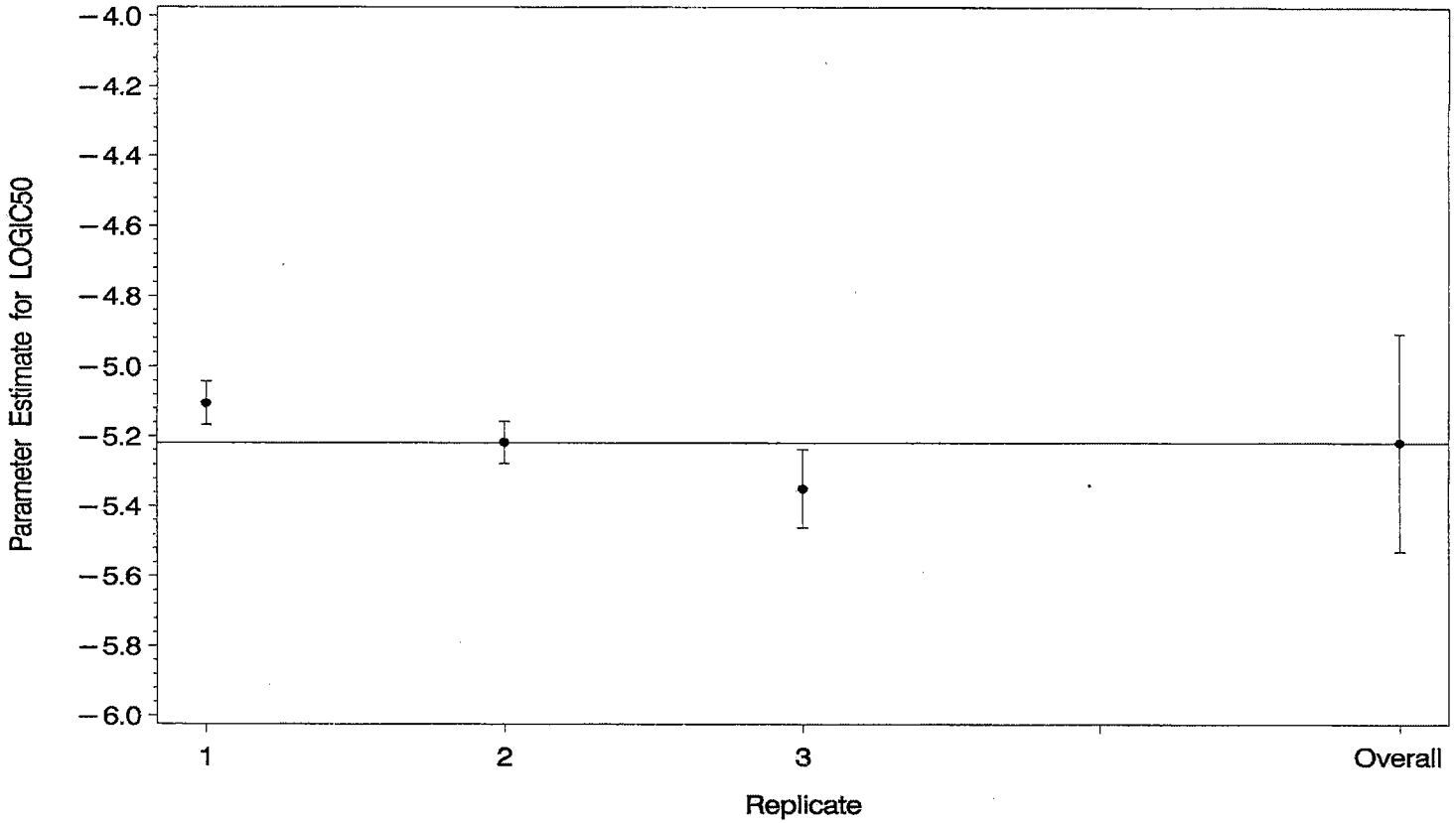


Figure 4-3. Chemical 2/D: $Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.

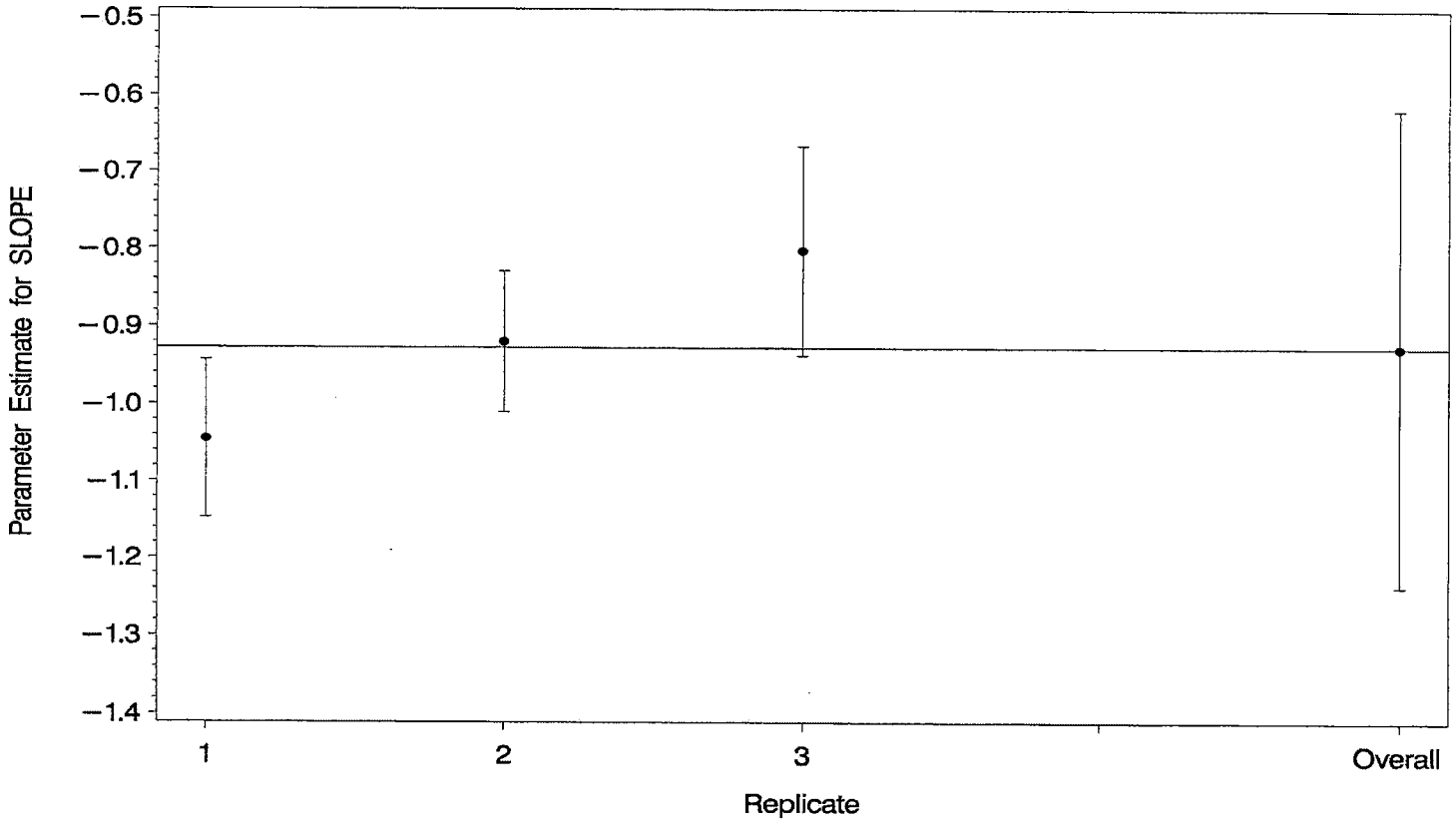


Figure 4-4. Chemical 2/D: Slope Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates.

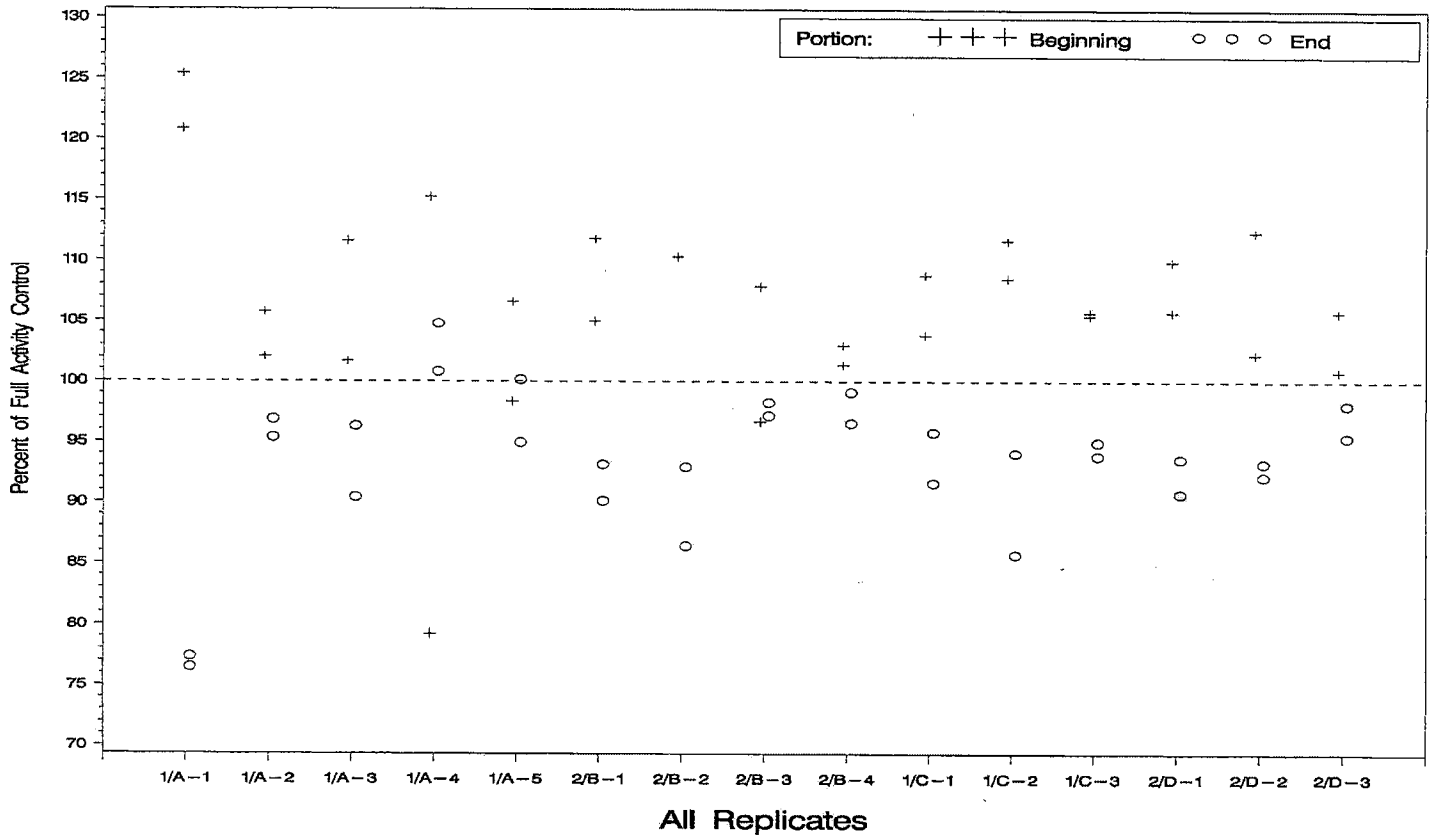


Figure 5. Full Enzyme Activity Controls. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%.

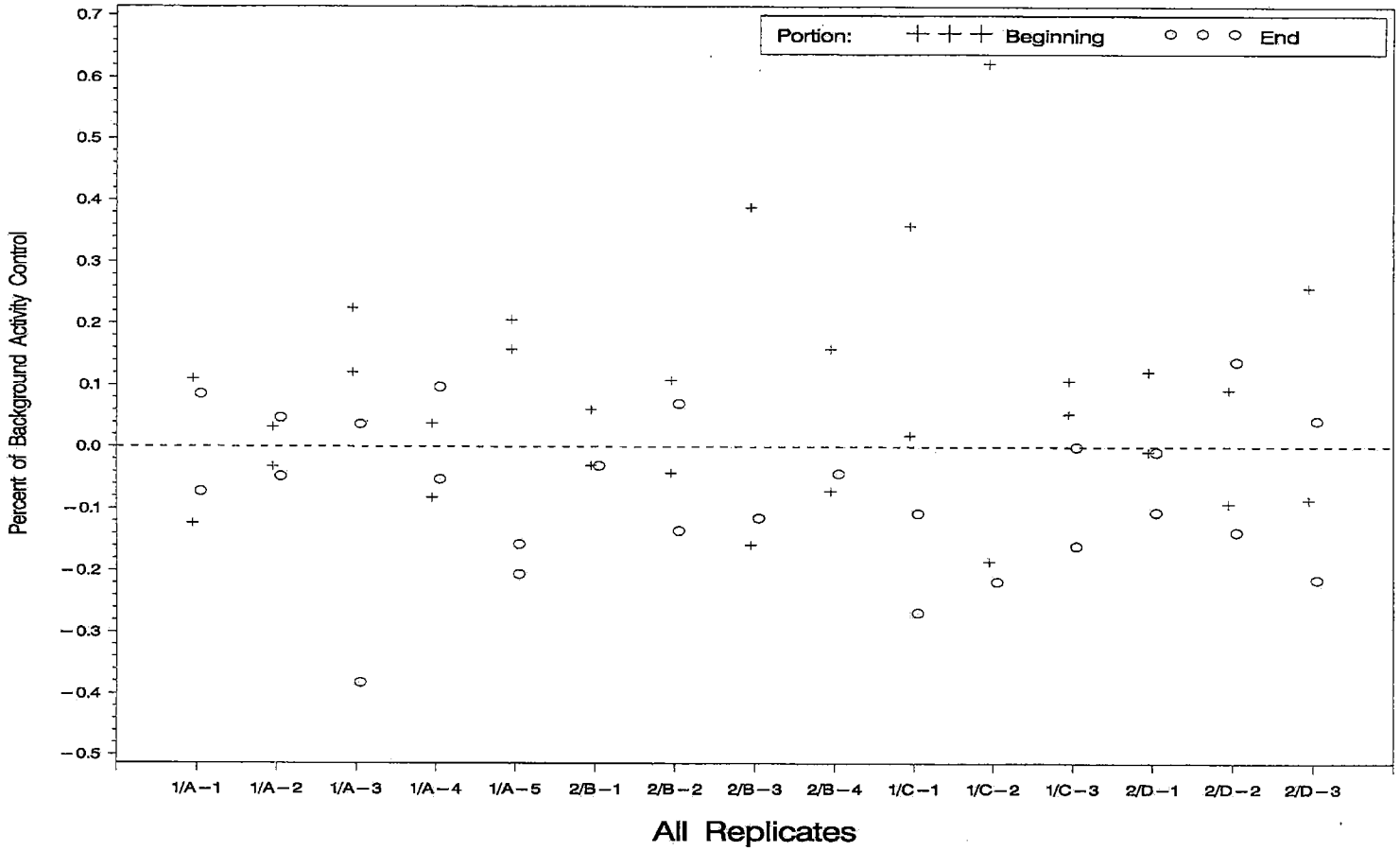


Figure 6. Background Activity Controls. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 0%.

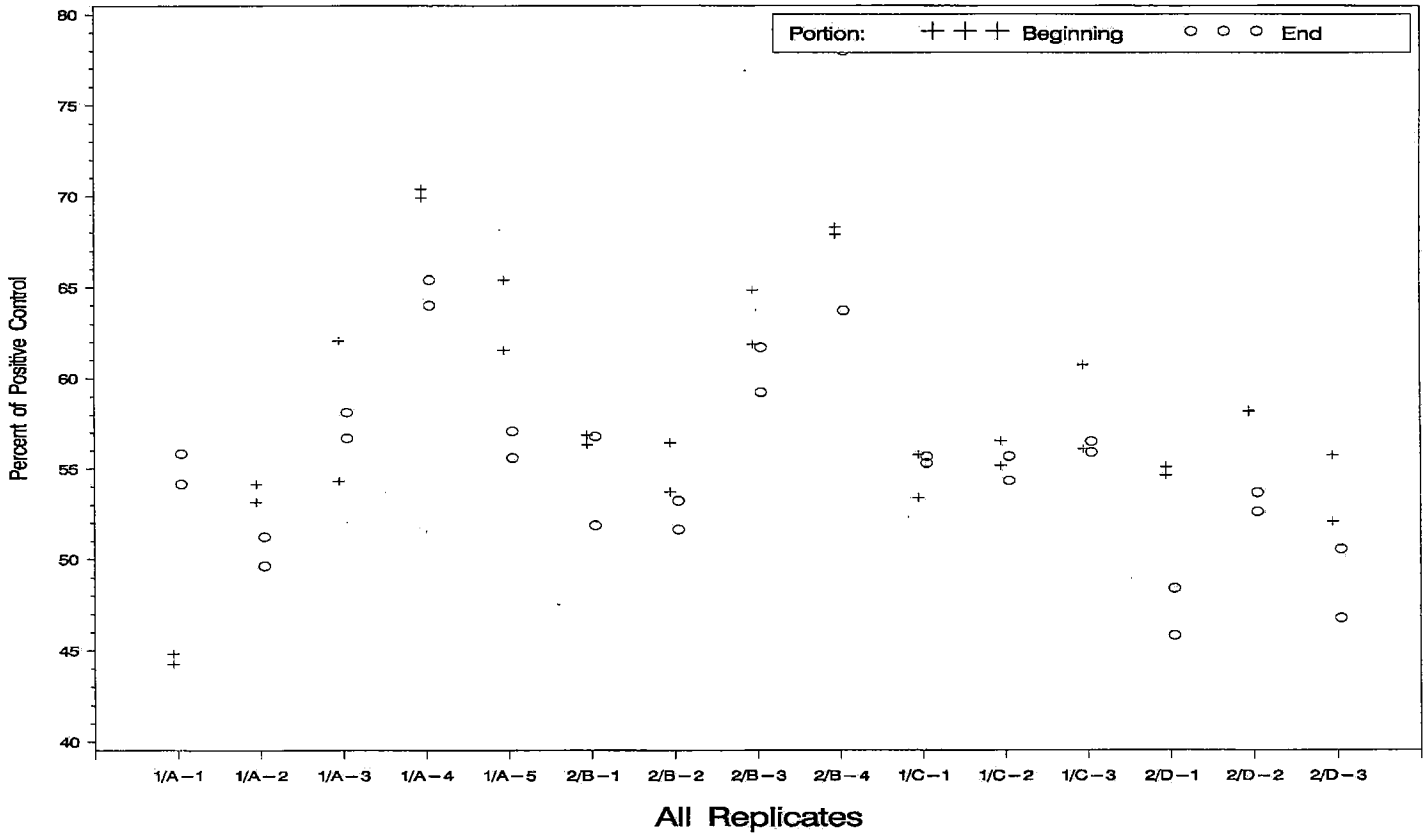


Figure 7. Positive Control Data. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay.

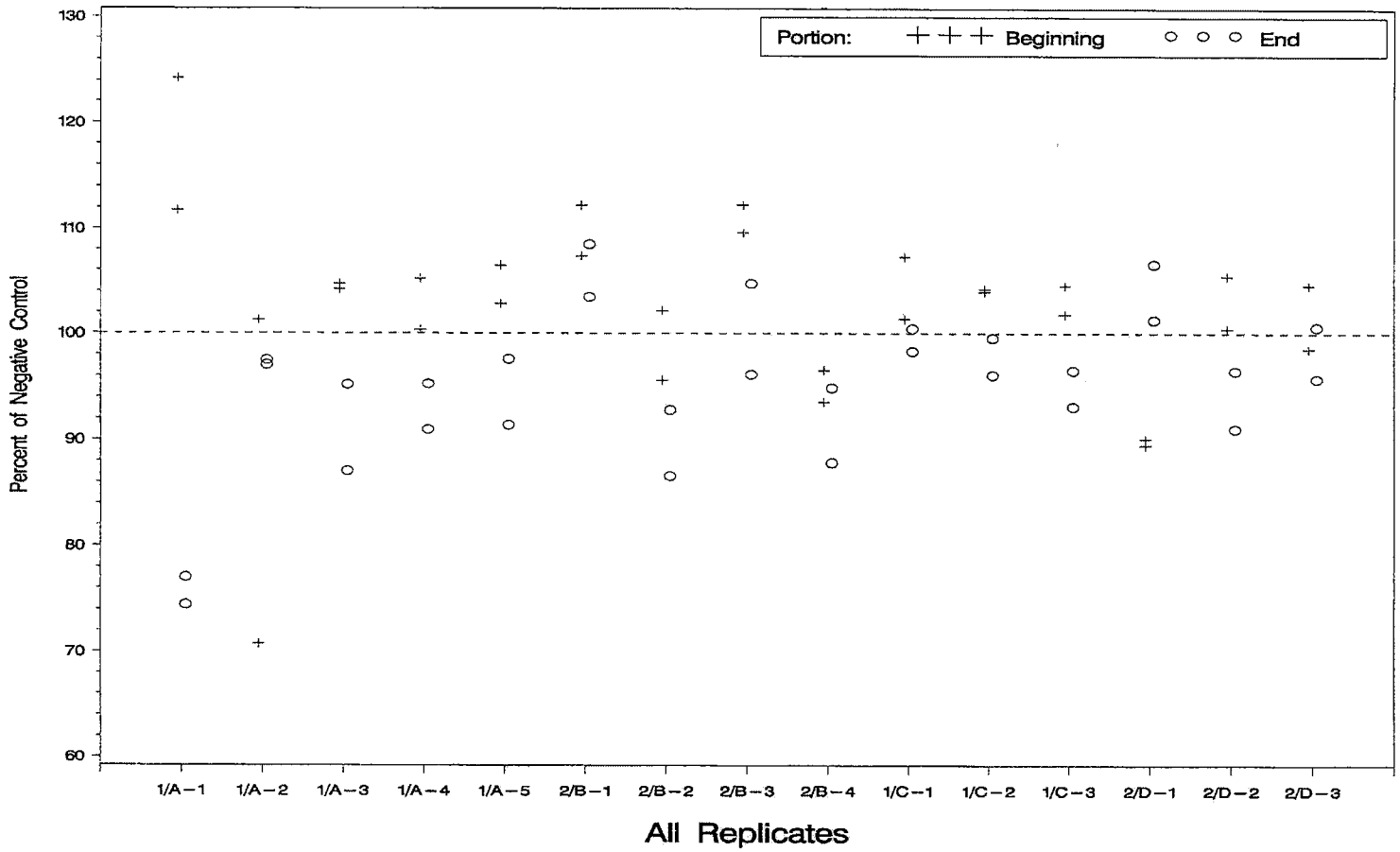


Figure 8. Negative Controls. Percent of Control by Replicate within Reference Chemical and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%.

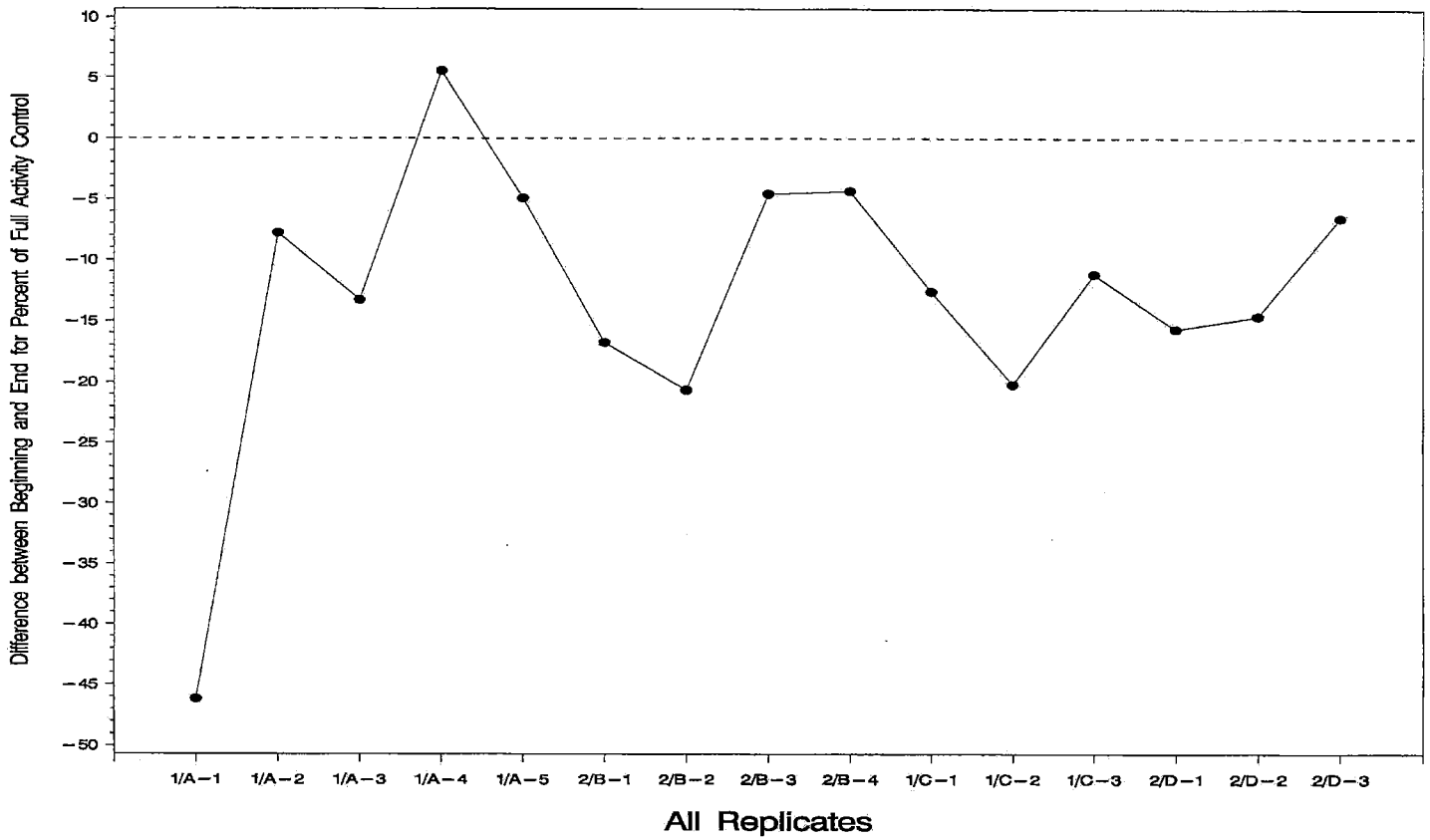


Figure 9. Full Enzyme Activity Controls. Difference Between the Averages of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Placental Aromatase Assay. Reference Line at 0%.

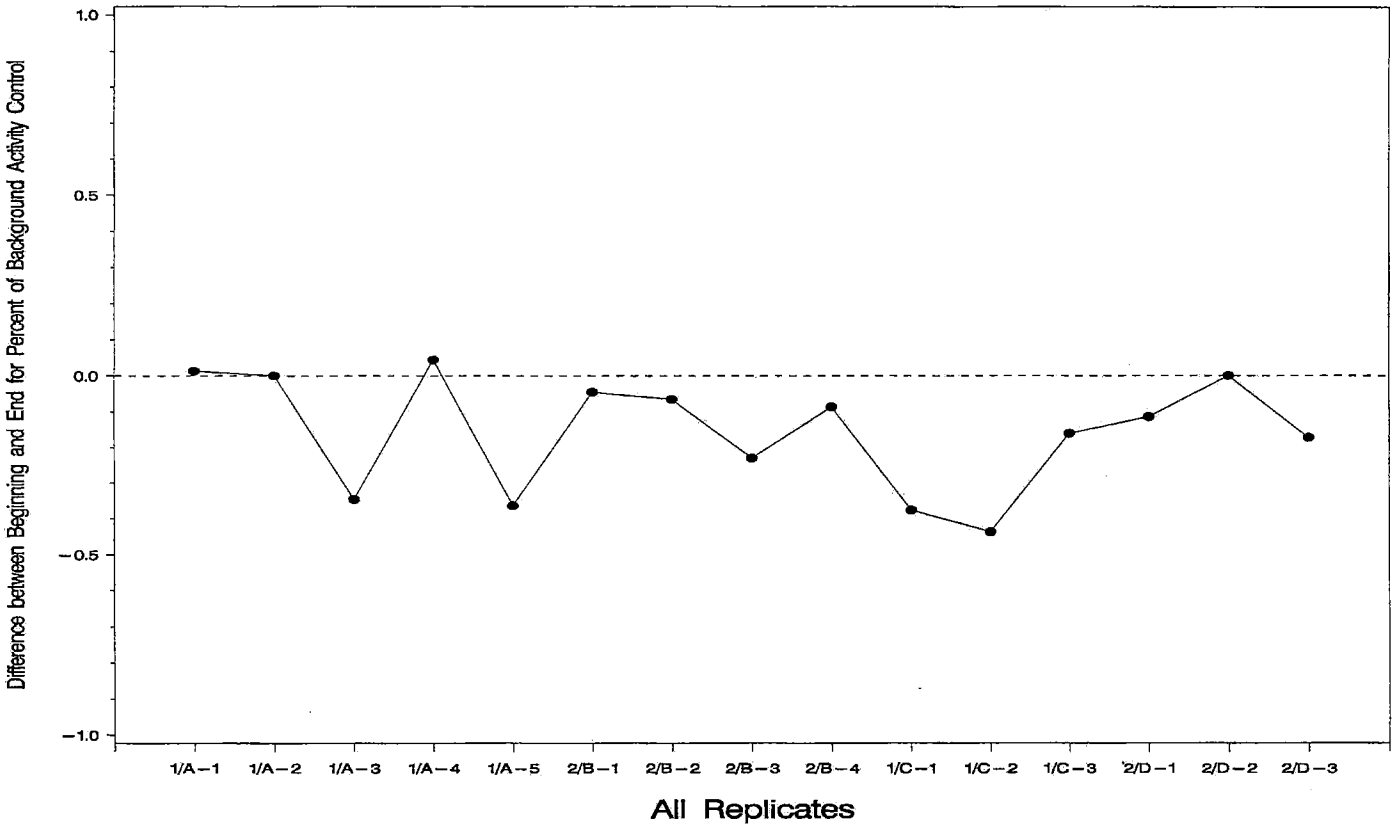


Figure 10. Background Activity Controls. Difference Between the Averages of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Placental Aromatase Assay. Reference Line at 0%.

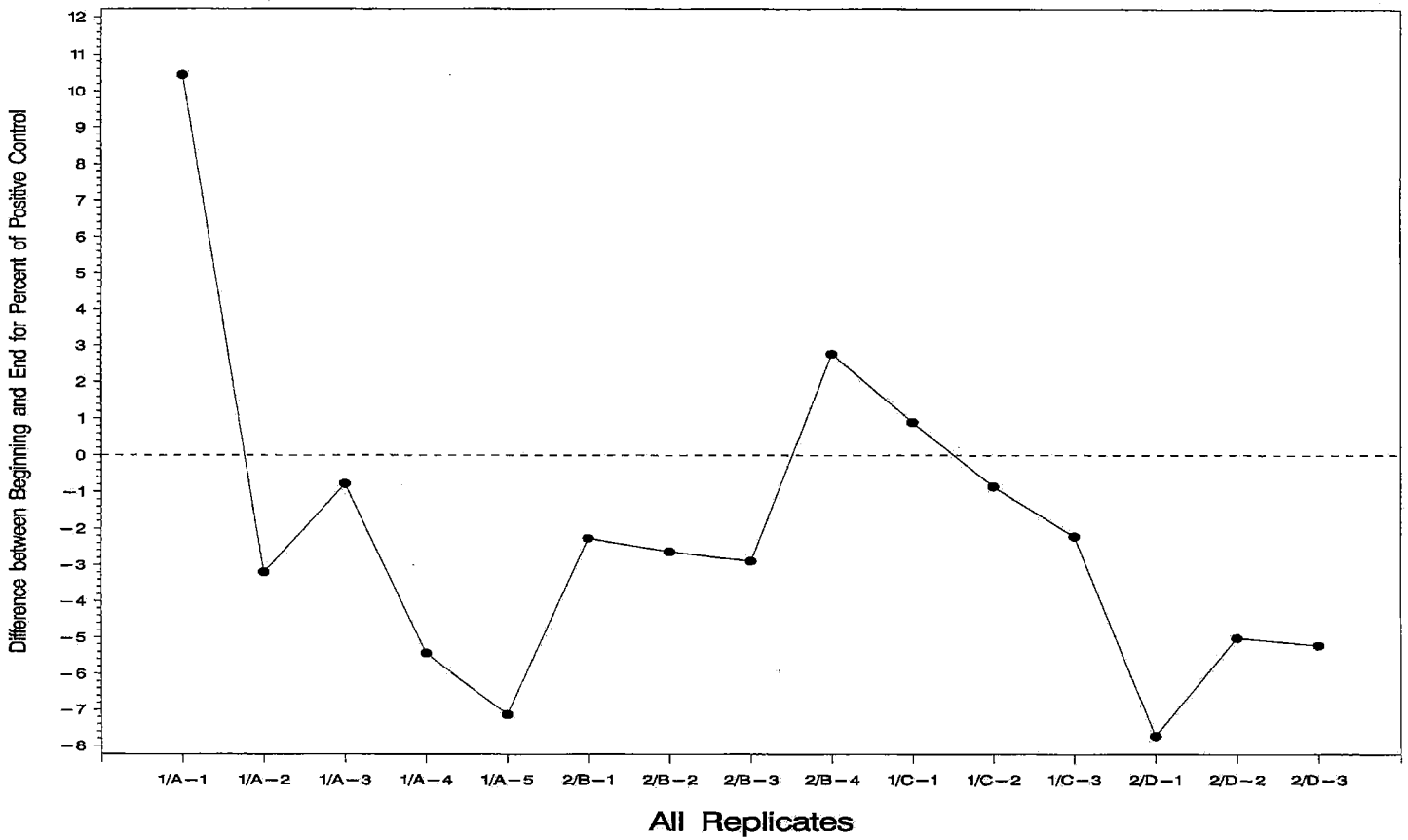


Figure 11. Positive Controls. Difference Between the Averages of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Placental Aromatase Assay. Reference Line at 0%.

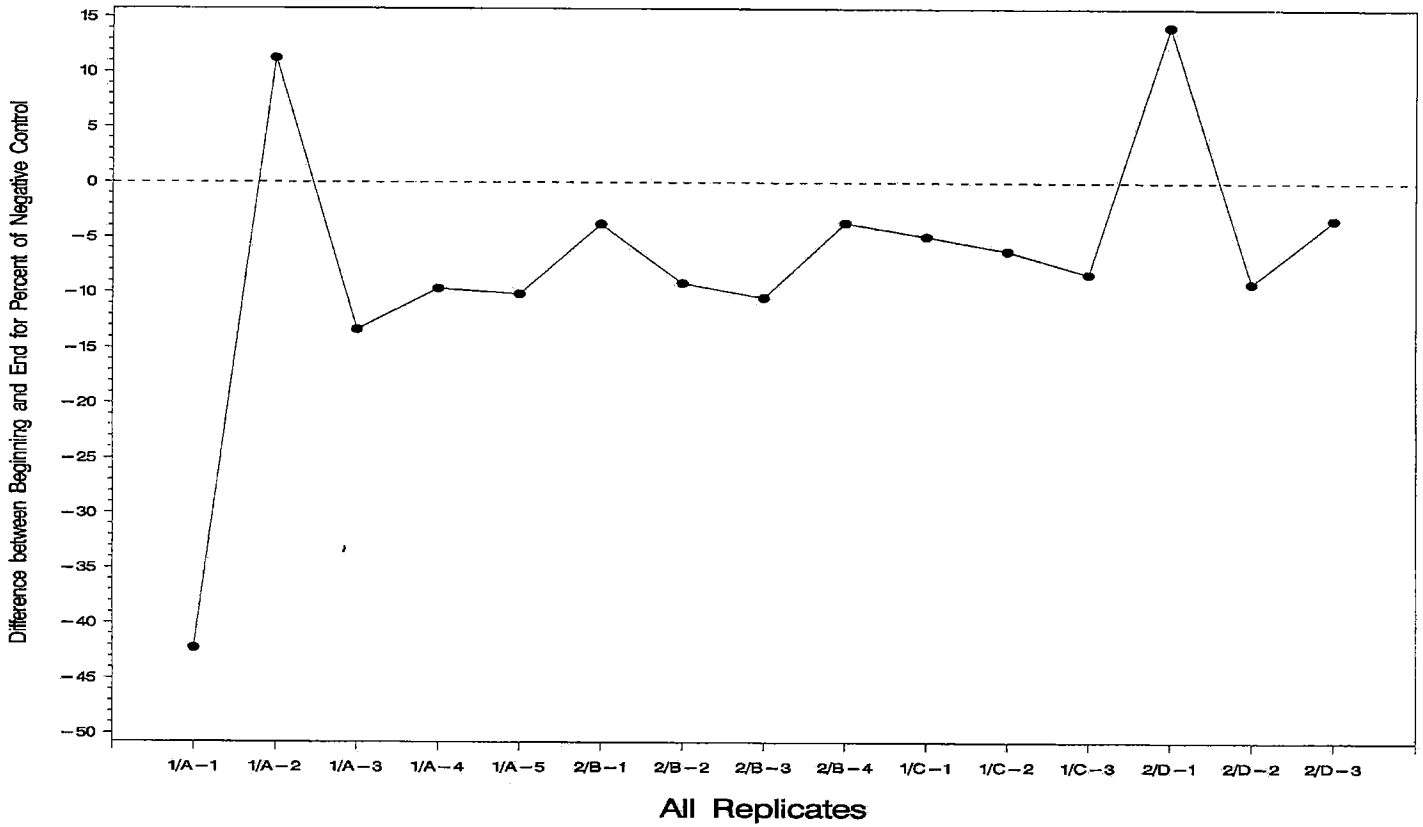


Figure 12. Negative Controls. Difference Between the Averages of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Placental Aromatase Assay. Reference Line at 0%.

Table A-1a. Chemical 1/A. Percent of Control Activity in Placental Aromatase Assay by Replicate, Concentration Within Replicate and Repetition Within Concentration.

Replicate	Log_{10} [Chem 1/A]	Percent of Control		
		Repetition 1	Repetition 2	Repetition 3
1	-3.00	0.22	0.56	0.23
	-4.00	3.10	3.36	3.14
	-5.00	25.61	24.72	23.92
	-6.00	75.98	78.06	79.71
	-7.00	108.28	102.43	92.77
	-8.00	103.24	105.49	103.49
	-9.00	102.86	103.13	96.44
	-10.00	80.75	77.04	75.64
2	-3.00	0.21	0.11	0.25
	-4.00	2.39	2.33	2.38
	-5.00	19.31	19.09	18.31
	-5.30	31.67	31.42	31.24
	-5.60	46.96	46.43	46.02
	-6.00	66.32	67.33	66.22
	-7.00	89.46	90.51	92.17
	-8.00	94.74	94.53	96.48
3	-3.00	0.29	0.62	0.33
	-4.00	3.83	3.85	4.60
	-5.00	25.89	26.89	26.99
	-5.30	42.69	41.08	42.46
	-5.60	60.94	57.26	59.10
	-6.00	81.14	81.43	78.67
	-7.00	49.35	94.89	89.58
	-8.00	101.21	102.62	103.52
4	-3.00	0.66	0.22	0.54
	-4.00	6.75	7.14	7.61
	-5.00	40.48	42.80	44.21
	-5.30	56.55	55.06	59.36
	-5.60	75.61	76.33	73.49
	-6.00	80.38	84.05	82.38
	-7.00	93.15	96.64	96.10
	-8.00	92.79	101.32	91.81
5	-3.00	0.50	0.52	0.59
	-4.00	8.29	7.93	7.59
	-5.00	40.24	38.60	39.83
	-5.30	54.53	57.14	56.71
	-5.60	74.47	72.17	67.19
	-6.00	90.74	88.20	85.51
	-7.00	100.21	91.27	96.19
	-8.00	99.26	105.85	99.24

Table A-1b. Chemical 2/B. Percent of Control Activity in Placental Aromatase Assay by Replicate, Concentration Within Replicate, and Repetition Within Concentration.

Replicate	Log_{10} [Chem 2/B]	Percent of Control		
		Repetition 1	Repetition 2	Repetition 3
1	-4.00	24.83	24.70	29.04
	-5.00	14.69	16.48	15.62
	-6.00	64.44	60.88	54.95
	-6.60	78.95	80.92	92.95
	-7.00	92.61	94.25	90.06
	-8.00	95.45	96.44	96.53
	-9.00	94.25	95.90	95.14
	-10.00	95.10	94.10	97.71
2	-4.00	22.70	27.38	19.03
	-5.00	26.46	26.14	28.16
	-5.60	55.26	56.23	60.14
	-6.00	74.75	75.61	76.02
	-6.30	82.56	86.64	78.23
	-6.60	89.69	93.13	90.70
	-7.00	94.59	88.08	88.62
	-8.00	102.54	104.47	100.71
3	-4.00	31.60	32.54	26.84
	-5.00	31.67	31.14	31.75
	-5.60	68.14	64.12	60.02
	-6.00	80.03	82.82	82.73
	-6.30	89.50	94.62	91.73
	-6.60	92.82	91.49	95.49
	-7.00	99.12	99.22	97.80
	-8.00	102.04	101.19	98.79
4	-4.00	35.63	40.09	37.01
	-5.00	46.07	43.74	42.04
	-5.60	73.60	73.14	73.48
	-6.00	89.77	87.35	83.38
	-6.30	87.93	91.20	91.69
	-6.60	83.61	90.92	95.35
	-7.00	95.23	88.04	93.36
	-8.00	95.17	95.92	99.23

Table A-1c. Chemical 1/C. Percent of Control Activity in Placental Aromatase Assay by Replicate, Concentration Within Replicate, and Repetition Within Concentration.

Replicate	Log_{10} [Chem 1/C]	Percent of Control		
		Repetition 1	Repetition 2	Repetition 3
1	-3.00	-0.27	-0.23	-0.23
	-4.00	-0.45	-0.27	-0.25
	-5.00	-0.02	-0.36	-0.25
	-6.00	-0.14	0.09	0.18
	-7.00	1.75	2.00	2.22
	-8.00	18.38	17.85	17.97
	-9.00	70.80	71.62	71.07
	-10.00	93.30	96.43	98.13
2	-6.00	0.09	-0.11	0.09
	-6.00	0.59	0.38	0.23
	-7.00	1.64	1.69	1.61
	-7.30	3.63	3.22	3.03
	-7.60	6.55	6.37	6.37
	-8.00	15.94	12.59	15.87
	-8.60	44.32	45.90	45.71
	-9.00	72.74	68.89	69.30
3	-6.00	0.41	0.05	0.37
	-7.00	1.74	1.90	2.13
	-8.00	15.09	15.59	15.10
	-8.30	26.08	25.71	25.51
	-8.60	44.53	43.11	43.36
	-9.00	65.92	68.00	69.51
	-9.60	92.82	88.49	86.98
	-10.00	90.58	89.63	94.28

Table A-1d. Chemical 2/D. Percent of Control Activity in Placental Aromatase Assay by Replicate, Concentration Within Replicate, and Repetition Within Concentration.

Replicate	Log_{10} [Chem 2/D]	Percent of Control		
		Repetition 1	Repetition 2	Repetition 3
1	-4.00	6.02	6.43	6.43
	-5.00	46.01	46.01	42.10
	-6.00	87.78	85.88	86.48
	-6.60	101.45	98.91	99.15
	-7.00	96.80	98.46	99.19
	-8.00	94.36	94.31	93.58
	-9.00	94.31	94.29	91.11
	-10.00	93.14	94.06	91.89
2	-4.00	5.38	5.63	5.91
	-4.60	22.99	19.94	23.11
	-5.00	42.81	40.29	40.52
	-5.30	57.69	55.99	56.43
	-5.60	72.68	69.90	70.75
	-6.00	84.41	84.09	66.87
	-6.60	86.18	88.02	89.63
	-7.00	91.08	89.81	89.03
3	-4.00	6.08	6.21	6.29
	-4.60	21.63	22.00	21.91
	-5.00	42.02	39.85	40.19
	-5.30	57.59	55.66	25.22
	-5.60	72.24	68.07	66.76
	-6.00	76.90	73.53	73.62
	-6.60	93.27	85.43	88.48
	-7.00	92.13	90.29	92.18

Table A-2a. Chemical 1/A. Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control Background Adjusted Aromatase Activity, and Percent of Control Data by Replicate and Portion (Beginning or End). Placental Aromatase Assay.

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
Full Activity Control	1	Beginning	0.08968	120.8
		Beginning	0.09306	125.4
		End	0.05677	76.5
		End	0.05742	77.4
	2	Beginning	0.04591	105.7
		Beginning	0.04432	102.1
		End	0.04206	96.86
		End	0.04141	95.35
	3	Beginning	0.05463	111.6
		Beginning	0.04976	101.7
		End	0.04715	96.32
		End	0.04426	90.41
	4	Beginning	0.03335	115.2
		Beginning	0.02293	79.22
		End	0.02917	100.8
		End	0.03033	104.8
	5	Beginning	0.04550	106.6
		Beginning	0.04198	98.34
		End	0.04054	94.95
		End	0.04275	100.1
Background Control	1	Beginning	0.00008	0.110
		Beginning	-0.00009	-0.123
		End	0.00006	0.086
		End	-0.00005	-0.072
	2	Beginning	-0.00001	-0.032
		Beginning	0.00001	0.032
		End	0.00002	0.048
		End	-0.00002	-0.048
	3	Beginning	0.00006	0.120
		Beginning	0.00011	0.225
		End	0.00002	0.037
		End	-0.00019	-0.382
	4	Beginning	0.00001	0.037
		Beginning	-0.00002	-0.082
		End	0.00003	0.097
		End	-0.00002	-0.052
	5	Beginning	0.00007	0.157
		Beginning	0.00009	0.206
		End	-0.00007	-0.157
		End	-0.00009	-0.206
Positive Control	1	Beginning	0.03327	44.82
		Beginning	0.03286	44.23

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
		End	0.04144	55.3
		End	0.04019	54.14
	2	Beginning	0.02351	54.13
		Beginning	0.02307	53.14
		End	0.02225	51.23
		End	0.02155	49.63
	3	Beginning	0.02658	54.31
		Beginning	0.03038	62.07
		End	0.02775	56.70
		End	0.02846	58.14
	4	Beginning	0.02038	70.39
		Beginning	0.02024	69.92
		End	0.01894	65.41
		End	0.01853	64.01
	5	Beginning	0.02627	61.55
		Beginning	0.02792	65.40
		End	0.02436	57.07
		End	0.02373	55.59
Negative Control	1	Beginning	0.08295	111.7
		Beginning	0.09221	124.2
		End	0.05720	77.05
		End	0.05524	74.42
	2	Beginning	0.04398	101.3
		Beginning	0.03071	70.72
		End	0.04214	97.03
		End	0.04233	97.48
	3	Beginning	0.05100	104.2
		Beginning	0.05125	104.7
		End	0.04262	87.07
		End	0.04658	95.17
	4	Beginning	0.03045	105.2
		Beginning	0.02904	100.3
		End	0.02633	90.94
		End	0.02757	95.24
	5	Beginning	0.04544	106.5
		Beginning	0.04387	102.8
		End	0.04165	97.57
		End	0.03901	91.37

a. Percent of control values were calculated by dividing the corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplying by 100 percent.

Table A-2b. Chemical 2/B. Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control Background Adjusted Aromatase Activity, and Percent of Control Data by Replicate and Portion (Beginning or End). Placental Aromatase Assay.

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
Full Activity Control	1	Beginning	0.07146	111.8
		Beginning	0.06710	105.0
		End	0.05761	90.12
		End	0.05953	93.12
	2	Beginning	0.05957	110.3
		Beginning	0.05962	110.4
		End	0.04669	86.44
		End	0.05020	92.93
	3	Beginning	0.05623	107.9
		Beginning	0.05040	96.69
		End	0.05122	98.26
		End	0.05065	97.17
	4	Beginning	0.03766	103.0
		Beginning	0.03707	101.4
		End	0.03625	99.12
		End	0.03531	96.56
Background Control	1	Beginning	-0.00002	-0.03
		Beginning	0.00004	0.06
		End	-0.00002	-0.03
		End	NA	NA
	2	Beginning	0.00006	0.108
		Beginning	-0.00002	-0.042
		End	-0.00007	-0.136
		End	0.00004	0.07
	3	Beginning	0.00020	0.388
		Beginning	-0.00008	-0.158
		End	-0.00006	-0.115
		End	-0.00006	-0.115
	4	Beginning	0.00006	0.158
		Beginning	-0.00003	-0.072
		End	-0.00002	-0.043
		End	-0.00002	-0.043
Positive Control	1	Beginning	0.03634	56.85
		Beginning	0.03601	56.33
		End	0.03316	51.87
		End	0.03630	56.79
	2	Beginning	0.02900	53.69
		Beginning	0.03049	56.44
		End	0.02875	53.22
		End	0.02789	51.63
	3	Beginning	0.03380	64.85
		Beginning	0.03225	61.87

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
		End	0.03216	61.70
		End	0.03087	59.23
	4	Beginning	0.02484	67.90
		Beginning	0.02498	68.31
		End	0.02331	63.73.
		End	0.02853	78.00
Negative Control	1	Beginning	0.06862	107.3
		Beginning	0.07171	112.2
		End	0.06934	108.5
		End	0.06612	103.4
	2	Beginning	0.05518	102.1
		Beginning	0.05164	95.60
		End	0.04678	86.61
		End	0.05014	92.81
	3	Beginning	0.05711	109.58
		Beginning	0.05849	112.2
		End	0.05459	104.7
		End	0.05010	96.12
	4	Beginning	0.03530	96.53
		Beginning	0.03421	93.535
		End	0.03213	87.84
		End	0.03469	94.86

a. Percent of control values were calculated by dividing the corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplied by 100 percent

Table A-2c. Chemical 1/C. Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control Background Adjusted Aromatase Activity, and Percent of Control Data by Replicate and Portion (Beginning or End). Placental Aromatase Assay.

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
Full Activity Control	1	Beginning	0.04903	103.8
		Beginning	0.05140	108.8
		End	0.04525	95.79
		End	0.04328	91.62
	2	Beginning	0.06172	108.5
		Beginning	0.06351	111.7
		End	0.05351	94.08
		End	0.04876	85.73
	3	Beginning	0.06431	105.4
		Beginning	0.06451	105.8
		End	0.05725	93.8
		End	0.05793	94.97
Background Control	1	Beginning	0.00001	0.018
		Beginning	0.00017	0.358
		End	-0.00005	-0.107
		End	-0.00013	-0.269
	2	Beginning	0.00035	0.622
		Beginning	-0.00011	-0.186
		End	-0.00012	-0.218
		End	-0.00012	-0.218
	3	Beginning	0.00007	0.107
		Beginning	0.00003	0.053
		End	-0.00010	-0.16
		End	0	0
Positive Control	1	Beginning	0.02634	55.76
		Beginning	0.02522	53.38
		End	0.02612	55.30
		End	0.02629	55.66
	2	Beginning	0.03137	55.16
		Beginning	0.03214	56.52
		End	0.03166	55.66
		End	0.03090	54.32
	3	Beginning	0.03705	60.74
		Beginning	0.03420	56.06
		End	0.03409	55.88
		End	0.03446	56.49
Negative Control	1	Beginning	0.05067	107.3
		Beginning	0.04788	101.3
		End	0.04745	100.4
		End	0.04645	98.31
	2	Beginning	0.05925	104.2

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
		Beginning	0.05911	103.9
		End	0.05465	96.08
		End	0.05663	99.57
	3	Beginning	0.06375	104.5
		Beginning	0.06209	101.8
		End	0.05888	96.52
		End	0.05680	93.12

a. Percent of control values were calculated by dividing the corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplied by 100 percent.

Table A-2d. Chemical 2/D. Full Enzyme Activity Control, Background Activity Control, Positive Control, and Negative Control Background Adjusted Aromatase Activity, and Percent of Control Data by Replicate and Portion (Beginning or End). Placental Aromatase Assay.

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
Full Activity Control	1	Beginning	0.05000	109.9
		Beginning	0.04809	105.7
		End	0.04257	93.60
		End	0.04126	90.73
	2	Beginning	0.04949	112.4
		Beginning	0.04502	102.2
		End	0.04106	93.24
		End	0.04059	92.16
	3	Beginning	0.03588	100.8
		Beginning	0.03763	105.7
		End	0.03395	95.40
		End	0.03490	98.06
Background Control	1	Beginning	0.00006	0.122
		Beginning	0	-0.008
		End	0	-0.008
		End	-0.00005	-0.105
	2	Beginning	0.00004	0.092
		Beginning	-0.00004	-0.092
		End	-0.00006	-0.138
		End	0.00006	0.138
	3	Beginning	-0.00003	-0.086
		Beginning	0.00009	0.258
		End	-0.00008	-0.215
		End	0.00002	0.043
Positive Control	1	Beginning	0.02505	55.08
		Beginning	0.02484	54.61
		End	0.02084	45.83
		End	0.02201	48.41
	2	Beginning	0.02563	58.20
		Beginning	0.02559	58.11
		End	0.02363	53.65
		End	0.02317	52.61
	3	Beginning	0.01983	55.72
		Beginning	0.01852	52.05
		End	0.01664	46.76
		End	0.01799	50.55
Negative Control	1	Beginning	0.04069	89.46
		Beginning	0.04097	90.08
		End	0.04605	101.3
		End	0.04846	106.6
	2	Beginning	0.04643	105.4

Aromatase Activity	Replicate	Portion	Background Adjusted Aromatase Activity	% of Control[a]
		Beginning	0.04422	100.4
		End	0.04249	96.48
		End	0.04009	91.03
	3	Beginning	0.03507	98.56
		Beginning	0.03720	104.5
		End	0.03580	100.6
		End	0.03407	95.74

a. Percent of control values were calculated by dividing the corrected aromatase activity values by the average of the four full enzyme activity control values within the same replicate and multiplied by 100 percent.

APPENDIX C

WIL REPORT

AUDITED DRAFT TASK REPORT

VALIDATION OF THE PLACENTAL AROMATASE ASSAY FOR ENDOCRINE DISRUPTOR SCREENING (WA 4-16, TASK 5)

WA 4-16 Task 5: Conduct Multiple Chemical Studies with Centrally Prepared Microsomes

EPA Contract Number 68-W-01-023
Work Assignment 4-16
WIL-431007

Sponsor:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

Performing Laboratory:

WIL Research Laboratories, LLC
1407 George Road
Ashland, OH 44805-9281

DRAFT REPORT

Title:	VALIDATION OF THE PLACENTAL AROMATASE ASSAY FOR ENDOCRINE DISRUPTOR SCREENING (WA 4-16, TASK 5)
Task 5:	Conduct Multiple Chemical Studies with Centrally Prepared Microsomes
Author:	Jennifer A. Thomas-Wohlever, Ph.D.
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Sponsor's Representatives:	David P. Houchens, Ph.D. EDSP Program Manager Battelle Jerry D. Johnson, Ph.D. Diplomate, A.B.T. Work Assignment Leader/Study Monitor Battelle
Study Initiation Date:	January 31, 2005
Experimental Start Date:	February 1, 2005
Experimental Termination Date:	April 1, 2005
Study Completion Date:	
Draft Task Report Date	June 25, 2005

Quality Assurance Statements

The study was conducted in compliance and audited in accordance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the United States EPA Good Laboratory Practice Standards (40 CFR Part 792), September 18, 1989; the standard operating procedures of WIL Research Laboratories, LLC, and the protocol as approved by the Sponsor, with the following exceptions. The Sponsor has assured GLP compliance of the initial chemical analyses (Appendix E) of the bulk chemicals for identity and purity and the preparation of stock formulations.

Intralaboratory data requiring statistical analysis were analyzed by BioSTAT Consultants, Inc., following the current procedural guidelines of BioSTAT Consultants, Inc. BioSTAT Consultants, Inc. provided a statistical analysis report, which is included as Appendix G. Quality Assurance auditing of the statistical report (for internal consistency with the study report) was conducted under the direction of the Quality Assurance Unit of WIL Research Laboratories, LLC.

Quality Assurance findings, derived from the inspections during the conduct of the study and from the inspections of the raw data and draft report are documented and have been reported to the Study Director. A status report is submitted to management monthly. This report accurately reflects the data generated during the study. The methods and procedures used in the study were those specified in the protocol, its amendments and the standard operating procedures of WIL Research Laboratories, LLC.

The raw data and draft report were audited by the WIL Quality Assurance Unit prior to submission to the Sponsor to assure that the Final Report accurately describes the conduct and the findings of the study. Quality control (QC) and quality assurance (QA) procedures followed those outlined in the Quality Assurance Project Plan (QAPP) that was prepared for this study (Appendix C).

Phases Inspected

Date of Inspection	Phases Inspected	Date(s) Findings Reported to Study Director	Date(s) Findings Reported to Management	Auditors
03-Feb-2005	Test & Control Articles & Analytical Chemistry / Aromatase Assay Sample Preparation 2/3/05	03-Feb-2005	25-Mar-2005	A.Deppe
03-Feb-2005	Test & Control Articles & Analytical Chemistry / Microsomal Preparation for Aromatase Assay 2/3/05	03-Feb-2005	25-Mar-2005	A.Deppe
03-Feb-2005	Test & Control Articles & Analytical Chemistry / Test Article dilution for Aromatase Assay 2/3/05	03-Feb-2005	25-Mar-2005	A.Deppe
26-Jul-2005, 06-Aug-2005, 08-Aug-2005, 09-Aug-2005	Reports / (Draft Metabolism Report)	09-Aug-2005	22-Sep-2005	E.Crawford
19-Apr-2005, 22-Apr-2005	Study Records / B-1, B-2 and Spreadsheet (Results of Assay)	22-Apr-2005	28-May-2005	E.Crawford
25-Jan-2005	Protocol / Protocol Amendment Review / Protocol Review	25-Jan-2005	16-Feb-2005	L.Goodrich

Approval

This study was inspected according to the criteria discussed above.

Report Audited By:

Elizabeth S. Crawford, B.S. Date
Compliance Specialist

Report Released By:

Heather L. Osborn, B.S., RQAP-GLP Date
Manager, Quality Assurance

Compliance Statement

This study, designated WIL-431007, was conducted in compliance with the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 160), October 16, 1989; the United States Environmental Protection Agency (EPA) Good Laboratory Practice Standards (40 CFR Part 792), September 18, 1989; the standard operating procedures of WIL Research Laboratories, LLC, and the protocol and protocol amendment as approved by the sponsor.

Author:

_____ _____

Jennifer Thomas-Wohlever, Ph.D. Date
Research Scientist, Metabolism
Study Director

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1.0 Executive Summary

The placental aromatase assay combines human placental microsomes, substrate, appropriate cofactors and reference chemicals in a common reaction vessel under optimized conditions for the enzyme. The effect of the reference chemicals on microsomal enzyme activity is evaluated by measuring the amount of product formed by the enzyme-catalyzed substrate oxidation. The aromatase assay is conducted over a range of reference chemical concentrations such that a dose response curve can be developed and an IC_{50} calculated to determine the amount of reference chemical required to inhibit aromatase activity by 50%. The general purpose of this assay is to screen chemicals for their ability to inhibit aromatase activity, an indication of the chemical's potential to disrupt endocrine function. This specific study was undertaken to demonstrate the conduct and responsiveness of the placental microsome aromatase assay at WIL Research Laboratories, LLC (a participating laboratory in the inter-laboratory validation) using the known aromatase inhibitor 4-hydroxyandrostenedione as a positive control, lindane as a negative control, and four reference chemicals whose IC_{50} was determined in the course of this Task (WA 4-16, Task 5).

Complete dose response curves were achieved consistently with the reference chemicals chrysin, aminoglutethimide and ketoconazole. Generally, reference chemical concentrations ranging from $1x10^{-3}$M to $1x10^{-10}$M resulted in sigmoidal dose response curves ranging from no inhibition (100 percent of Full Enzyme Activity Control) to almost full inhibition (approximately 0-5% percent of Full Enzyme Activity Control), respectively. The inhibition curves and IC_{50} for the reference chemicals are consistent with the published values reviewed in the pre-validation summary plan (WA 2-5, Task 12). The Full Enzyme and Background Activity Controls demonstrated that the conditions were constant throughout each successful replicate reference and that there was no Background Activity that might interfere with the interpretation of the results. During the testing of the fourth reference chemical, econazole, the Full Enzyme Activity Control was variable and the enzyme was much less active. For this reason, an IC_{50} for econazole could be estimated from a single replicate, but further efforts to confirm the result were not successful. Based on these results, the response of human placental aromatase to known aromatase inhibitors was successfully validated in this laboratory, although the econazole evaluation was limited to one replicate.

2.0 Introduction

2.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, *e.g.*, a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Advisory Committee (EDMVAC) provides advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are

biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed 1) searching the literature databases 2) contacting individuals to obtain information on unpublished research, and evaluating the literature and personal communication (Work Assignment (WA) 2-7).

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens such as testosterone and androstenedione into the estrogens estradiol and estrone, respectively. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450arom and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is also found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970s and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be used as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay using human placental microsomes is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant recombinant tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human recombinant microsomal assay system, with IC_{50} values for aromatase inhibition ranging from 0.04 mM to greater than 50 mM (WA 2-7, Task 2).

The human placental microsomal aromatase assay has been recommended as an *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on placental aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta to demonstrate the utility of the microsomal assay to detect known aromatase inhibitors and compare to the performance of a recombinant microsomal assay system. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10). The placental microsome aromatase assay was previously validated at WIL Research Laboratories using the known aromatase inhibitor 4-hydroxyandrostenedione (4-OH ASDN) as a positive control (WIL-431006, WA 4-16, Task 4). As part of the continuing validation process, four reference chemicals known to inhibit placental microsomal aromatase have been selected by EDMVAC. As

discussed in the pre-validation study plan (WA 2-5, Task 12), these chemicals are readily available and have IC_{50} values in the µM to nM range. The full inhibition curve can thus be investigated by determining aromatase inhibition at a series of concentrations ranging from 10^{-3} M to 10^{-10} M.

2.2 Task Description and Objectives

WIL Research Laboratories, LLC (as one of the participating laboratories) was charged with demonstrating the responsiveness of the human placental microsomal aromatase assay to a series of reference chemicals: chrysin, aminoglutethimide, ketoconazole and econazole. For inter- and intra-laboratory variability assessment, each reference chemical was tested via three repetitions on each of three days (replicates). Each replicate included Full Enzyme Activity Controls, Background Activity Controls and Positive and Negative controls to support the validation of the human placental microsomal aromatase assay. This report provides the intra-laboratory data for WIL Research Laboratories, LLC.

3.0 Materials and Methods

3.1 Substrate

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot # 024K0809) was obtained from Sigma (St. Louis, MO) by the Sponsor's Chemical Repository and was then distributed to the participating laboratories. It had a reported purity of 100%. The radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN, Lot # 3538-496), was obtained from Perkin Elmer Life Science (Boston, MA) and had a reported specific activity of 25.30 Ci/mmol. Radiochemical purity was reported by the supplier to be > 97%. Radiochemical purity was assessed by high pressure liquid chromatography (HPLC) by the lead laboratory. The results of this analysis are presented in the report contained in Appendix D and radiochemical purity was found to be 97%.

A mixture of ASDN and [^{3}H]ASDN was made such that the final concentration of ASDN in the assay was 100 nM, and each assay contained 0.1 µCi. This was accomplished by preparing a 100-fold dilution of the radiolabeled stock in buffer. In addition, a 1 mg/mL solution of ASDN in ethanol was prepared, and then dilutions of stock ASDN were made in buffer to a final concentration of 1 µg/mL. To make 8 mL of substrate solution (enough for 80 tubes), 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL buffer were combined. For accuracy, the weight of each component added to the substrate solution was also recorded. To determine the specific activity of the ASDN substrate, aliquots of substrate solution (ca 20 µL, weighed) were combined with scintillation cocktail for radiochemical content analysis.

3.2 Reference and Control Chemicals

The Sponsor's Chemical Repository was responsible for chemistry activities required to perform this study. Their responsibilities included chemical procurement, solubility, formulation stability assessment, formulation preparation, formulation analysis and shipment of stock formulation to the participating laboratories. These chemistry activities and results are described in the Sponsor's Chemistry report which is appended to this document (Appendix E).

When reference chemicals arrived at WIL, they were assigned a unique identification number (e.g. MET-XXXXY), which was recorded and dated on the log-in sheet as specified in WIL standard operating procedures. Also recorded on the log-in sheet were the label identification information, quantity received, storage conditions, storage location and a physical

description of the material. Any documents accompanying the shipment were filed. The following table summarized reference chemical information, including the WIL identification number assigned.

Reference Chemical Information								
Chemical name	Chemical code	Mfr. Purity	CAS No.	Molecular formula	M.W.	Stock Solution (WIL ID)	Vehicle	Storage
4-Androsten-4-ol-3,17 dione Lot: 063K4069	4-OH ASDN (Positive Control)	99%	566-48-3	$C_{19}H_{26}O_3$	302.4	MET-0264A	95% Ethanol	~5°C
Lindane Lot: 14419EB	Negative Control	99.6%	58-89-9	$C_6H_6Cl_6$	290.8	MET-0266A	DMSO Lot # 2969A2 4437	~5°C
Chrysin Lot:10101DC	Reference Chemical 1	98.2-101.4 %	480-40-0	$C_{15}H_{10}O_4$	254.2	MET-0267A	DMSO Lot # 2969Y3 0428	~5°C
Aminoglute-thimide Lot: 043K0939	Reference Chemical 2	>99%	125-84-8	$C_{13}H_{16}N_2O_2$	232.3	MET-0265A	DMSO Lot # 2969A2 4437	~5°C
Ketoconazole Lot: 121H0524	Reference Chemical 3	>99%	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	531.4	MET-0272A	DMSO Lot # 2969A2 4437	~5°C
Econazole Lot: 123K1220	Reference Chemical 4	98%	24169-02-6	$C_{18}H_{15}Cl_3N_2O{\cdot}HNO_3$	444.7	MET-0271A	DMSO Lot # 2969Y3 0428	~5°C

4-OH ASDN was received as a 0.01 M stock solution in 95% ethanol. This solution was used to create a 5×10^{-6} M working stock solution by first diluting 10 µL to 1 mL in 95% ethanol, then diluting again by adding 50 µL to an additional 950 µL of 95% ethanol. Lindane was received as a 0.1 M stock solution in DMSO. The stock solution was diluted 100-fold (10 µL in 990 µL DMSO) and then ten-fold (100 µL in 900 µL DMSO) to create a 1×10^{-4} M solution. The concentration of 4-OH ASDN was determined from a previous work assignment (WA 4-16, Task 4, WIL-431006) and published values. The concentration of lindane was chosen because it was within the range of the concentration of reference chemicals, although no concentration was expected to inhibit aromatase.

The reference chemicals were received as 0.01 M or 0.1 M stock solutions in DMSO. The reference chemicals were diluted in DMSO according to the schemes below. In addition, the reference chemicals were blinded by staff not involved in the Task prior to distribution to the staff performing each assay.

Reference Chemical Dilutions – 0.01 M Stock Solution (Chrysin, Ketoconazole)					
	Reference Chemical Stock solution		Diluent (µL DMSO)	Solution Concentration (M)	Target Concentration in Assay (M)
	µL	M			
Concentration 1	200	1×10^{-2}	0	1×10^{-2}	1×10^{-4}
Concentration 2	100	1×10^{-2}	900	1×10^{-3}	1×10^{-5}
Concentration 3	100	1×10^{-3}	900	1×10^{-4}	1×10^{-6}
Concentration 4	100	1×10^{-4}	900	1×10^{-5}	1×10^{-7}
Concentration 5	500	1×10^{-5}	500	5×10^{-6}	5×10^{-8}

Concentration 6	100	1×10^{-5}	900	1×10^{-6}	1×10^{-8}
Concentration 7	100	1×10^{-6}	900	1×10^{-7}	1×10^{-9}
Concentration 8	100	1×10^{-7}	900	1×10^{-8}	1×10^{-10}

Reference Chemical Dilutions – 0.1 M Stock Solution (Aminoglutethimide, Econazole)					
	Reference Chemical Stock solution		Diluent (µL DMSO)	Solution Concentration (M)	Target Concentration in Assay (M)
	µL	M			
Concentration 1	200	1×10^{-1}	0	1×10^{-1}	1×10^{-3}
Concentration 2	100	1×10^{-1}	900	1×10^{-2}	1×10^{-4}
Concentration 3	100	1×10^{-2}	900	1×10^{-3}	1×10^{-5}
Concentration 4	100	1×10^{-3}	900	1×10^{-4}	1×10^{-6}
Concentration 5	100	1×10^{-4}	900	1×10^{-5}	1×10^{-7}
Concentration 6	100	1×10^{-5}	900	1×10^{-6}	1×10^{-8}
Concentration 7	100	1×10^{-6}	900	1×10^{-7}	1×10^{-9}
Concentration 8	100	1×10^{-7}	900	1×10^{-8}	1×10^{-10}

After completion of the first replicate for each reference chemical, the data was reviewed and the concentration of reference chemical used in the second and third replicates were adjusted to better define the dose-response curve. The decision whether to adjust test concentrations was made by the Study Director. The decision was based on the results from the first replicate with the following guidelines in mind:

- If insolubility was observed at the high concentration (10^{-3} M), then the highest concentration for the second and third replicates was set at the highest concentration that appeared to be soluble (limited to 10^{-4} or 10^{-5} M). The highest concentration tested was never lower than 10^{-5} M.
- When the highest concentration to be tested was lowered to 10^{-4} M, then mid-log concentration(s) were added near the estimated IC_{50} based on the replicate one results in order to keep eight concentrations in the test set.
- The lowest concentration tested was 10^{-10} M.

3.3 Microsomes

Human placental microsomes were received as multiple frozen aliquots (approximately 500 µL) from RTI International. Upon receipt, the sample code number MET-0255A was assigned. For the fifth replicate (last replicate) of reference chemical 4 (econazole), two additional tubes of human placental microsomes were obtained from RTI. These microsomes were assigned sample code number MET-0275A.

Microsomes were stored between -70 and -80°C. On the day of the assay, microsomes were thawed rapidly in a 37 ± 1°C water bath, re-homogenized and then kept on ice until used. For reference chemical 1 (chrysin) Replicate 1, a volume of microsome preparation in excess of the amount needed was transferred to a Potter-Elvejhem homogenizer. Once the microsomes

were suspended, the amount needed was removed for the assay and the remainder flash frozen and returned to -70 to -80°C storage.

At the request of the Sponsor, all remaining aliquots were thawed, pooled, homogenized and re-distributed into smaller (~100 µL) single-use aliquots. The purpose of having smaller aliquots was to prevent the need to re-freeze microsomes after thawing.

During the microsome preparation for the reference chemical 1 (chrysin) Replicate 2, the necessary 90 µL of the 100 µL used in the homogenizer was not recovered, therefore the necessary 90 µL of microsomes were obtained from two 100 µL aliquots, homogenized and quantitatively transferred to the assay. However, the protein concentration in these preparations was lower than expected by approximately half. Therefore, following consultation with the Sponsor, subsequent microsome preparations (reference chemicals 2 through 4) were homogenized in the cryotube via brief (5 second) vortex mixing.

Aliquots of microsomes used for each assay were either never thawed, or only thawed once during the pooling/re-distributing into 100 µL aliquots (aliquots were never used for more than one replicate). The reported protein concentration from RTI International was 14 mg/mL. This concentration was used for calculating the initial dilution of microsomes. A 50-fold dilution was made to give a concentration of approximately 0.28 mg/mL. The actual protein concentration of this dilution was determined using the protein assay described in Section 3.5. A second dilution was made to achieve the final 0.025 mg/mL working stock of microsomes.

3.4 Other assay components

Assay Reagents - Information		
Chemical	Supplier	Lot Number
NADPH	Sigma-Aldrich	103K7046
Propylene glycol	J.T. Baker	Y41659
Sodium phosphate dibasic	J.T. Baker	A11H37
Sodium phosphate monobasic	J.T. Baker	A28H21
Test/control vehicle A – Ethanol, 95%	Sponsor	04B104B
Test/control vehicle B – Dimethylsulfoxide (DMSO)	Sponsor	Lot # 2969A24437 Lot # 2969Y30428

3.4.1 NADPH

Nicotinamide adenine dinucleotide phosphate (NADPH, reduced form tetrasodium salt) is a required cofactor for aromatase activity. As such, it was included in excess in the aromatase assay. First, 0.025 g NADPH was weighed and transferred into a 5-mL volumetric flask to make a 5 mg/mL solution in phosphate buffer (see Section 3.4.2). Adding 100 µL of this NADPH solution to the reaction mixture resulted in a final assay concentration of 0.3 mM. NADPH was prepared fresh every assay day and was stored on ice until adding to the reaction mixture.

3.4.2 Assay Buffer

Sodium phosphate monobasic and sodium phosphate dibasic solutions (0.1 M each) were combined in an approximate 2:8 ratio to create a final 0.1 M pH 7.4 solution. The assay buffer was stored refrigerated for up to one month.

3.5 Protein Determination

The protein concentration of the microsome preparation was determined on each day of use of the microsomes in the aromatase assay. A 6-point standard curve was prepared, ranging

from approximately 0.13 to 1.5 mg protein/mL. The protein standards were made from bovine serum albumin (BSA) obtained from BioRad (Hercules, CA). At the request of the Sponsor, quality control samples were included in assays beginning 23 February 2005. These were commercially available BSA standards (Pierce, Rockford, IL) with a concentration of 0.125, 0.5 and 1.0 mg/mL. Total protein in standards, quality controls, and samples of the microsome preparation were analyzed using a DC Protein Assay kit, also purchased from BioRad. To a 25-µL aliquot of unknown or standard, 125 µL of BioRad DC Protein Kit Reagent A was added and vortex mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B was added to each standard or unknown and the samples were vortex mixed again. The samples were allowed to sit at room temperature for at least 15 minutes to allow for color development. The absorbance values were stable for about 1 hour. Each sample (microsome samples, QC and standards) was transferred to an appropriately labeled cuvette and the absorbance at 750 nm was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed from the protein standards.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

Aromatase activity was determined via an *in vitro* screening assay utilizing human placental microsomes supplied by RTI International. The assays were performed in 13x100 mm test tubes. Each test tube was uniquely identified with replicate, repetition, and group information summarized in the table below as necessary to differentiate the tubes. In addition to tubes containing inhibitor, Full Enzyme Activity Controls (includes vehicle but no inhibitor) and Background Activity Controls (tubes contain no NADPH cofactor) were utilized to determine 100% and 0% activity. Positive controls using the known aromatase inhibitor 4-hydroxyandrostenedione and the known non-inhibitor lindane were also included in the assay design. The following table demonstrates the assay design with a 0.1 M stock of reference chemical. The reference chemical concentrations were adjusted for 0.01 M reference chemical stocks, as shown in Section 3.2. Following the first replicate of each reference chemical, reference chemical concentrations were adjusted at the discretion of the Study Director, as described in Section 3.2.

Assay Design – First Replicate – 0.1 M Reference Chemical			
Sample Type/Group	Repetitions (test tubes)	Description	Reference Chemical Concentration (M final)
Full Enzyme Activity Control-Beginning	2	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control-Beginning	2	Complete assay with reference chemical vehicle control omitting NADPH	N/A
Positive Control – Beginning	2	Complete assay with 4-OH ASDN added	5×10^{-8}
Negative Control – Beginning	2	Complete assay with Lindane added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}
Full Enzyme Activity Control-End	2	Complete assay with reference chemical vehicle control	N/A
Background Activity Control-End	2	Complete assay with reference chemical vehicle control omitting NADPH	N/A
Positive Control – End	2	Complete assay with 4-OH ASDN added	5×10^{-8}
Negative Control – End	2	Complete assay with Lindane added	1×10^{-6}

[a]The complete assay contains buffer, propylene glycol, microsomal protein, substrate and NADPH

Propylene glycol (100 µL), ^{3}H-ASDN substrate solution (100 µL), NADPH (100 µL, excluded from background control), and vehicle or inhibitor (20 µL) were added to the appropriate test tube with buffer to make 1 mL total volume. Microsomes were diluted to the appropriate concentration as detailed in Section 3.3. Both the reaction mixture and the microsomes were incubated at 37 ± 1°C independently for at least 5 minutes with shaking. After the addition of 1 mL of microsomes to the first test tube containing the reaction mixture, the remaining assays were initiated at 15 second intervals. Each assay was incubated at 37 ± 1°C for 15 minutes. At the conclusion of the reaction time, tubes were quenched with 2.0 mL of methylene chloride in the order in which microsomes had been added, one every 15 seconds. The tubes were vortex-mixed for approximately 5 seconds and placed on ice until all tubes were quenched. The tubes

were then vortex-mixed an additional 20-25 seconds. The tubes were centrifuged for 10 minutes at approximately 162 x g to facilitate separation of the organic and aqueous layers. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Perkin Elmer, approximately 10 mL) was added to each counting vial and shaken to mix the solution. Analysis of the samples was performed using a Beckman Coulter LS6500 liquid scintillation counter (LSC).

Radiolabel found in the aqueous fractions represents ^3H_2O formed. One ^3H_2O molecule is released per molecule of radiolabeled ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of estrogen product formed is determined by dividing the total amount of ^3H_2O (in dpm) formed by the specific activity of the [^{3}H]ASDN substrate (expressed in dpm/nmol). Results are presented as the activity (velocity) of the enzyme reaction. The activity of the enzyme reaction is expressed in nmol/mg protein/min and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used and incubation time, e.g. 15 minutes.

Each assay replicate was performed on the day shown in the following table. The same technician performed each replicate for a given reference chemical. As discussed in Section 2.2, study design and objectives were to be met by completing three repetitions on each of three replicate days. In the table below, five replicates of reference chemical 4, econazole, were completed. Replicate 1 gave the expected inhibition curve. Replicates 2 through 4 had extremely low and erratic Full Enzyme Activity. Replicates 3 and 4 were separated by one successful ketoconazole replicate (3), indicating that the problem was probably not with the microsomes. New microsomes were used to confirm this hypothesis in the fifth econazole replicate, and again the results were poor. Further investigation of this problem was left to future Tasks. For the purposes of this report, statistical analyses were performed on all control data and then on the data from the first three reference chemicals only to show that the problems were isolated to reference chemical 4 (econazole). Where an IC_{50} value is given for econazole, it was determined for the first replicate only.

Assay Dates by Technician		
Replicate	Date	Technician
Chrysin Rep 1	2/1/2005	JG
Chrysin Rep 2	2/3/2005	JG
Chrysin Rep 3	2/4/2005	JG
Aminoglutethimide Rep 1	2/9/2005	JG
Aminoglutethimide Rep 2	2/14/2005	JG
Aminoglutethimide Rep 3	2/15/2005	JG
Ketoconazole Rep 1	2/17/2005	JRH
Ketoconazole Rep 2	2/18/2005	JRH
Ketoconazole Rep 3	3/8/2005	JRH
Econazole Rep 1	2/23/2005	JG
Econazole Rep 2	2/28/2005	JG
Econazole Rep 3	3/1/2005	JG
Econazole Rep 4	3/18/2005	JG
Econazole Rep 5	4/1/2005	JG

3.7 Data Analysis

Relevant data were entered into the latest version of the Microsoft® Excel spreadsheet Aromatase_Master_Version x.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. For each reference chemical, three

independent replicates of the concentration response curve fit were carried out (except econazole).

For each replicate two repeat tubes of the Full Enzyme Activity Controls, the Background Activity Controls, and the Positive and Negative controls were run prior to the varying concentrations of the reference chemical. Also, two repeat tubes of each control were run following the reference chemical tubes. Three repetitions (tubes) were prepared for each concentration of the reference chemical.

The data recorded for each replicate included assay date and run number, technician, chemical and log chemical concentration, total DPM minus background DPM, and calculated percent activity. The spreadsheet calculated DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion after extraction. Total initial DPM was calculated by the multiplication of the volume of substrate solution added to the incubation by the substrate solution radiochemical content (DPM/mL) and yields the total DPM present in the assay tube at initiation. Background DPM was calculated as the average of the DPMs present in the aqueous portion for the Background Activity Control tubes, and was subtracted from actual DPMs for all samples to provide DPMs for calculating aromatase activity. This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). The activity of the enzyme reaction is expressed in nmol/mg protein/min and is calculated by dividing the amount of estrogen formed (nmol) by the product of mg microsomal protein used and the incubation time.

The average activity in the four background-corrected Full Enzyme Activity Control samples for a given replicate was calculated. Percent of Full Enzyme Activity remaining in the presence of various inhibitor concentrations was calculated by dividing the aromatase activity at a given concentration by the average Full Enzyme Activity Control and multiplying by 100. Thus, the average percent activity across the four Background Activity Control repeat tubes must necessarily equal 0 within each replicate and the average percent activity across the four Full Enzyme Activity repeat tubes must necessarily equal 100 within each replicate. Although percent of control values ideally vary between 0% near high inhibitor concentrations and 100% near low inhibitor concentrations, individual experimental percent of control activity values will sometimes extend below 0% or above 100%. For curve fitting, observed individual percent activity values above 100% were set to 99.5%, and values below 0% were set to 0.5%

In several instances, percent of Full Enzyme Activity Control values within a replicate appeared to have an outlier. These values were subjected to a Q-test as described by Dean and Dixon (1951). Briefly, with an n of 3, if the quotient of the difference between the outlier (x_1) and the next closest value (x_2), and the range of values (w) exceeded 0.94, the value could be rejected with 90% confidence.

$$\frac{(x_2 - x_1)}{w} = Q$$

Percent of control activity data was exported to Prism (GraphPad, San Diego) for curve fitting. IC_{50} was calculated using Prism (v. 4.02) software to fit the percent of control activity versus log concentration data to a curve using the following equation:

$$Y = 100/(1+10^{((\mathrm{Log}IC_{50}-X)*\mathrm{Hill\ Slope})})+\varepsilon$$

where X is the logarithm of inhibitor concentration and Y is the percent of activity. ε is the variation among repetitions, distributed with mean 0 and variance proportional to D_{avg} (based on Poisson distribution theory for radiation counts. The software incorporated a weighting factor of 1/Y for the least squares nonlinear regression analysis of the percent of activity vs. log reference chemical concentration. As shown, the curve fitting equation uses the fixed value of 100 as the numerator. An alternative is to use a four-parameter equation that estimates the top and bottom

plateau from the percent of control activity values. Fixing the top and bottom boundary permits estimation of the IC_{50} value on inhibition curves that do not span the entire inhibition range from 100% to 0%. The status of each response curve was classified in the following manner: complete curves show inhibition from 0-100% for any particular chemical, incomplete curves show at least 50% inhibition but do not span the entire range, and chemicals that result in less than 80% inhibition across the inhibitor concentration range are not inhibitors. The addition of inhibitor resulted in a complete inhibition curve for all reference chemicals, and the concentration response fits were carried out for each replicate. The status is displayed in a table with the resultant μ (log IC_{50}) and β (slope) that summarize the extent of aromatase inhibition (see Section 4.6). Concentration response models were fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate, the extent of aromatase inhibition was summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for a reference chemical is a (weighted) geometric mean across the replicates. The estimated overall standard error was based on the standard errors within each replicate and the replicate-to-replicate variability.

Slope and log IC_{50} were also compared across replicates within reference chemicals based on random effects analysis of variance, treating the replicates as random effects with mean β_{avg} and μ_{avg}. β and μ are estimated, separately within each replicate, and plotted along with the average across replicates, where

$$Y_{avg} = 100/[1+10^{(\mu_{avg}-X)\beta_{avg}}].$$

The associated 95% confidence interval from the analysis of variance across replicates is also displayed on separate plots for β and μ.

Microsoft® Excel using full floating decimal point calculations was used to determine mathematical averages, standard deviations, and standard errors of the mean in order to assess the variation between repetitions (within a single replicate) and between replicates. Calculations were performed on the data as collected or as displayed or output by instruments, with no censoring for quantitation limits or significant figures. Slightly different results can be expected if calculations are based on the values as presented in the tables because some numbers have been rounded for display.

Within each replicate, quadruplicate repetitions (repeat tubes) were made of the Background Activity Control tubes, the Full Enzyme Activity Control tubes, the Positive control tubes and the Negative control tubes. Half the repetitions were carried out at the beginning of the replicate and half at the end. The enzyme activity in the control tubes at the beginning of the sample set was equivalent to the enzyme activity in the control tubes at the end of the sample set. The average of the four Background Activity Control samples within a replicate must necessarily be 0 and the average of the four Full Enzyme Activity Controls within a replicate must necessarily be 100. The two beginning controls and the two end controls were plotted by replicate with plotting symbols distinguishing between beginning and end, and with test line 0% (Background Activity) or 100% (Full Enzyme Activity Control) respectively. These plots display the extent of consistency across replicates with respect to average value and variability and provide comparisons of beginning versus end of each replicate. Plots of Positive and Negative control values, by replicate for each reference chemical, illustrate consistent % of control with test lines at 50% and 100%, respectively.

Three-factor mixed effects analysis of variance models were fitted separately for the Full Enzyme Activity Control, the Background Activity Control and the Positive and Negative control tubes. The fixed effect factors in the analysis of variance were

- reference chemical
- portion (beginning or end)
- portion by reference chemical interaction.

The random effects were

- replicate nested within reference chemical
- portion by replicate within reference chemical interaction.

The residual error variation corresponds to repetition within reference chemical, replicate, and portion. Statistical analysis was performed on the data reported as "Percent of Full Enzyme Activity Control". Because the average of the repetitions for the Background and Full Enzyme Activity Controls within a reference chemical and replicate were constrained to be 0 and 100, respectively, and the way in which "percent of control" is defined, the variation associated with the reference chemical effect and the replication within reference chemical effect were both necessarily constrained to be 0.

If the daily replicates were in control the portion main effect and the portion by replicate within reference chemical interaction would be non-significant. If the portion by replicate within reference chemical interaction was significant, the nature of the effect would be assessed by comparing the portion effect within each replicate within reference chemical to the portion effect averaged across replicates within reference chemical, adjusting for simultaneity by Bonferroni's method. Analysis of variance for Full Enzyme Activity Controls, Background Activity Controls, positive controls, and negative controls were completed for reference chemicals 1 through 4. Due to the nature of the analysis, poor Full Enzyme Activity Control values in reference chemical 4 reflected poorly in the values for reference chemicals 1, 2, and 3. In order to better describe the results of the successful replicates using reference chemicals 1, 2, and 3 the statistical analyses were repeated with only that data.

3.8 Data Retention

The Sponsor has title to all documentation records, raw data, specimens or other work product generated during the performance of the study. All work product generated by WIL Research Laboratories, LLC, including raw paper data and pertinent electronic storage media, are retained in the Archives at WIL Research Laboratories, LLC, as specified in the study protocol. Data generated by BioSTAT Consultants, Inc. are maintained in the Archives at WIL Research Laboratories, LLC. Data generated by the Sponsor reference chemical formulation data are maintained and archived as defined in the Sponsor's applicable standard operating procedures. Pertinent electronic storage media and the original final report are retained in the Archives at WIL Research Laboratories, LLC, in compliance with regulatory requirements.

4.0 Results

4.1 Radiochemical Purity

Purity Report: Appendix D

The radiochemical purity of the [^{3}H]ASDN was determined by RTI International to be 97%. The concentration and specific activity of the substrate was used to calculate the aromatase activity in the assay.

Assay Substrate Analysis Results					
Replicate	*Radiochemical Code*	*Radiochemical ID*	*Radiochemical Stock Concentration (µCi/g)*	*Assay Substrate Final Concentration (µg/g) (ASDN + ^{3}H ASDN)*	*Substrate Solution Specific Activity (dpm/nmol)*
Chrysin Rep 1	[^{3}H]-ASDN	MET-0251A	0.680	0.568	760803
Chrysin Rep 2	[^{3}H]-ASDN	MET-0251A	0.711	0.570	792701
Chrysin Rep 3	[^{3}H]-ASDN	MET-0251A	0.703	0.587	761105
Aminoglutethimide Rep 1	[^{3}H]-ASDN	MET-0251A	0.590	0.574	653396
Aminoglutethimide Rep 2	[^{3}H]-ASDN	MET-0251A	0.677	0.569	755709
Aminoglutethimide Rep 3	[^{3}H]-ASDN	MET-0251A	0.701	0.576	773276
Ketoconazole Rep 1	[^{3}H]-ASDN	MET-0251A	0.747	0.573	828933
Ketoconazole Rep 2	[^{3}H]-ASDN	MET-0251A	0.631	0.573	700748
Ketoconazole Rep 3	[^{3}H]-ASDN	MET-0251A	0.696	0.575	768497
Econazole Rep 1	[^{3}H]-ASDN	MET-0251A	0.695	0.582	759207
Econazole Rep 2	[^{3}H]-ASDN	MET-0251A	0.683	0.570	761611
Econazole Rep 3	[^{3}H]-ASDN	MET-0251A	0.918	0.572	1021330
Econazole Rep 4	[^{3}H]-ASDN	MET-0251A	0.670	0.575	740239
Econazole Rep 5	[^{3}H]-ASDN	MET-0251A	0.816	0.577	899707

4.2 Stock Formulation Analysis

Chemistry Report: Appendix E

Stock formulation analysis was performed by the sponsor, as reported in Appendix E. Briefly, solubility and formulation analyses showed that the stock formulations provided to the laboratories for this study were within the acceptance criteria for both average concentration and percent relative standard deviation between analyses. In addition, the formulations were found to be stable for the duration of their time of use.

Reference Chemical Stock Solution Results				
Reference Chemical Code	Reference Chemical ID	Target Concentration (mg/mL)	Measured Concentration (mg/mL)	Stock Solution Expiration Date
4-OH ASDN	MET-0264A	3.02	3.07	4/18/2005
Lindane	MET-0266A	29.08	29.9	4/18/2005
Chrysin	MET-0267A	2.54	2.47	5/5/2005
Aminoglutethimide	MET-0265A	23.2	22.9	3/24/2005
Ketoconazole	MET-0272A	5.31	5.13	4/5/2005
Econazole	MET-0271A	44.4	46.1	4/4/2005

4.3 Protein Analysis (Microsomes)

The protein concentration of each microsomal preparation was determined on the day that the microsomes were used in the assay. Variation in the handling of microsomes during dilution (*e.g.*, homogenization procedure, see Section 3.3) was likely the cause of the variability in the protein concentrations shown in the table below, as all the microsomes (except econazole Replicate 5) were from the same source.

Microsomal Protein Analysis Results		
Replicate	Assay Date	Protein stock concentration (mg/mL, measured)
Chrysin Rep 1	2/1/2005	13.244
Chrysin Rep 2	2/3/2005	4.352
Chrysin Rep 3	2/4/2005	7.087
Aminoglutethimide Rep 1	2/9/2005	5.793
Aminoglutethimide Rep 2	2/14/2005	12.509
Aminoglutethimide Rep 3	2/15/2005	9.934
Ketoconazole Rep 1	2/17/2005	12.585
Ketoconazole Rep 2	2/18/2005	12.834
Ketoconazole Rep 3	3/8/2005	10.026
Econazole Rep 1	2/23/2005	11.367
Econazole Rep 2	2/28/2005	12.471
Econazole Rep 3	3/1/2005	11.045
Econazole Rep 4	3/18/2005	8.174
Econazole Rep 5	4/1/2005	13.559

Quality Control standards for protein assay were included by protocol amendment between Ketoconazole Replicate 2 and replicate 3; no QC protein data was generated for Ketoconazole Replicates 1 or 2 or Aminoglutethimide Replicates 1, 2 or 3. Inter-laboratory differences in protein concentration could be a factor in varying aromatase activity among laboratories. The results from the determination of protein concentration for BSA standards (0.125, 0.5 to 1 mg/mL are summarized below. These results will be compared with that of the other participating laboratories by the Sponsor to determine protein assay reproducibility.

Quality Control Protein Analysis Results					
Replicate	Actual mg/mL BSA	Experimental mg/mL BSA		Mean	% Difference
		Tube1	Tube 2		
Ketoconazole Rep 3	0.125	0.053	0.059	0.056	-55.36
Econazole Rep 1	0.125	0.181	0.128	0.155	23.66
Econazole Rep 2	0.125	0.095	0.123	0.109	-12.69
Econazole Rep 3	0.125	0.115	0.115	0.115	-7.8
Econazole Rep 4	0.125	0.116	0.120	0.118	-5.73
Econazole Rep 5	0.125	0.115	0.119	0.117	-6.25

Quality Control Protein Analysis Results					
Replicate	Actual mg/mL BSA	Experimental mg/mL BSA		Mean	% Difference
		Tube1	Tube 2		
Ketoconazole Rep 3	0.5	0.556	0.548	0.552	10.37
Econazole Rep 1	0.5	0.581	0.595	0.588	17.58
Econazole Rep 2	0.5	0.590	0.598	0.594	18.85
Econazole Rep 3	0.5	0.569	0.586	0.578	15.51
Econazole Rep 4	0.5	0.576	0.595	0.586	17.13
Econazole Rep 5	0.5	0.581	0.578	0.580	15.97
Ketoconazole Rep 3	1	1.166	1.113	1.140	13.96
Econazole Rep 1	1	1.075	1.119	1.097	9.67
Econazole Rep 2	1	1.123	1.092	1.107	10.75
Econazole Rep3	1	1.119	1.141	1.130	13.01
Econazole Rep 4	1	1.120	1.169	1.144	14.43
Econazole Rep 5	1	1.066	1.114	1.090	9.02
OVERALL	Mean*	sd	sem	% CV	% Difference
0.125 mg/mL BSA	0.112	0.033	0.010	29.5	-10.69
0.5 mg/mL BSA	0.576	0.016	0.004	2.69	15.90
1 mg/mL BSA	1.118	0.031	0.009	2.79	11.81
* = mean of tubes					

4.4 *Control Aromatase Activity*

Figure 1a-d – Summary of Full Enzyme Activity Results
Figure 2a-d – Summary of Background Activity Results
Figure 3a-d – Summary of Positive Control Activity Results
Figure 4a-d – Summary of Negative Control Activity Results
Appendix F – Individual Replicate Spreadsheets

Full Enzyme Activity Controls were conducted in duplicate tubes at the beginning and end of each replicate. As shown in the table below, the percent coefficient of variation (%CV) within each replicate of reference chemical 1, 2 and 3 was less than 10%. Therefore, the intra-replicate enzymatic activity was consistent throughout the assay. By contrast, the inter-assay variability within a reference chemical was higher, *e.g.* the %CV for reference chemical 3 was 51.5%. Because there is no correlation between activity (calculated per mg protein) and the varying protein concentration previously discussed, this variability was likely due to the presence of inactive enzyme. Inactive enzyme contributed to the microsomal protein mass in the assay, but did not react with substrate. This is also indicated in reference chemical 3 by the low Full Enzyme Activity (0.018 nmol/mg protein/min).

In the case of reference chemical 4, both intra- and inter-assay variability was extensive, and the percent of control activity could not be accurately determined from these Full Enzyme Activity Results. For this report, Replicate 1 alone was used for estimation of inhibition by econazole.

Statistical evaluation of the Full Enzyme Activity was based on values normalized to a percent of Full Enzyme Activity Control. This data, summarized in the Statistician's Report in Appendix G, shows no portion effect on the Full Enzyme Activity for reference chemicals 1 -3.

Full Enzyme Activity Control Aromatase Activity - Chrysin						
Replicate	Beginning (nmol/mg protein/min)	End (nmol/mg protein/min)	Within Replicate Mean (±sd, sem)	% CV	Overall Mean (±sd, sem)	Overall % CV
1	0.069	0.072	0.071 (0.005, 0.002)	7.00	0.069 (0.005, 0.001)	7.0
2	0.071	0.066	0.068 (0.003, 0.001)	4.30		
3	0.073	0.061	0.067 (0.007, 0.003)	9.75		
Full Enzyme Activity Control Aromatase Activity - Aminoglutethimide						
Replicate	Beginning (nmol/mg protein/min)	End (nmol/mg protein/min)	Within Replicate Mean (±sd, sem)	% CV	Overall Mean (±sd, sem)	Overall % CV
1	0.070	0.066	0.068 (0.003, 0.001)	3.78	0.059 (0.016, 0.005)	27.7
2	0.075	0.069	0.072 (0.004, 0.002)	5.47		
3	0.038	0.037	0.037 (0.001 ,0.001)	3.64		
Full Enzyme Activity Control Aromatase Activity - Ketoconazole						
Replicate	Beginning (nmol/mg protein/min)	End (nmol/mg protein/min)	Within Replicate Mean (±sd, sem)	% CV	Overall Mean (±sd, sem)	Overall % CV
1	0.014	0.013	0.014 (0.001, 0.000)	4.56	0.018 (0.009, 0.003)	51.5
2	0.010	0.010	0.010 (0.000, 0.000)	4.79		
3	0.029	0.031	0.030 (0.003, 0.001)	9.42		

Full Enzyme Activity Control Aromatase Activity - Econazole						
Replicate	Beginning (nmol/mg protein/min)	End (nmol/mg protein/min)	Within Replicate Mean (±sd, sem)	% CV	Overall Mean (±sd, sem)	Overall % CV
1	0.027	0.030	0.028 (0.002, 0.001)	8.44	0.023 (0.019, 0.004)	80.9
2	0.016	0.054	0.035 (0.024, 0.012)	69.03		
3	0.019	0.051	0.035 (0.021, 0.010)	58.30		
4	0.000	0.006	0.003 (0.004, 0.002)	112.78		
5	0.003	0.027	0.015 (0.014, 0.007)	92.79		

Background Activity Controls were conducted in duplicate at the beginning and end of each assay. Figure 2a-d (Summary of Background Activity Results) presents the individual background aromatase activity values and the percent of control values from each replicate and a graphical representation of the data. The aromatase activity in these control samples was negligible, indicating that there was no background activity (potentially caused by nonspecific turnover of reactant to product, or unintentional NADPH contamination) that might interfere with the interpretation of the results. There were negligible differences between the beginning and end background aromatase activity values per replicate, indicating that the conditions were constant throughout each replicate test (see Appendix F, Individual Replicate Spreadsheets).

Positive and Negative controls were also included, split between the beginning and end of each assay. Positive controls contained the known aromatase inhibitor 4-OH ASDN. The 4-OH ASDN concentration used was expected to inhibit 50% of the enzyme activity (IC_{50}). Negative controls contained lindane, a chemical known for its inability to inhibit aromatase activity. Figures 3a-d and 4a-d illustrate the Positive and Negative control results, respectively, across all four reference chemicals. The Positive and Negative controls behaved as expected during chrysin, aminoglutethimide, and ketoconazole assay days. In contrast, the experiments completed for econazole reflect the variability in the Full Enzyme Activity controls previously discussed.

4.5 Reference Chemical Aromatase Activity

Figure 5a-d – Concentration Response Curves – Chrysin
Figure 6a-d – Concentration Response Curves – Aminoglutethimide
Figure 7a-d – Concentration Response Curves – Ketoconazole
Figure 8 – Concentration Response Curve - Econazole

Four reference chemicals were tested for aromatase inhibition at eight concentrations each. Increasing the concentration of the reference chemical increased aromatase inhibition in a dose responsive manner. Low intra-assay variability was characterized by a coefficient of variance of less than 11% across triplicate samples (tubes) at all concentrations and replicates, except at the highest inhibitor concentrations, which resulted in percent of control activities near 0%. Small deviations in small results can result in large errors, for example the first aminoglutethimide replicate had unusually high variability at the highest concentrations. For all the replicates, outliers were rejected with a 90% confidence level using the Q-test described by Dean and Dixon (1951). Decreasing concentrations of reference chemical resulted in decreased enzyme inhibition characterized by increased percent of control activity. This inhibition was characterized by a sigmoidal dose response (See Figures 5 through 9). Complete curves

(inhibition in the full range of approximately 0-100%) were obtained in all cases. The daily fluctuation in Full Enzyme Activity discussed in Section 4.4 did not affect the percent inhibition, or the shape or fit of the dose-response curve, because the data are reported as a percent of Full Enzyme Control Activity.

Reference Chemical Aromatase Activity – Percent of Full Enzyme Control Activity									
Reference chemical	Rep	Log [reference chemical]	Percent of Full Enzyme Control Activity			Overall			
			Tube 1	Tube 2	Tube 3	Mean	s.d.	s.e.m.	%CV
CHRYSIN									
1	1	-4.00	5.73	6.29	5.37	5.80	0.46	0.27	7.96
1	1	-5.00	27.92	28.74	28.06	28.24	0.44	0.26	1.57
1	1	-6.00	84.57	82.88	85.95	84.47	1.54	0.89	1.82
1	1	-7.00	105.94	107.22	106.01	106.39	0.72	0.41	0.68
1	1	-7.30	109.36	107.96	109.27	108.86	0.78	0.45	0.72
1	1	-8.00	112.84	113.53	109.12	111.83	2.37	1.37	2.12
1	1	-9.00	111.00	109.67	111.20	110.62	0.83	0.48	0.75
1	1	-10.00	106.86	107.42	109.43	107.90	1.35	0.78	1.25
1	2	-4.00	5.39	4.23	3.86	4.50	0.80	0.46	17.82
1	2	-5.00	24.85	27.05	25.73	25.88	1.11	0.64	4.28
1	2	-5.30	37.41	40.72	42.91	40.34	2.77	1.60	6.87
1	2	-5.60	58.05	57.39	56.16	57.20	0.96	0.55	1.68
1	2	-6.00	77.10	~~123.39*~~	76.56	76.83	0.38	0.27	0.49
1	2	-6.30	87.45	82.33	84.66	84.81	2.56	1.48	3.02
1	2	-7.00	96.92	92.89	95.14	94.98	2.02	1.17	2.13
1	2	-8.00	95.08	97.89	100.55	97.84	2.74	1.58	2.80
1	3	-4.00	37.45	6.30	3.24	15.67	18.93	10.93	120.84
1	3	-5.00	21.87	22.80	21.98	22.22	0.51	0.29	2.29
1	3	-5.30	37.37	35.13	~~80.50*~~	36.25	1.58	1.12	4.37
1	3	-5.60	61.05	50.47	53.38	54.96	5.47	3.16	9.94
1	3	-6.00	70.74	77.94	68.93	72.54	4.76	2.75	6.57
1	3	-6.30	102.03	125.04	113.84	113.64	11.50	6.64	10.12
1	3	-7.00	90.86	86.23	88.84	88.64	2.33	1.34	2.62
1	3	-8.00	91.08	89.36	90.11	90.18	0.86	0.50	0.96
AMINOGLUTETHIMIDE									
2	1	-3.00	-0.36	-0.66	-0.57	-0.53	0.15	0.09	-29.39
2	1	-4.00	4.09	2.87	2.93	3.30	0.69	0.40	20.95
2	1	-5.00	30.21	29.80	28.16	29.39	1.08	0.63	3.69
2	1	-6.00	80.66	76.40	78.93	78.66	2.14	1.24	2.72
2	1	-7.00	97.34	97.83	96.34	97.17	0.76	0.44	0.78
2	1	-8.00	99.14	97.24	99.85	98.74	1.35	0.78	1.37
2	1	-9.00	97.99	99.18	95.70	97.62	1.77	1.02	1.81
2	1	-10.00	95.93	98.24	99.63	97.93	1.87	1.08	1.91
2	2	-3.00	5.43	-0.05	3.10	2.83	2.75	1.59	97.24
2	2	-4.00	4.23	4.12	3.98	4.11	0.12	0.07	3.00
2	2	-5.00	29.58	28.57	29.88	29.34	0.69	0.40	2.35
2	2	-5.12	38.94	34.48	35.67	36.36	2.31	1.33	6.34
2	2	-5.60	63.51	60.22	60.43	61.39	1.84	1.06	3.00
2	2	-6.00	76.05	77.71	76.38	76.71	0.88	0.51	1.15
2	2	-7.00	89.03	91.67	95.96	92.22	3.50	2.02	3.79
2	2	-9.00	92.86	92.64	90.39	91.96	1.37	0.79	1.48
2	3	-3.00	0.72	0.35	0.20	0.42	0.27	0.16	63.77
2	3	-4.00	5.19	5.14	4.89	5.07	0.16	0.09	3.24

2	3	-5.00	32.95	32.92	33.42	33.10	0.28	0.16	0.86
2	3	-5.12	39.40	39.90	39.68	39.66	0.25	0.14	0.63
2	3	-5.60	65.00	67.24	65.80	66.01	1.13	0.65	1.72
2	3	-6.00	83.74	83.04	80.32	82.37	1.80	1.04	2.19
2	3	-7.00	97.62	96.42	96.42	96.82	0.70	0.40	0.72
2	3	-9.00	99.41	97.44	96.91	97.92	1.32	0.76	1.35
KETOCONAZOLE									
3	1	-4.00	~~83.48*~~	8.39	7.23	7.81	0.82	0.58	10.48
3	1	-5.00	47.50	46.58	48.63	47.57	1.02	0.59	2.15
3	1	-6.00	89.91	88.93	87.01	88.62	1.48	0.85	1.66
3	1	-7.00	97.67	97.64	97.49	97.60	0.09	0.05	0.09
3	1	-7.30	97.57	91.98	100.33	96.63	4.25	2.46	4.40
3	1	-8.00	102.49	104.80	98.65	101.98	3.11	1.79	3.05
3	1	-9.00	97.09	94.32	98.61	96.67	2.17	1.25	2.25
3	1	-10.00	92.36	96.64	96.37	95.12	2.39	1.38	2.52
3	2	-4.00	8.91	9.12	8.26	8.76	0.45	0.26	5.11
3	2	-4.40	23.80	22.39	22.80	23.00	0.72	0.42	3.15
3	2	-4.80	41.44	39.73	41.55	40.91	1.02	0.59	2.50
3	2	-5.19	63.17	61.88	63.54	62.86	0.87	0.50	1.39
3	2	-5.60	79.69	80.47	80.88	80.35	0.61	0.35	0.76
3	2	-6.00	90.14	85.28	88.71	88.04	2.50	1.44	2.84
3	2	-7.30	97.26	113.53	105.64	105.48	8.13	4.70	7.71
3	2	-9.00	92.94	97.92	94.72	95.19	2.52	1.46	2.65
3	3	-4.00	3.20	5.09	17.50	8.60	7.77	4.49	90.40
3	3	-4.40	13.73	13.03	13.46	13.41	0.35	0.20	2.61
3	3	-4.80	30.88	30.67	30.46	30.67	0.21	0.12	0.69
3	3	-5.19	51.60	49.88	49.70	50.39	1.05	0.60	2.08
3	3	-5.60	71.09	72.18	71.13	71.47	0.62	0.36	0.87
3	3	-6.00	84.65	83.42	82.82	83.63	0.93	0.54	1.11
3	3	-7.30	97.51	96.34	97.53	97.13	0.68	0.39	0.70
3	3	-9.00	99.58	100.86	98.95	99.79	0.97	0.56	0.97

* Outliers are identified by $(x_2 - x_1)/w > Q_{critical}$. $Q_{critical}$ = 0.94 with n=3. (See Section 3.7)

The following table illustrates the inter-assay variability between aromatase inhibition effected by the same chemical. Like the intra-assay variability, the coefficient of variation in percent of control between replicates was less at lower reference chemical concentrations. The average %CV for all reference chemicals at concentrations less than 1 x 10^{-5}M was 7.2%. Inter-assay results are shown for chrysin, aminoglutethimide, and ketoconazole in Figures 5d, 6d, and 7d, respectively.

Mean Reference Chemical Aromatase Activity – Percent of Control								
Reference Chemical	Log [reference Chemical]	Mean Percent of Control			Overall			
		Rep 1	Rep 2	Rep 3	Mean	s.d.	s.e.m.	% CV
Chrysin	-4.00	5.80	4.50	15.67	8.65	6.11	3.53	70.59
	-5.00	28.24	25.88	22.22	25.44	3.03	1.75	11.92
	-5.30	*	40.34	36.25	38.30	2.89	2.05	7.55
	-5.60	*	57.20	54.96	56.08	1.58	1.12	2.82
	-6.00	84.47	76.83	72.54	77.95	6.04	3.49	7.75
	-6.30	*	84.81	113.64	99.22	20.38	14.41	20.54
	-7.00	106.39	94.98	88.64	96.67	8.99	5.19	9.30
	-7.30	108.86	*	*	108.86	*	*	*

	-8.00	111.83	97.84	90.18	99.95	10.98	6.34	10.98
	-9.00	110.62	*	*	110.62	*	*	*
	-10.00	107.90	*	*	107.90	*	*	*
Aminoglutethimide	-3.00	-0.53	2.83	0.42	0.91	1.73	1.00	190.45
	-4.00	3.30	4.11	5.07	4.16	0.89	0.51	21.38
	-5.00	29.39	29.34	33.10	30.61	2.15	1.24	7.04
	-5.12	*	36.36	39.66	38.01	2.33	1.65	6.13
	-5.60	*	61.39	66.01	63.70	3.27	2.31	5.14
	-6.00	78.66	76.71	82.37	79.25	2.87	1.66	3.62
	-7.00	97.17	92.22	96.82	95.40	2.76	1.59	2.89
	-8.00	98.74	*	*	98.74	*	*	*
	-9.00	97.62	91.96	97.92	95.84	3.36	1.94	3.50
	-10.00	97.93	*	*	97.93	*	*	*
Ketoconazole	-4.00	7.81	8.76	8.60	8.39	0.51	0.29	6.06
	-4.40	*	23.00	13.41	18.20	6.78	4.79	37.25
	-4.80	*	40.91	30.67	35.79	7.24	5.12	20.23
	-5.00	47.57	*	*	47.57	*	*	*
	-5.19	*	62.86	50.39	56.63	8.82	6.23	15.57
	-5.60	*	80.35	71.47	75.91	6.28	4.44	8.27
	-6.00	88.62	88.04	83.63	86.76	2.73	1.57	3.14
	-7.00	97.60	*	*	97.60	*	*	*
	-7.30	96.63	105.48	97.13	99.74	4.97	2.87	4.99
	-8.00	101.98	*	*	101.98	*	*	*
	-9.00	96.67	95.19	99.79	97.22	2.35	1.36	2.42
	-10.00	95.12	*	*	95.12	*	*	*

* Empty cells result from change in reference chemical concentration following examination of the results of the first replicate. (See Section 3.2)

4.6 IC_{50} and Slope Determination

The dose-response equation presented in Section 3.7 was fitted to the percent of Full Enzyme Activity Control data versus varying reference chemical concentration plots discussed in Section 4.5. The individual curves fit to the data from each of the three replicates resulted in values for each reference chemical of μ (log IC_{50}) and β (Hill Slope).The results for all four reference chemicals are summarized in the table below. Arithmetic calculation of the mean IC_{50} resulted in values of 0.0020 μM (econazole, estimated from replicate 1) to 8.672 μM (ketoconazole). The inter-assay variability (%CV) of the arithmetic IC_{50} determination varied according to reference chemical from 13.3 – 25.0%. There was not sufficient data for the determination of an average IC_{50} from econazole.

IC_{50} and Slope Results									
Reference chemical	Repl	Log [IC_{50}]	s.e. log[IC_{50}]	IC_{50} (μM)	Slope	s.e. slope	Status*	Mean IC_{50} (μM) (±sd, sem)	Overall %CV
CHRYSIN									
1	1	-5.369	0.022	4.276	-0.9651	0.0315	Complete	3.534 (0.661, 0.382)	18.7%
	2	-5.479	0.011	3.319	-0.9219	0.0195	Complete		
	3	-5.522	0.063	3.006	-0.9308	0.114	Complete		
AMINOGLUTETHIMIDE									
2	1	-5.400	0.017	3.981	-1.011	0.023	Complete	4.277 (0.568, 0.328)	13.3%
	2	-5.407	0.031	3.917	-0.9202	0.047	Complete		
	3	-5.307	0.009	4.932	-0.9984	0.015	Complete		

KETOCONAZOLE									
3	1	-5.055	0.022	8.810	-1.001	0.037	Complete	8.672 (2.165, 1.250)	25.0%
	2	-4.968	0.014	10.765	-0.9947	0.025	Complete		
	3	-5.191	0.032	6.442	-1.009	0.049	Complete		
ECONAZOLE									
4	1	-8.711	0.037	0.0020	-0.9756	0.044	Complete	0.0020	-

*Status = codes as described in the statistical analysis section that describe the curve fit that led to the IC_{50} calculation.

4.7 Statistical Analysis

Figure 9a-c – Average Aromatase Inhibition
Figure 10a-c – Response Curve Summary Table – Hill Slope (β_{avg})
Figure 11a-c – Response Curve Summary Table – Log IC_{50}
Figure 12a-d – Three-factor Mixed Effects Analysis of Variance Across Chemicals

The Log IC_{50} (μ) and slope (β) were subjected to random effects analysis of variance to determine the within replicate standard error (see table in Section 4.6, IC_{50} and Slope Determination). Log IC_{50} and slope were also compared across replicates based on random effects analysis of variance using μ_{AVG} and β_{AVG}. From the statistical analysis described in Section 3.7, the overall IC_{50} based on μ_{AVG} and β_{AVG} was between 3.57 and 8.53 µM for reference chemicals 1-3 (see table below, and Figures 9a-c, average aromatase inhibition curves). The β (slope) and 95% confidence interval is also plotted per replicate and across replicates in Figures 10a-c. The Log IC_{50} (μ) and 95% confidence interval is plotted per replicate and across replicates (*e.g.* per chemical) for each reference chemical in Figures 11a-c.

Reference Chemical	μ_{avg}	IC_{50} (µM)	β_{avg}
Chrysin	-5.448	3.57	-0.936
Aminoglutethimide	-5.368	4.29	-0.997
Ketoconazole	-5.069	8.53	-0.998

The Full Enzyme Activity and Background Activity Control values among replicates were analyzed by three-factor mixed effects analysis of variance, as described in Section 3.7, with the percent of control aromatase activity as the response variable. Replicate by portion (beginning and end) interactions were not significant for chrysin, aminoglutethimide, or ketoconazole. Probabilities for replicate, portion, and replicate by portion are presented in Appendix G (Statistical Analysis Report). In addition, estimates for the LSMeans and 95% Confidence intervals are presented for percent of Full Enzyme Activity Control aromatase activity across replicates.

5.0 Discussion

The results of the validation study indicated that human placental microsomes respond in a predictable manner to the reference chemicals applied, and that the microsomal experiment can be repeated within and between laboratories with acceptable reproducibility in many cases. The response of the human placental microsomes to three of the four reference chemicals were in accord with the reported inhibition constants from the prevalidation study plan and study protocol for the Aromatase Assay (WA 2-5, Task 12). Generally treating the microsomes with 10^{3} to 10^{-10} M inhibitor resulted in a complete sigmoidal dose response curve ranging from no inhibition to full inhibition of aromatase enzyme activity. This illustrates the responsiveness of the placental aromatase assay for screening reference chemicals for their ability to inhibit placental aromatase activity as an indication of the chemicals' potential to disrupt endocrine function *in vivo*. Quantitatively, the IC_{50} for the reference chemicals in this report were within or near the

reported values in the pre-study review. For aminoglutethimide, the IC_{50} of 4.3 µM was within the reported range of 1 to 6 µM. Chrysin IC_{50} was 3.6 µM, which is between the reported values of 0.1 to 10 µM. Ketoconazole experiments had greater variability between replicates, and also had an IC_{50} that was lower than reported (8.7 µM versus 65.0 µM). The least successful test of aromatase inhibition occurred with econazole. However, the result of the single successful replicate (IC_{50} = 2 nM), did reflect the potent inhibition reported in the review (IC_{50} = 30 - 50 nM).

The Full Enzyme and Background Enzyme Activity Controls included in the assays demonstrated that the intra-assay conditions were constant throughout each successful replicate test and that there were no artifacts that occurred over the time course of the assay. In addition, there was no Background Activity resulting from non-enzymatic reaction of the substrate that may interfere with the interpretation of the results. Variability in the replicate to replicate Full Enzyme Activity did not cause a portion effect or change the inhibition curve for chrysin, aminoglutethimide, or ketoconazole. Reference chemical 4 (econazole) had a significant portion effect, primarily in the Full Enzyme Activity Controls, that prevented data processing of all but the first replicate. The negative control, lindane, did not affect aromatase activity. As expected, the positive control, 4-OH ASDN, was a potent inhibitor of aromatase activity.

6.0 Conclusion

The response of the human placental aromatase to known aromatase inhibitors was successfully validated in this laboratory, although the econazole evaluation was limited to one replicate due to problems with the control assay data. No definitive cause could be found for the anomalous response of the assay in the presence of econazole. Additional testing of this placental microsomal assay for aromatase inhibition using ten reference chemicals will be evaluated under WA 4-16, Task 7, WIL-431009, including additional testing of econazole.

7.0 References

Bowman, C.J. Validation of the Placental Aromatase Assay: Positive Control Study (WA 4-16, Task 4). (Study No. WIL-431006). WIL Research Laboratories, LLC, Ashland, OH, Draft

Pre-Optimization Experiments for Substrate Characterization for Human Recombinant and Human Placental Microsomes (WA 2-24). Battelle Memorial Institute, Columbus,OH, 2003

Pre-Validation Study Plan and Study Protocol for the Aromatase Assay Using Human, Bovine, and Porcine Placenta, and Human Recombinant Microsomes (WA 2-5, Task 12) Battelle Memorial Institute, Columbus, OH, 2002

Final Detailed Review Paper on Aromatase, (WA 2-7 and 5-5, Task 2), Battelle Memorial Institute, Columbus, OH, 2005

Microsomal Aromatase Prevalidation Study Reports (WA 4-10), Battelle Memorial Institute, Columbus, OH, 2004.

Dean, R. B. and Dixon, W. J. Simplified Statistics for Small Numbers of Observations. Analytical Chemistry v.23 no.4, 636-638 (1951)

8.0 Deviations From The Protocol

This study was conducted in accordance with the protocol and protocol amendments, except for the following:

- **Section 7** During the Aromatase Assay for reference chemical 1 (MET-0267A) on study day 2, the test tube containing Background Activity Control replicate 2 post sample sequence was inadvertently spilled. As a result, only a 0.3 mL of the aliquots of the aqueous layer were transferred into LSC vial number 155, not the 0.5 mL required in the protocol. No aliquot of the aqueous layer was placed into the duplicate vial (number 156). This vial (number 156) only contained 10 mL of liquid scintillation cocktail.

- **Section 7** During the Aromatase Assay for reference chemical 1 (MET-0267A) on study day 1, 100 µL of NADPH was inadvertently not placed into the test tubes containing negative control, pre and post sample sequence, replicates 1 and 2.

- **Section 5.2** During the Aromatase Assay for reference chemical 1 (MET-0267A) between replicates 1 and 2, the total amount of placental microsomes (MET-0255A) remaining were pooled, homogenized and re-aliquoted into smaller volumes at the request of the sponsor (followed up in Protocol Amendment I). The smaller aliquots were not sufficient to recover the quantity of protein required due to coating of the protein on the walls of the homogenizer, but the requisite 90 µL could still not be recovered. Therefore, the procedure used for replicates 2 and 3 was to add 90 µL of the microsomes to the homogenizer, then quantitively transfer the homogenized microsomes to the dilution vial, using the diluent as the transfer agent. However, upon quantification of the protein in solution by DC Protein Assay, the actual concentration was approximately ½ that required by the protocol. Because the activity of the protein present was consistent with the activity seen on previous days, it was determined that the enzyme concentration

was not critical and the replicates did not need to be repeated at this time. All of the above activities were either requested by or approved by the sponsor.

These deviations did not negatively impact the quality or integrity of the data nor the outcome of the study.

9.0 Key Personnel and Report Submission

Report Submitted By:

Jennifer A. Thomas-Wohlever, Ph.D. Research Scientist, Metabolism Study Director	Date

Report Reviewed By:

Terry L. Johnson, Ph.D. Associate Director, Metabolism	Date
Mark D. Nemec, B.S., D.A.B.T. Director, Developmental and Reproductive Toxicology	Date
Daniel W. Sved, Ph.D. Director, Metabolism and Analytical Chemistry	Date
Christopher J. Bowman, Ph.D. Staff Toxicologist, Developmental and Reproductive Toxicology	Date
Robert A. Wally, B.S., RAC Acting Manager, Reporting and Regulatory Technical Services	Date

FIGURES 1a–12d

Figure 1a. Chrysin - Summary of Full Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0732	103.7			0.0705	103.3			0.0728	108.7				
	0.0649	92.0	0.0690	97.8	0.0710	104.0	0.0707	103.6	0.0723	108.1	0.0725	108.4	0.0708	103.2
End	0.0682	96.7			0.0651	95.4			0.0609	91.1				
	0.0759	107.6	0.0721	102.2	0.0664	97.3	0.0658	96.4	0.0616	92.1	0.0613	91.6	0.0664	96.8
	Average % Activity:	100				100				100				

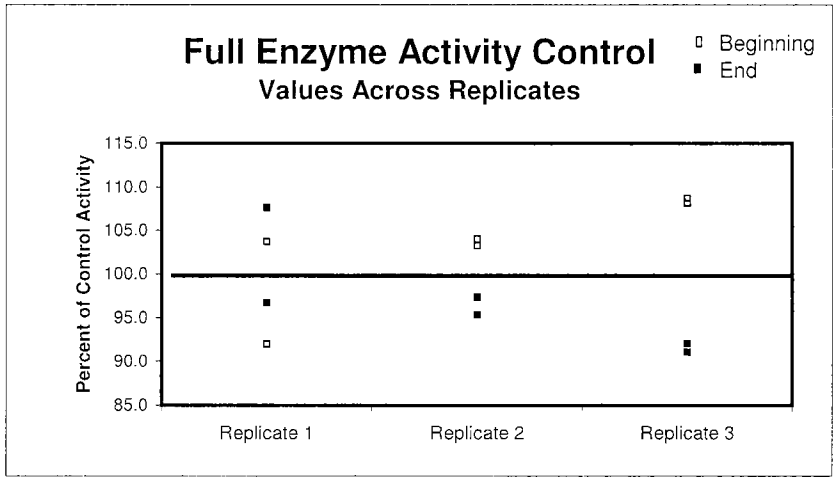


Figure 1b. Aminoglutethimide - Summary of Full Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0716	105.5			0.0765	106.0			0.0362	96.9				
	0.0676	99.5	0.0696	102.5	0.0743	103.0	0.0754	104.5	0.0393	105.3	0.0378	101.1	0.0609	103.0
End	0.0661	97.4			0.0697	96.7			0.0369	98.7				
	0.0663	97.6	0.0662	97.5	0.0680	94.3	0.0688	95.5	0.0371	99.2	0.0370	98.9	0.0573	97.0
	Average % Activity:	100				100				100				

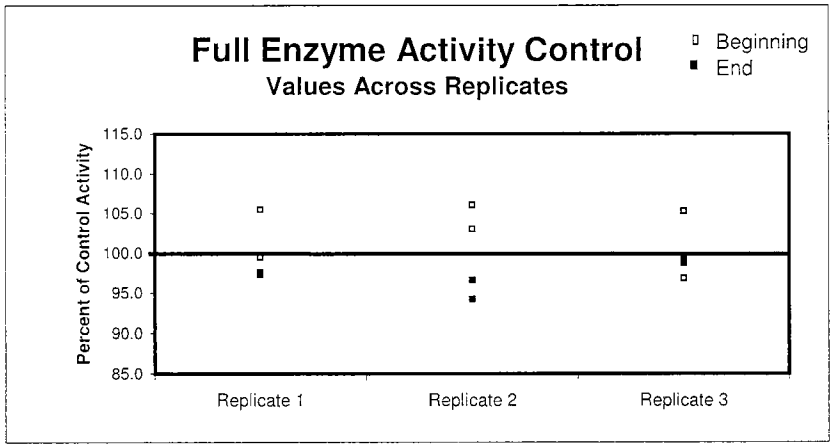


Figure 1c. Ketoconazole - Summary of Full Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0138	100.4			0.0107	104.2			0.0270	88.7				
	0.0146	106.3	0.0142	103.3	0.0102	99.1	0.0105	101.7	0.0319	104.7	0.0295	96.7	0.0180	99.3
End	0.0132	96.4			0.0106	103.1			0.0336	110.2				
	0.0133	96.9	0.0132	96.7	0.0096	93.6	0.0101	98.3	0.0294	96.4	0.0315	103.3	0.0183	100.7
	Average % Activity:	100				100				100				

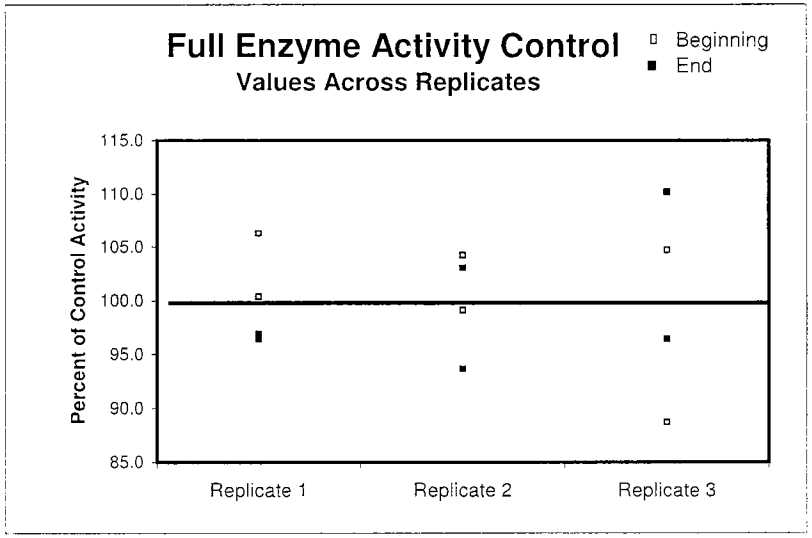


Figure 1d. Econazole - Summary of Full Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across 5 Replicates	Portion Percent of Control Across 5 Replicates
Begin	0.0286	101.8			0.0041	11.7			0.0294	83.5				
	0.0246	87.6	0.0266	94.7	0.0282	80.3	0.0161	46.0	0.0087	24.6	0.0191	54.0	0.0130	55.9
End	0.0294	104.6			0.0501	142.5			0.0523	148.4				
	0.0297	106.0	0.0296	105.3	0.0582	165.6	0.0541	154.0	0.0506	143.5	0.0515	146.0	0.0336	144.1
	Average % Activity:	100				100				100				

	Replicate 4				Replicate 5			
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control
Begin	0.0001	3.6			0.0011	7.7		
	0.0006	16.9	0.0003	10.3	0.0050	33.7	0.0031	20.7
End	0.0045	135.6			0.0285	192.1		
	0.0081	243.8	0.0063	189.7	0.0247	166.5	0.0266	179.3
	Average % Activity:	100				100		

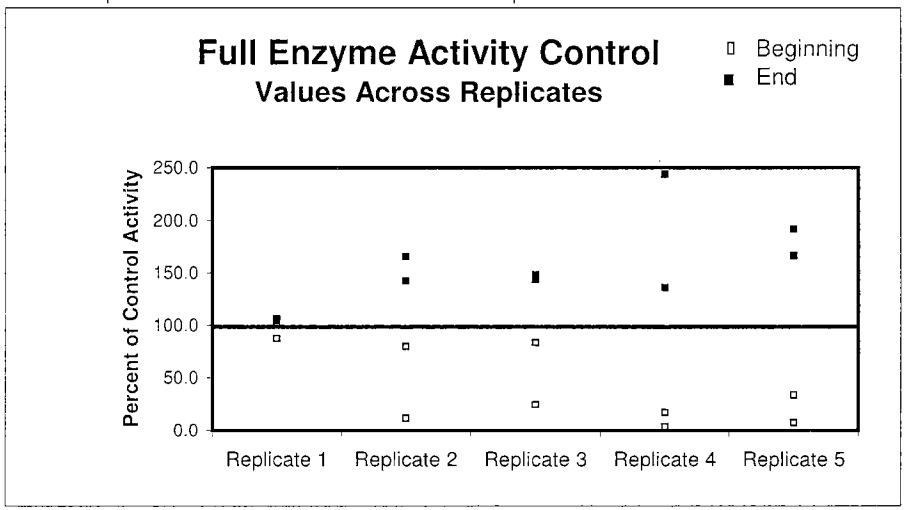


Figure 2a. Chrysin - Summary of Background Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	-4.40E-05	-0.062			-1.40E-04	-0.204			9.22E-04	1.378				
	1.23E-04	0.174	3.95E-05	0.056	1.24E-04	0.182	-7.67E-06	-0.011	-5.27E-04	-0.788	1.97E-04	0.295	7.64E-05	0.111
End	-1.29E-05	-0.018			-6.74E-05	-0.099			-1.14E-04	-0.170				
	-6.61E-05	-0.094	-3.95E-05	-0.056	8.28E-05	0.121	7.67E-06	0.011	-2.81E-04	-0.420	-1.97E-04	-0.295	-7.64E-05	-0.111
	Sum of Background Activity:	0				4.6E-16				0				

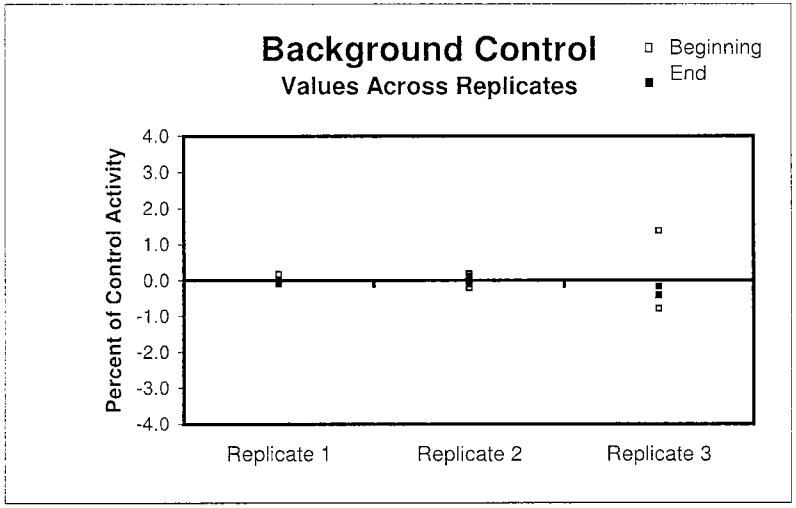


Figure 2b. Aminoglutethimide - Summary of Background Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	-4.69E-04	-0.690			-3.35E-04	-0.465			-8.95E-06	-0.024				
	2.30E-03	3.389	9.16E-04	1.349	8.45E-04	1.171	2.55E-04	0.353	-1.15E-05	-0.031	-1.02E-05	-0.027	3.87E-04	0.654
End	-7.91E-04	-1.165			-3.19E-04	-0.443			1.73E-05	0.046				
	-1.04E-03	-1.534	-9.16E-04	-1.349	-1.90E-04	-0.264	-2.55E-04	-0.353	3.14E-06	0.008	1.02E-05	0.027	-3.87E-04	-0.654
	Sum of Background Activity:	0				0				-2.5E-16				

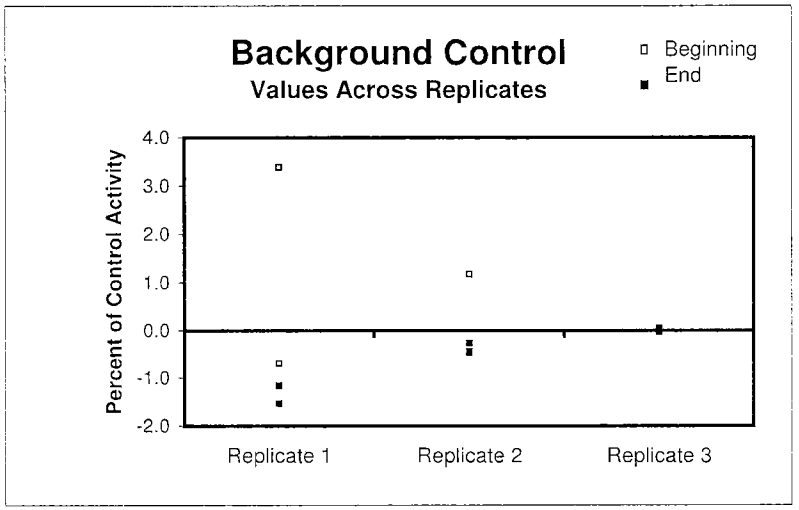


Figure 2c. Ketoconazole - Summary of Background Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	8.96E-05	0.654			1.45E-05	0.141			-1.66E-04	-0.545				
	8.71E-05	0.635	8.83E-05	0.645	-1.11E-05	-0.108	1.71E-06	0.017	-2.22E-04	-0.727	-1.94E-04	-0.636	-3.46E-05	-0.190
End	-6.29E-05	-0.459			2.20E-05	0.214			1.90E-04	0.624				
	-1.14E-04	-0.830	-8.83E-05	-0.645	-2.54E-05	-0.247	-1.71E-06	-0.017	1.97E-04	0.647	1.94E-04	0.636	3.46E-05	0.190
	Sum of Background Activity:	0				4.72E-16				0				

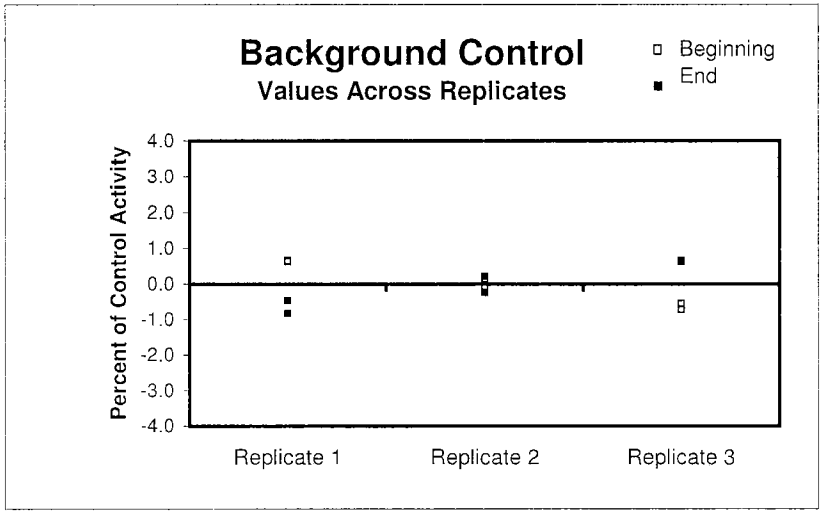


Figure 2d. Econazole - Summary of Background Enzyme Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across 5 Replicates	Portion Percent of Control Across 5 Replicates
Begin	-7.24E-05	-0.258			-4.21E-05	-0.120			1.71E-05	0.049				
	4.34E-05	0.155	-1.45E-05	-0.052	-1.44E-05	-0.041	-2.83E-05	-0.080	1.10E-05	0.031	1.41E-05	0.040	-1.28E-05	-0.055
End	4.99E-05	0.178			2.20E-05	0.062			-2.85E-05	-0.081				
	-2.09E-05	-0.074	1.45E-05	0.052	3.46E-05	0.098	2.83E-05	0.080	3.81E-07	0.001	-1.41E-05	-0.040	1.28E-05	0.055
	Sum of Background Activity:	-3.33E-16				-2.78E-16				4.03E-16				

	Replicate 4				Replicate 5			
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control
Begin	6.59E-05	1.981			3.01E-05	0.203		
	-1.26E-04	-3.776	-2.99E-05	-0.898	-4.05E-05	-0.273	-5.21E-06	-0.035
End	-2.54E-05	-0.762			-1.60E-05	-0.108		
	8.51E-05	2.557	2.99E-05	0.898	2.64E-05	0.178	5.21E-06	0.035
	Sum of Background Activity:	0				0		

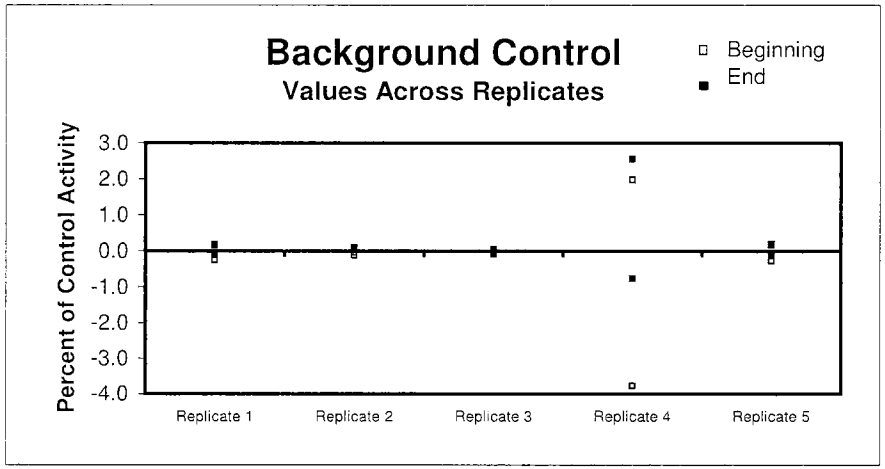


Figure 3a. Chrysin - Summary of Positive Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0296	41.9			0.0492	72.1			0.0334	50.0				
	0.0288	40.9	0.0292	41.4	0.0304	44.5	0.0398	58.3	0.0593	88.6	0.0463	69.3	0.0384	56.1
End	0.0338	47.9			0.0296	43.3			0.0287	42.8				
	0.0319	45.2	0.03288	46.6	0.0278	40.8	0.0287	42.0	0.0273	40.8	0.0280	41.8	0.0299	43.5
	Average of Positive Activity:	44.0				50.2				55.5				

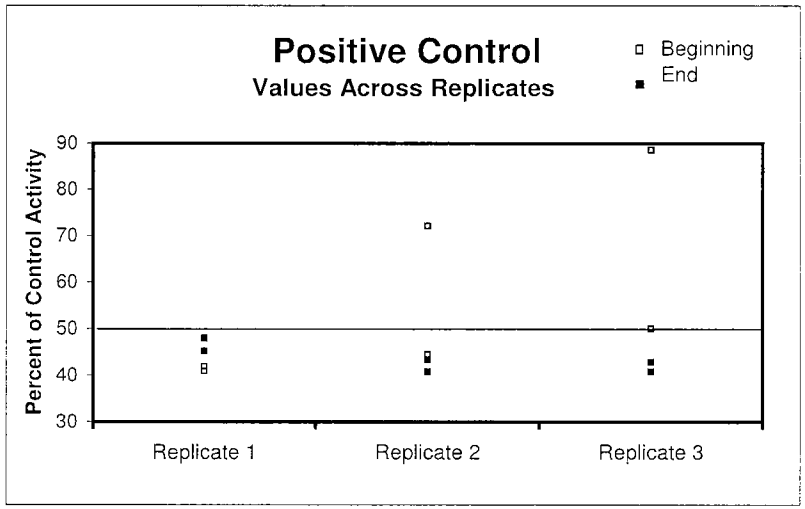


Figure 3b. Aminoglutethimide - Summary of Positive Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0360	53.1			0.0495	68.6			0.0178	47.6				
	0.0295	43.5	0.0328	48.3	0.0369	51.2	0.043204	59.9	0.0178	47.6	0.017804	47.6	0.0313	52.9
End	0.0295	43.4			0.0316	43.8			0.0163	43.7				
	0.0291	42.8	0.0293	43.1	0.0310	42.9	0.031281	43.4	0.0164	43.8	0.016364	43.8	0.0256	43.4
Average of Positive Activity:		45.7				51.6				45.7				

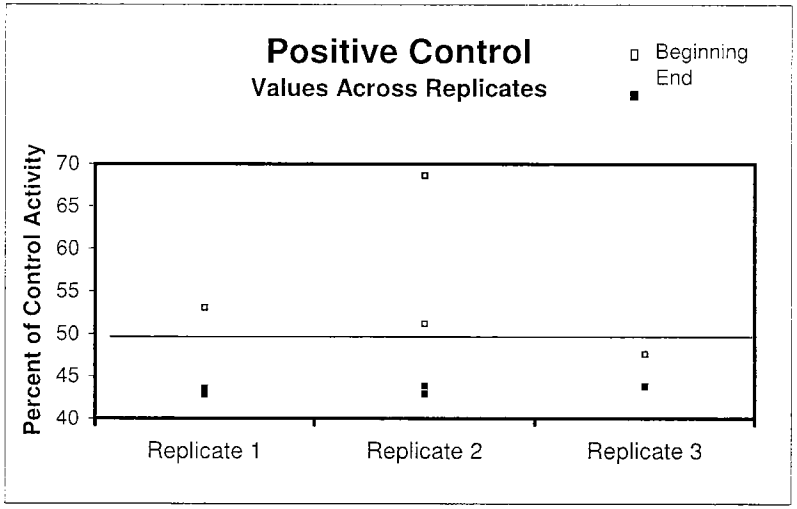


Figure 3c. Ketoconazole - Summary of Positive Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0075	54.7			0.0058	56.4			0.0123	40.2				
	0.0068	49.6	0.0071	52.1	0.0059	57.2	0.0058	56.8	0.0126	41.2	0.0124	40.7	0.0085	46.6
End	0.0064	46.7			0.0055	53.7			0.0115	37.6				
	0.0065	47.2	0.0064	47.0	0.0053	51.4	0.0054	52.6	0.0106	34.8	0.0110	36.2	0.0076	42.0
	Average of Positive Activity:	49.5				54.7				38.4				

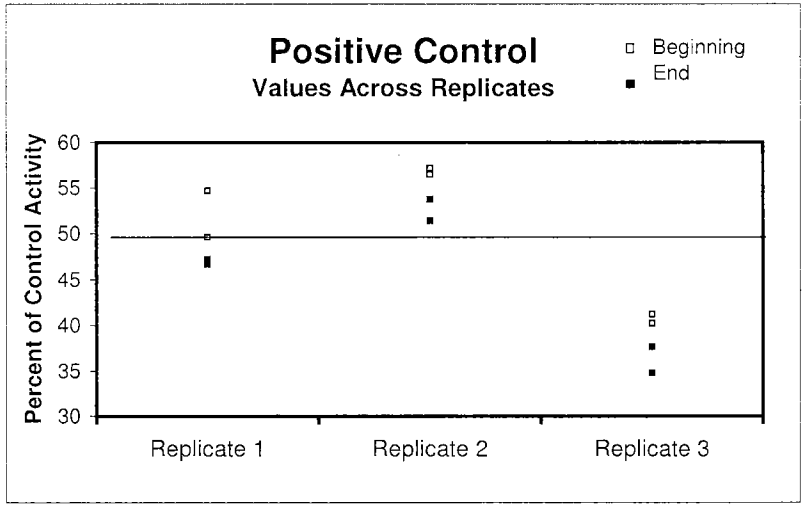


Figure 3d. Econazole - Summary of Positive Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across 5 Replicates	Portion Percent of Control Across 5 Replicates
Begin	0.0142	50.5			0.0311	88.6			0.0246	69.7				
	0.0149	53.1	0.0145	51.8	0.0242	68.8	0.0276	78.7	0.0257	72.9	0.025	71.3	0.0175	74.9
End	0.0133	47.5			0.0279	79.5			0.0205	58.1				
	0.0137	48.7	0.0135	48.1	0.0286	81.4	0.0283	80.5	0.0220	62.4	0.021	60.2	0.0178	76.3
	Average of Positive Activity:	49.9				79.6				65.8				

	Replicate 4				Replicate 5			
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control
Begin	0.0031	92.2			0.0161	108.5		
	0.0029	87.5	0.0030	89.8	0.0180	121.1	0.0170	114.8
End	0.0043	129.9			0.0210	141.7		
	0.0023	70.3	0.0033	100.1	0.0242	162.9	0.0226	152.3
	Average of Positive Activity:	95.0				133.5		

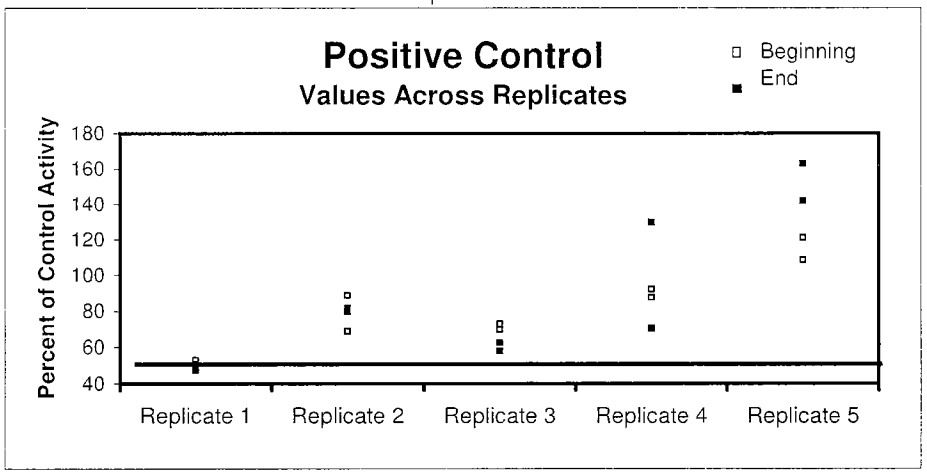


Figure 4a. Chrysin - Summary of Negative Control Activity Results

		Replicate 1			Replicate 2				Replicate 3				OVERALL	
		Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	no data					0.0695	101.8			0.0704	105.2				
	no data					0.0725	106.2	0.0710	104.0	0.0863	128.9	0.0783	117.1	0.0747	108.9
End	no data					0.0608	89.1			0.0597	89.2				
	no data					0.0625	91.6	0.0617	90.3	0.0574	85.8	0.0585	87.5	0.0601	87.6
	Average of Negative Activity:						97.2				102.3				

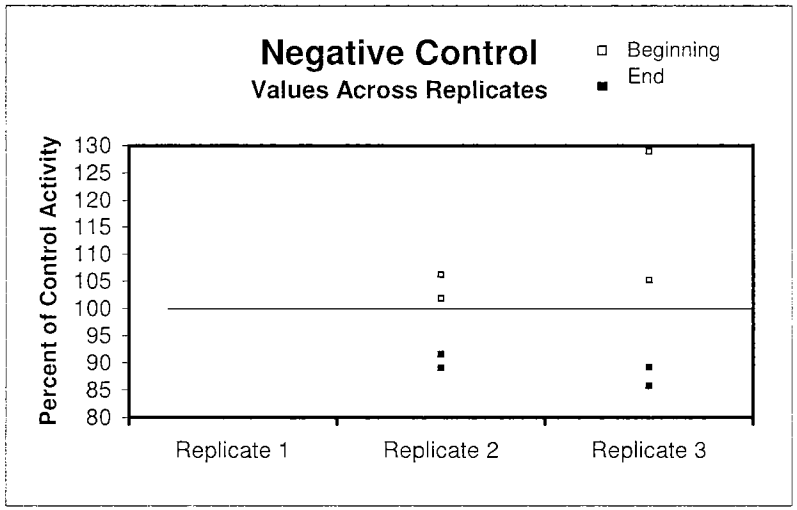


Figure 4b. Aminoglutethimide - Summary of Negative Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0683	100.6			0.0720	99.9			0.0388	103.9				
	0.0673	99.2	0.0678	99.9	0.0745	103.3	0.0733	101.6	0.0386	103.4	0.038747	103.7	0.0599	101.4
End	0.0621	91.5			0.0647	89.7			0.0353	94.6				
	0.0607	89.5	0.0614	90.5	0.0664	92.1	0.0655	90.9	0.0350	93.7	0.035181	94.1	0.0541	91.4
	Average of Negative Activity:	95.2				96.2				98.9				

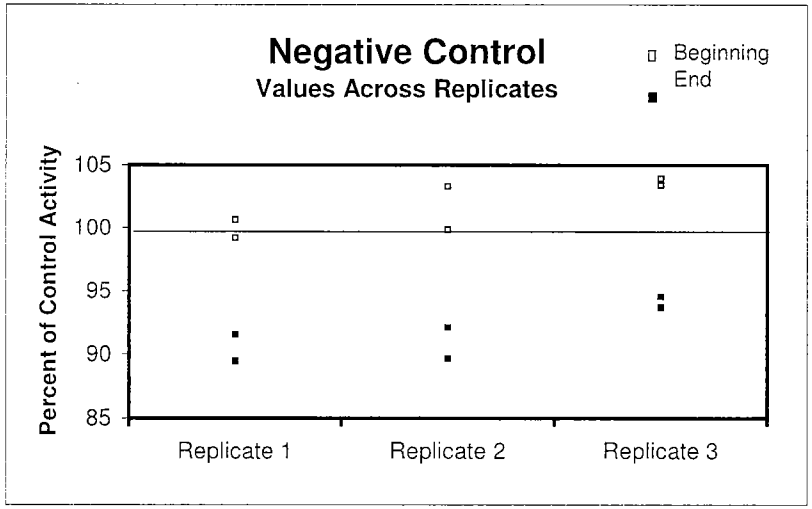


Figure 4c. Ketoconazole - Summary of Negative Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across Replicates	Portion Percent of Control Across Replicates
Begin	0.0136	99.4			0.0099	96.4			0.0289	94.9				
	0.0137	100.3	0.0137	99.9	0.0105	102.0	0.0102	99.2	0.0306	100.3	0.0298	97.6	0.0179	98.5
End	0.0126	92.0			0.0098	95.3			0.0285	93.5				
	0.0121	88.4	0.0124	90.2	0.0095	92.5	0.0097	93.9	0.0281	92.2	0.0283	92.9	0.0168	92.4
	Average of Negative Activity:	95.1				96.5				95.2				

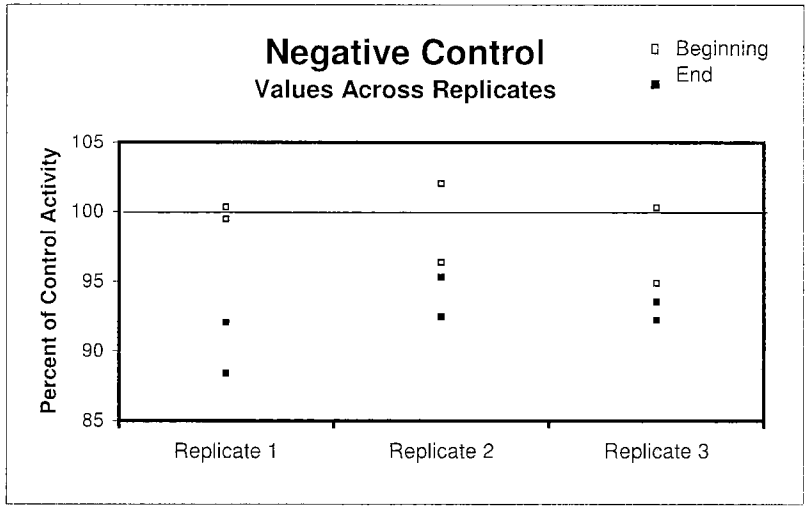


Figure 4d. Econazole - Summary of Negative Control Activity Results

	Replicate 1				Replicate 2				Replicate 3				OVERALL	
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Portion Mean Across 5 Replicates	Portion Percent of Control Across 5 Replicates
Begin	0.0299	106.5			0.0729	207.6			0.0616	174.7				
	0.0302	107.7	0.0301	107.1	0.0778	221.5	0.0754	214.5	0.0548	155.5	0.0582	165.1	0.0463	198.6
End	0.0269	96.0			0.0711	202.5			0.0590	167.3				
	0.0276	98.4	0.0273	97.2	0.0668	190.3	0.0690	196.4	0.0574	162.9	0.0582	165.1	0.0443	190.0
	Average of Negative Activity:	102.1				205.5				165.1				

	Replicate 4				Replicate 5			
	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control	Aromatase activity (nmol estrogen formed/mg protein/min)	% of Control	Portion Mean	Portion % of Control
Begin	0.0056	168.8			0.0595	401.0		
	0.0066	197.3	0.0061	183.0	0.0641	432.4	0.0618	416.7
End	0.0081	244.8			0.0603	406.7		
	0.0020	60.0	0.0051	152.4	0.0637	429.1	0.0620	417.9
	Average of Negative Activity:	167.7				417.3		

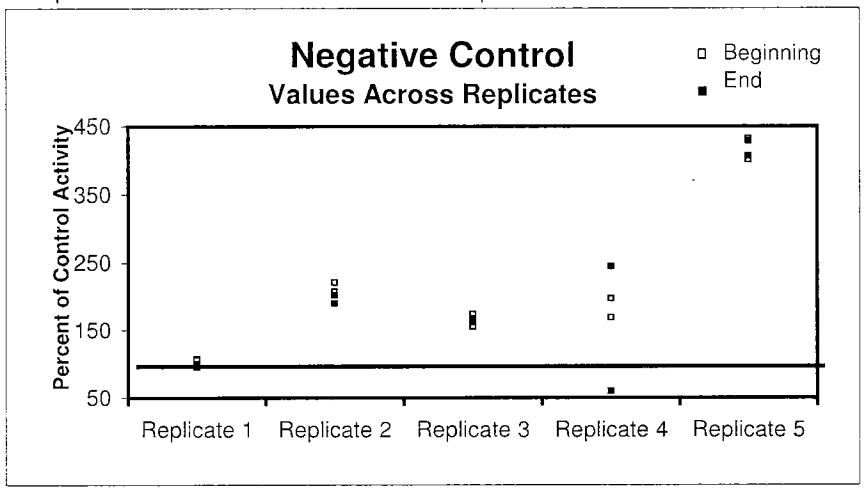
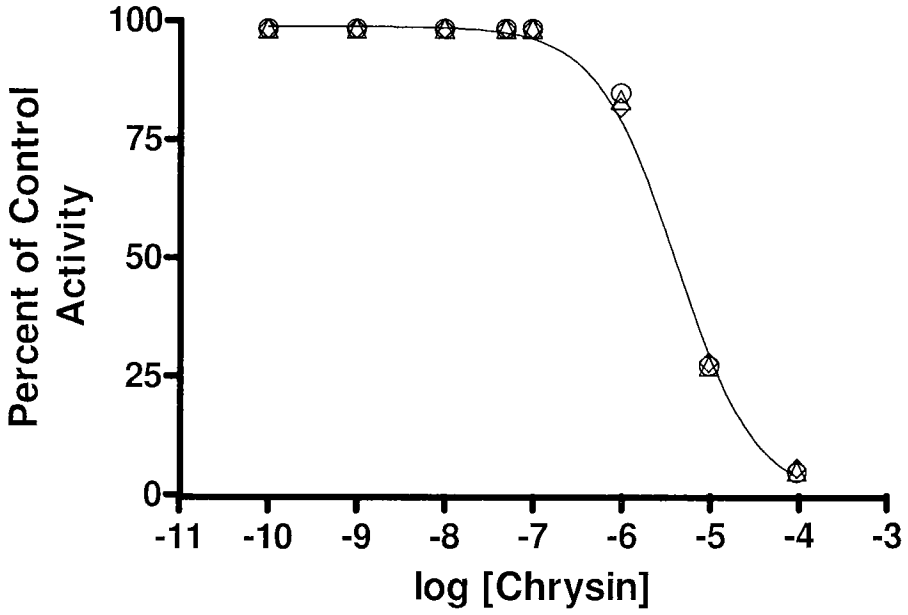


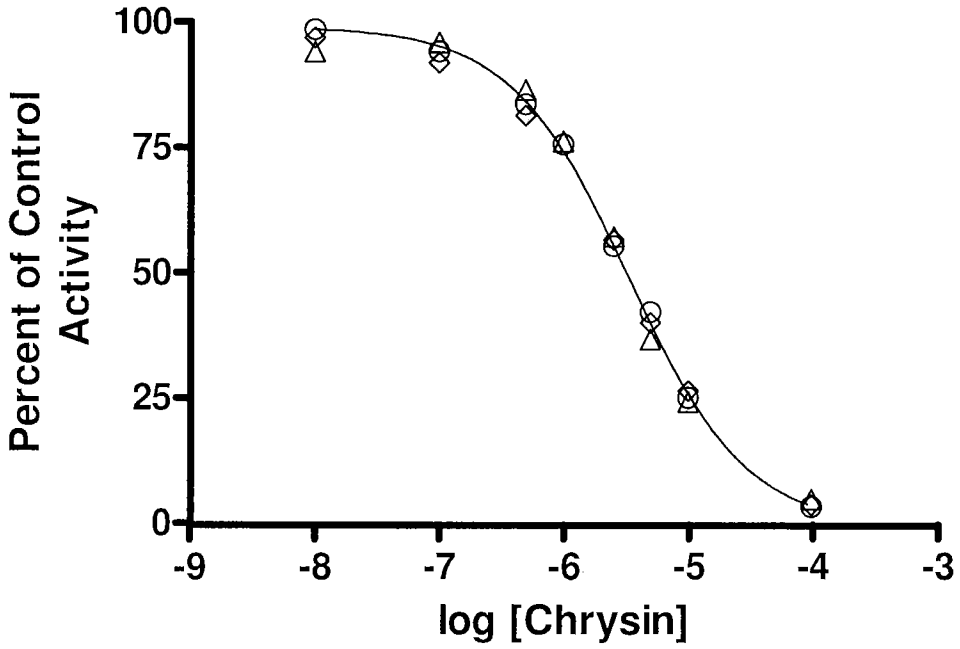
Figure 5a.
Chrysin Replicate 1



	Replicate 1
aromatase eqn	
Best-fit values	
LOGEC50	-5.369
HILLSLOPE	-0.9651
Std. Error	
LOGEC50	0.02155
HILLSLOPE	0.03148
95% Confidence Intervals	
LOGEC50	-5.414 to -5.324
HILLSLOPE	-1.030 to -0.8998
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9968
Weighted Sum of Squares (1/Y)	2.289
Absolute Sum of Squares	96.61
Sy.x	2.096
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

log [Chrysin]	Percent of Control Activity		
	Y1	Y2	Y3
-4.00	5.73	6.29	5.37
-5.00	27.92	28.74	28.06
-6.00	84.57	82.88	85.95
-7.00	99.50	99.50	99.50
-7.30	99.50	99.50	99.50
-8.00	99.50	99.50	99.50
-9.00	99.50	99.50	99.50
-10.00	99.50	99.50	99.50

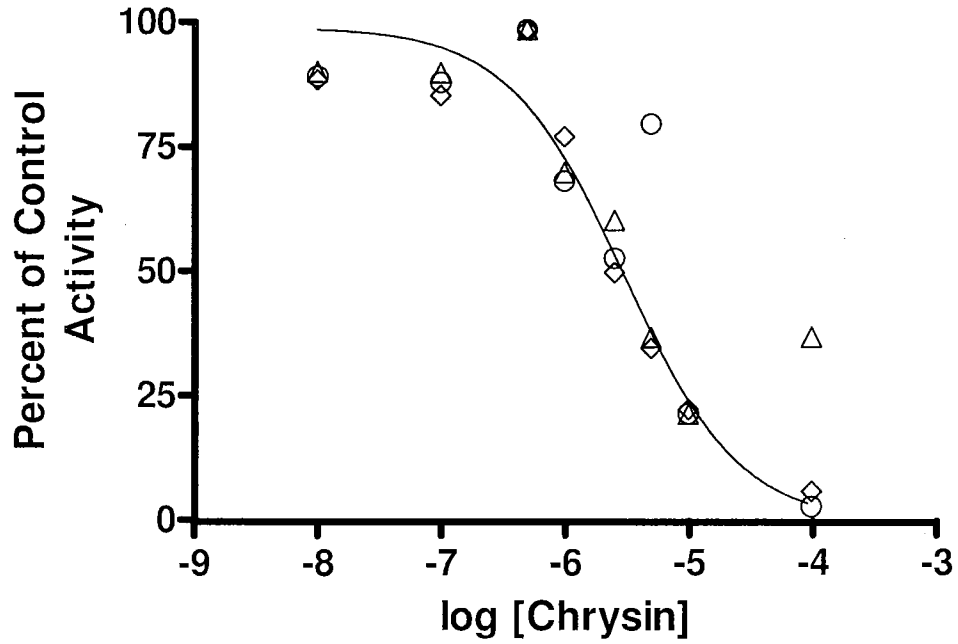
Figure 5b.
Chrysin Replicate 2



	Replicate 2
aromatase eqn	
Best-fit values	
LOGEC50	-5.479
HILLSLOPE	-0.9219
Std. Error	
LOGEC50	0.01148
HILLSLOPE	0.01947
95% Confidence Intervals	
LOGEC50	-5.503 to -5.456
HILLSLOPE	-0.9624 to -0.8814
Goodness of Fit	
Degrees of Freedom	21
R^2 (unweighted)	0.9967
Weighted Sum of Squares (1/Y)	1.531
Absolute Sum of Squares	79.22
Sy.x	1.942
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	23
Number of missing values	1

log [Chrysin]	Percent of Control Activity		
	Y1	Y2	Y3
-4.00	5.39	4.23	3.86
-5.00	24.85	27.05	25.73
-5.30	37.41	40.72	42.91
-5.60	58.05	57.39	56.16
-6.00	77.10	~~-123.39~~	76.56
-6.30	87.45	82.33	84.66
-7.00	96.92	92.89	95.14
-8.00	95.08	97.89	99.50

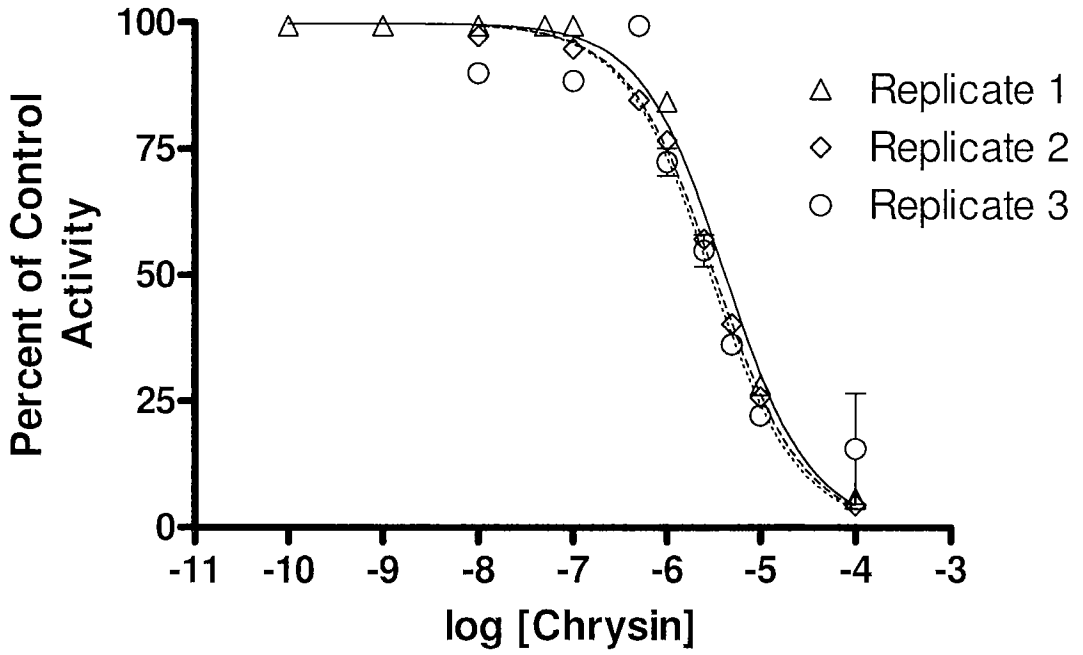
Figure 5c.
Chrysin Replicate 3



	Replicate 3
aromatase eqn	
Best-fit values	
LOGEC50	-5.522
HILLSLOPE	-0.9308
Std. Error	
LOGEC50	0.06295
HILLSLOPE	0.1140
95% Confidence Intervals	
LOGEC50	-5.653 to -5.391
HILLSLOPE	-1.168 to -0.6938
Goodness of Fit	
Degrees of Freedom	21
R^2 (unweighted)	0.8923
Weighted Sum of Squares (1/Y)	46.43
Absolute Sum of Squares	2429
Sy.x	10.75
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	23
Number of missing values	1

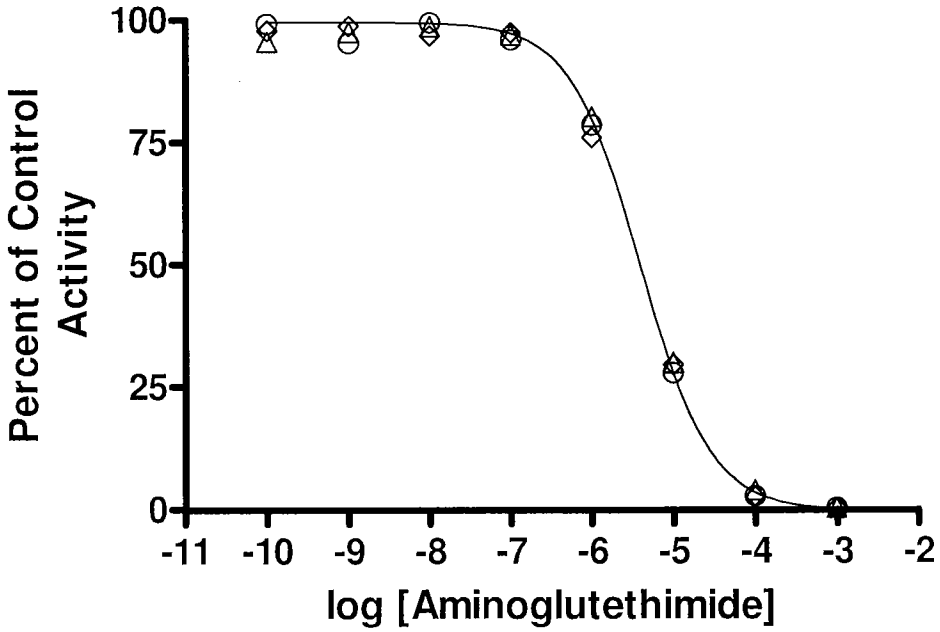
log [Chrysin]	Percent of Control Activity		
	Y1	Y2	Y3
-4.00	37.45	6.30	3.24
-5.00	21.87	22.80	21.98
-5.30	37.37	35.13	~~80.50~~
-5.60	61.05	50.47	53.38
-6.00	70.74	77.94	68.93
-6.30	99.50	99.50	99.50
-7.00	90.86	86.23	88.84
-8.00	91.08	89.36	90.11

Figure 5d.
Aromatase Inhibition by Chrysin



	Replicate 1	Replicate 2	Replicate 3
Best-fit values			
LOGEC50	-5.369	-5.479	-5.522
HILLSLOPE	-0.9651	-0.9219	-0.9308
Std. Error			
LOGEC50	0.02155	0.01148	0.06295
HILLSLOPE	0.03148	0.01947	0.1140
95% Confidence Intervals			
LOGEC50	-5.414 to -5.324	-5.503 to -5.456	-5.653 to -5.391
HILLSLOPE	-1.030 to -0.8998	-0.9624 to -0.8814	-1.168 to -0.6938
Goodness of Fit			
Degrees of Freedom	22	21	21
R^2 (unweighted)	0.9968	0.9967	0.8923
Weighted Sum of Squares (1/Y)	2.289	1.531	46.43
Absolute Sum of Squares	96.61	79.22	2429
Sy.x	2.096	1.942	10.75
Data			
Number of X values	8	16	16
Number of Y replicates	3	3	3
Total number of values	24	23	23
Number of missing values	0	25	25

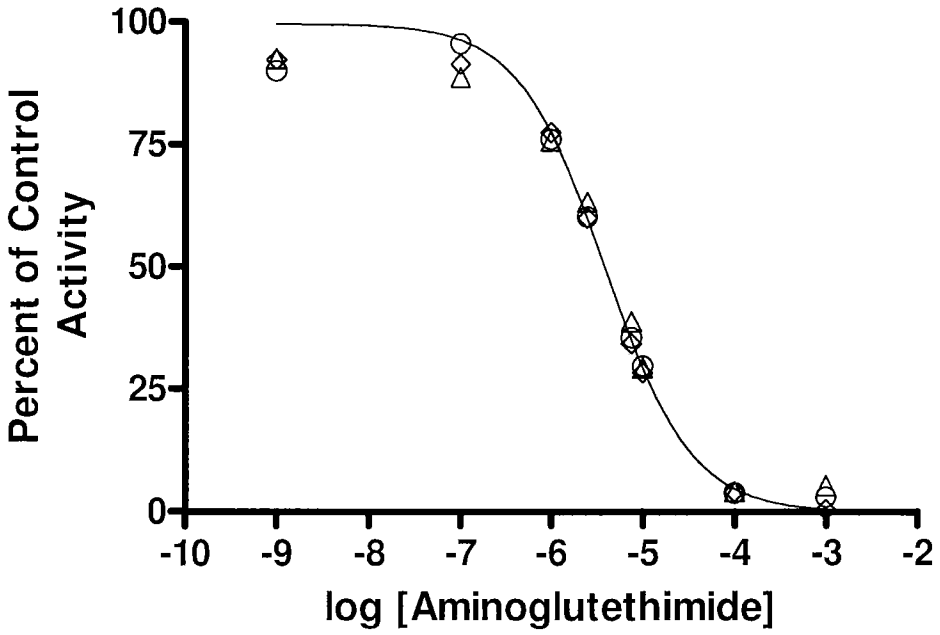
Figure 6a.
Aminoglutethimide Replicate 1



	Replicate 1
aromatase eqn	
Best-fit values	
LOGEC50	-5.400
HILLSLOPE	-1.011
Std. Error	
LOGEC50	0.01737
HILLSLOPE	0.02272
95% Confidence Intervals	
LOGEC50	-5.436 to -5.364
HILLSLOPE	-1.059 to -0.9643
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9982
Weighted Sum of Squares (1/Y)	1.520
Absolute Sum of Squares	74.96
Sy.x	1.846
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

log [Aminoglutethimide]	Percent of Control Activity		
	Y1	Y2	Y3
-3.00	0.50	0.50	0.50
-4.00	4.09	2.87	2.93
-5.00	30.21	29.80	28.16
-6.00	80.66	76.40	78.93
-7.00	97.34	97.83	96.34
-8.00	99.14	97.24	99.85
-9.00	97.99	99.18	95.70
-10.00	95.93	98.24	99.63

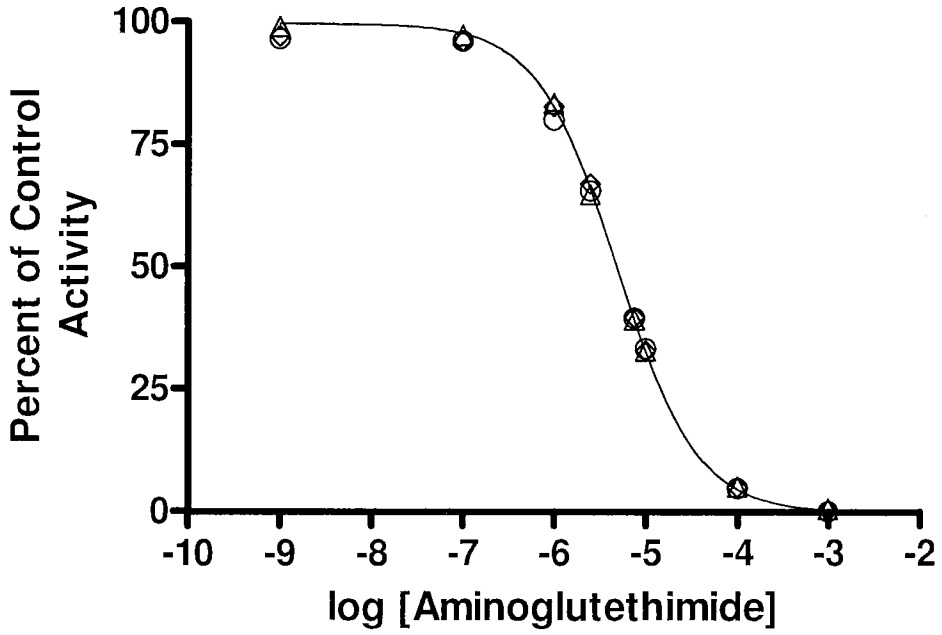
Figure 6b.
Aminoglutethimide Replicate 2



	Replicate 2
aromatase eqn	
Best-fit values	
LOGEC50	-5.407
HILLSLOPE	-0.9202
Std. Error	
LOGEC50	0.03082
HILLSLOPE	0.04691
95% Confidence Intervals	
LOGEC50	-5.471 to -5.343
HILLSLOPE	-1.018 to -0.8230
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9877
Weighted Sum of Squares (1/Y)	10.45
Absolute Sum of Squares	343.7
Sy.x	3.953
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

log [Aminoglutethimide]	Percent of Control Activity		
	Y1	Y2	Y3
-3.00	5.43	0.50	3.10
-4.00	4.23	4.12	3.98
-5.00	29.58	28.57	29.88
-5.12	38.94	34.48	35.67
-5.60	63.51	60.22	60.43
-6.00	76.05	77.71	76.38
-7.00	89.03	91.67	95.96
-9.00	92.86	92.64	90.39

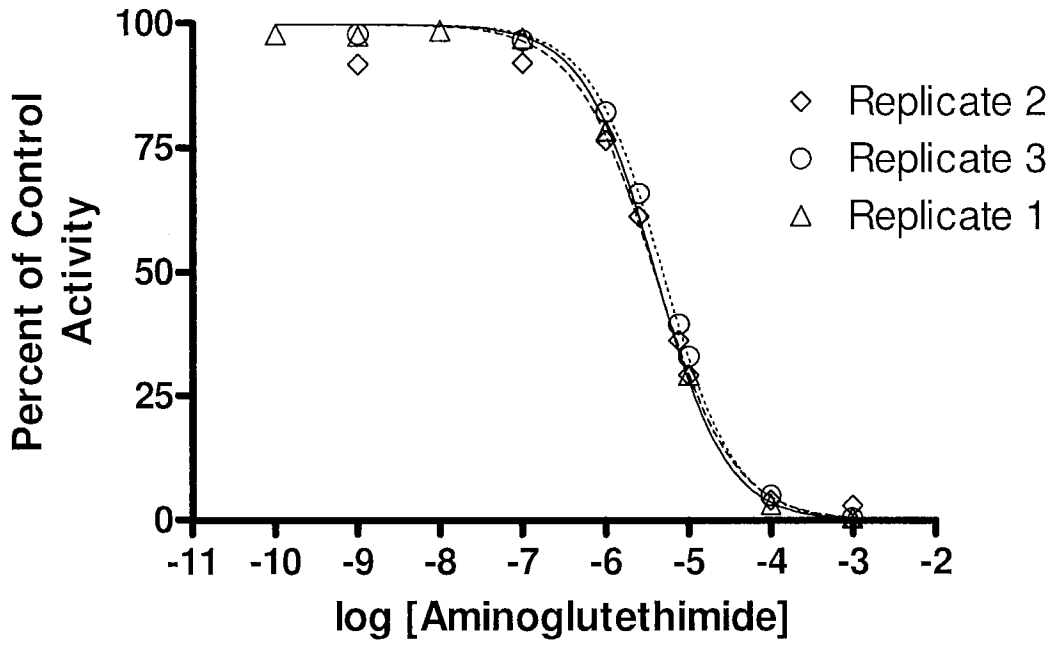
Figure 6c.
Aminoglutethimide Replicate 3



	Replicate 3
aromatase eqn	
Best-fit values	
LOGEC50	-5.307
HILLSLOPE	-0.9984
Std. Error	
LOGEC50	0.008930
HILLSLOPE	0.01546
95% Confidence Intervals	
LOGEC50	-5.326 to -5.289
HILLSLOPE	-1.030 to -0.9663
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9990
Weighted Sum of Squares (1/Y)	1.023
Absolute Sum of Squares	33.26
Sy.x	1.229
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

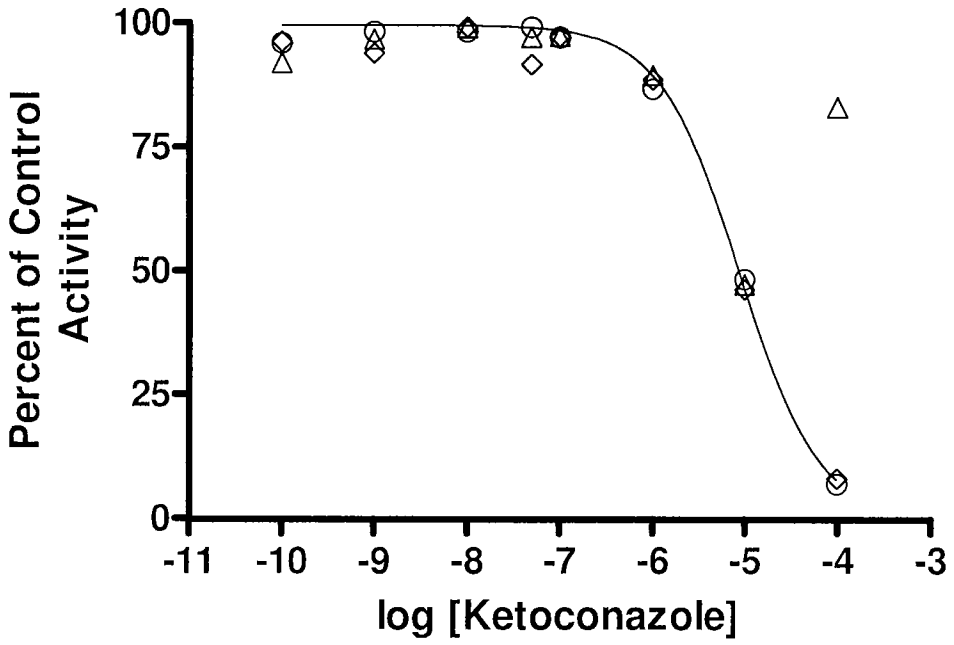
log [Aminoglutethimide]	Percent of Control Activity		
	Y1	Y2	Y3
-3.00	0.72	0.35	0.20
-4.00	5.19	5.14	4.89
-5.00	32.95	32.92	33.42
-5.12	39.40	39.90	39.68
-5.60	65.00	67.24	65.80
-6.00	83.74	83.04	80.32
-7.00	97.62	96.42	96.42
-9.00	99.41	97.44	96.91

Figure 6d. Aromatase Inhibition by Aminoglutethimide



	Replicate 1	Replicate 2	Replicate 3
aromatase eqn			
Best-fit values			
LOGEC50	-5.400	-5.407	-5.307
HILLSLOPE	-1.011	-0.9202	-0.9984
Std. Error			
LOGEC50	0.01737	0.03082	0.008930
HILLSLOPE	0.02272	0.04691	0.01546
95% Confidence Intervals			
LOGEC50	-5.436 to -5.364	-5.471 to -5.343	-5.326 to -5.289
HILLSLOPE	-1.059 to -0.9643	-1.018 to -0.8230	-1.030 to -0.9663
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9982	0.9877	0.9990
Weighted Sum of Squares (1/Y)	1.520	10.45	1.023
Absolute Sum of Squares	74.96	343.7	33.26
Sy.x	1.846	3.953	1.229
Data			
Number of X values	8	16	16
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	0	24	24

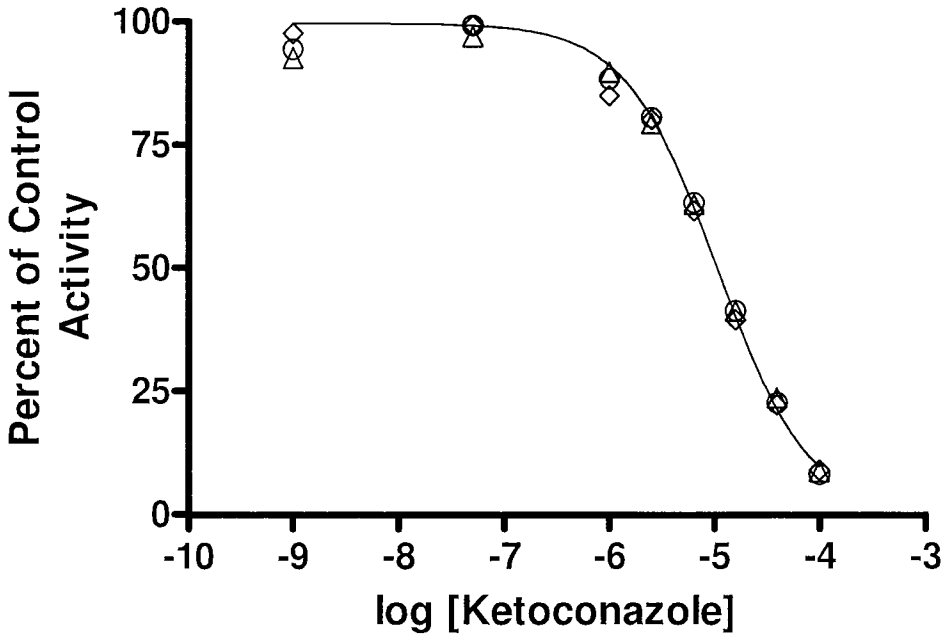
Figure 7a.
Ketoconazole Replicate 1



	Replicate 1
aromatase eqn	
Best-fit values	
LOGEC50	-5.055
HILLSLOPE	-1.001
Std. Error	
LOGEC50	0.02151
HILLSLOPE	0.03737
95% Confidence Intervals	
LOGEC50	-5.099 to -5.010
HILLSLOPE	-1.079 to -0.9233
Goodness of Fit	
Degrees of Freedom	21
R^2 (unweighted)	0.9887
Weighted Sum of Squares (1/Y)	2.330
Absolute Sum of Squares	204.4
Sy.x	3.120
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	23
Number of missing values	1

log [Ketoconazole]]	Percent of Control Activity		
	Y1	Y2	Y3
-4.00	~~83.48~~	8.39	7.23
-5.00	47.50	46.58	48.63
-6.00	89.91	88.93	87.01
-7.00	97.67	97.64	97.49
-7.30	97.57	91.98	99.50
-8.00	99.50	99.50	98.65
-9.00	97.09	94.32	98.61
-10.00	92.36	96.64	96.37

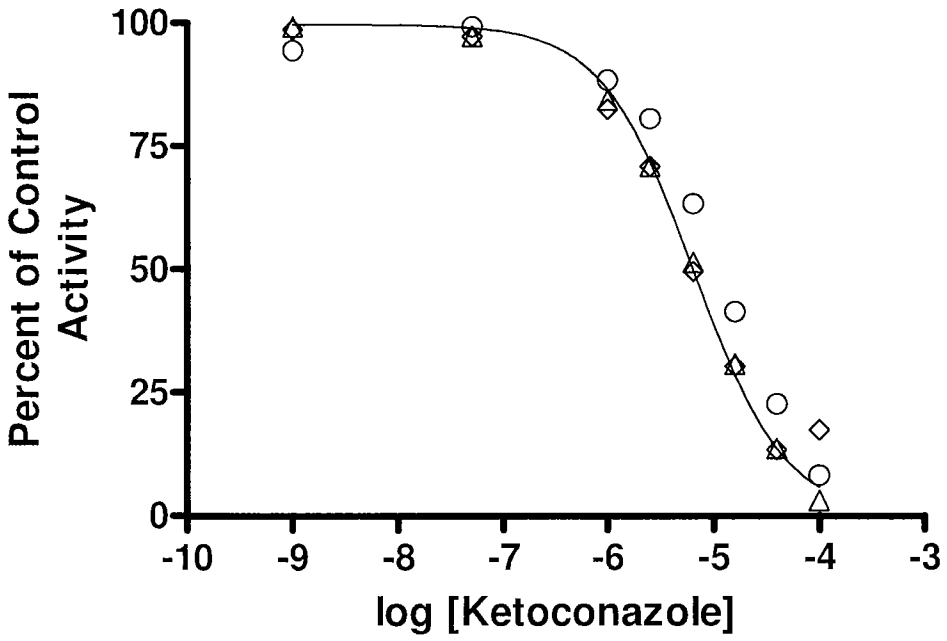
Figure 7b.
Ketoconazole Replicate 2



	Replicate 2
aromatase eqn	
Best-fit values	
LOGEC50	-4.968
HILLSLOPE	-0.9947
Std. Error	
LOGEC50	0.01390
HILLSLOPE	0.02458
95% Confidence Intervals	
LOGEC50	-4.997 to -4.939
HILLSLOPE	-1.046 to -0.9437
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9939
Weighted Sum of Squares (1/Y)	2.397
Absolute Sum of Squares	152.1
Sy.x	2.630
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

log [Ketoconazole]	Percent of Control Activity		
	Y1	Y2	Y3
-4.00	8.91	9.12	8.26
-4.40	23.80	22.39	22.80
-4.80	41.44	39.73	41.55
-5.19	63.17	61.88	63.54
-5.60	79.69	80.47	80.88
-6.00	90.14	85.28	88.71
-7.30	97.26	99.50	99.50
-9.00	92.94	97.92	94.72

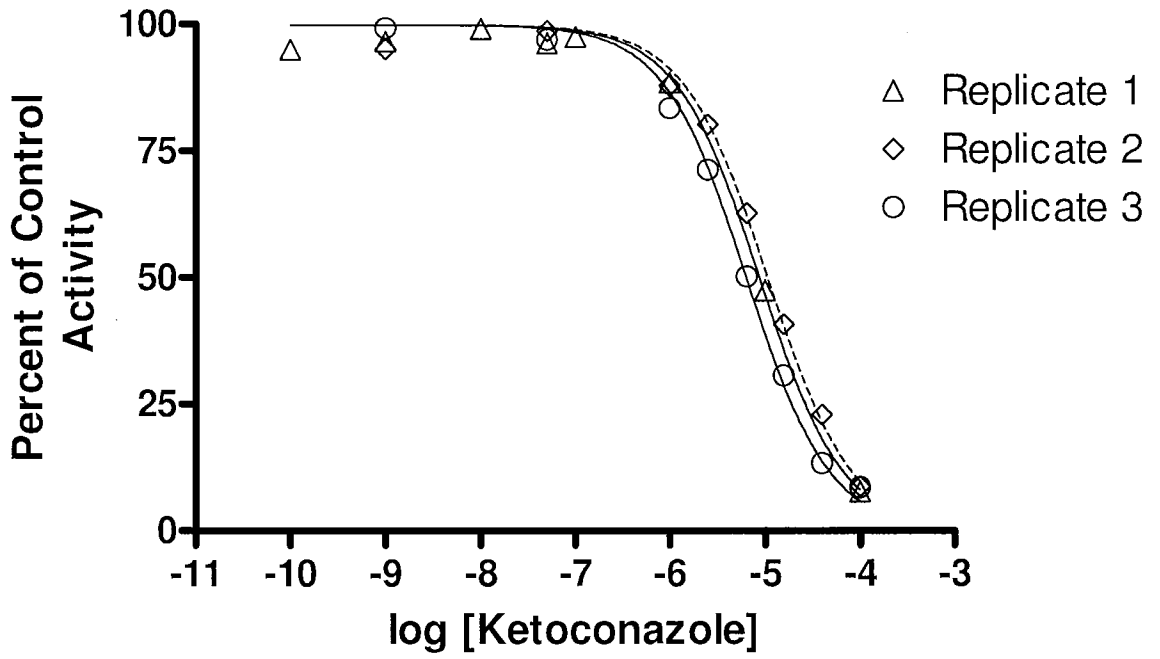
Figure 7c.
Ketoconazole Replicate 3



	Replicate 3
aromatase eqn	
Best-fit values	
LOGEC50	-5.191
HILLSLOPE	-1.009
Std. Error	
LOGEC50	0.03016
HILLSLOPE	0.04931
95% Confidence Intervals	
LOGEC50	-5.254 to -5.129
HILLSLOPE	-1.112 to -0.9072
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9926
Weighted Sum of Squares (1/Y)	11.14
Absolute Sum of Squares	206.7
Sy.x	3.065
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

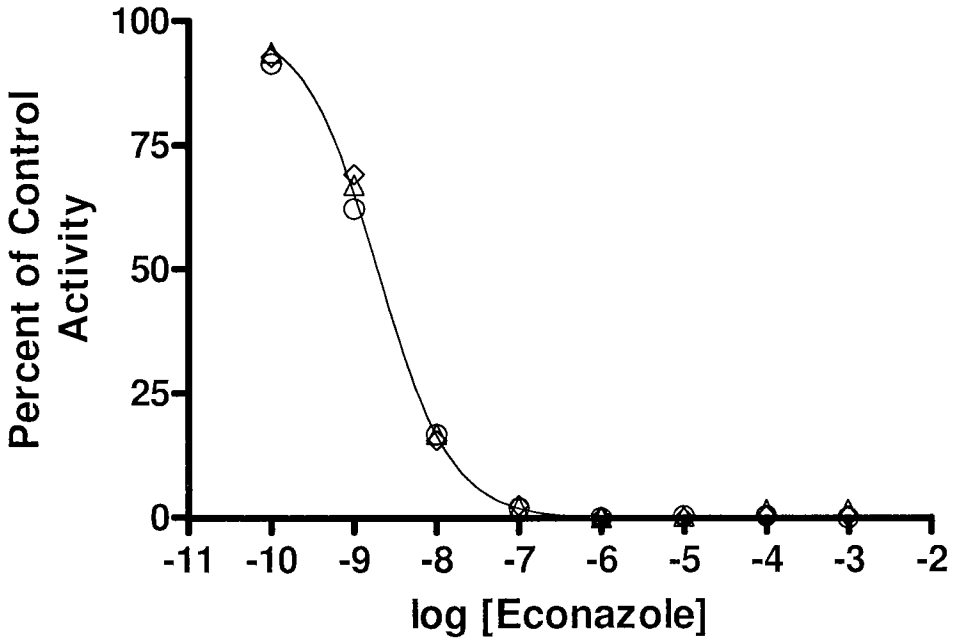
log [Ketoconazole]	Percent of Control Activity		
	Y1	Y2	Y3
-4.00	3.20	5.09	17.50
-4.40	13.73	13.03	13.46
-4.80	30.88	30.67	30.46
-5.19	51.60	49.88	49.70
-5.60	71.09	72.18	71.13
-6.00	84.65	83.42	82.82
-7.30	97.51	96.34	97.53
-9.00	99.58	99.50	98.95

Figure 7d.
Aromatase Inhibition by Ketoconazole



	Replicate 1	Replicate 2	Replicate 3
aromatase eqn			
Best-fit values			
LOGEC50	-5.055	-4.968	-5.191
HILLSLOPE	-1.001	-0.9947	-1.009
Std. Error			
LOGEC50	0.02151	0.01390	0.03016
HILLSLOPE	0.03737	0.02458	0.04931
95% Confidence Intervals			
LOGEC50	-5.099 to -5.010	-4.997 to -4.939	-5.254 to -5.129
HILLSLOPE	-1.079 to -0.9233	-1.046 to -0.9437	-1.112 to -0.9072
Goodness of Fit			
Degrees of Freedom	21	22	22
R^2 (unweighted)	0.9887	0.9939	0.9926
Weighted Sum of Squares (1/Y)	2.330	2.397	11.14
Absolute Sum of Squares	204.4	152.1	206.7
Sy.x	3.120	2.630	3.065
Data			
Number of X values	8	16	16
Number of Y replicates	3	3	3
Total number of values	23	24	24
Number of missing values	1	24	24

Figure 8.
Econazole Replicate 1



	Replicate 1
aromatase eqn	
Best-fit values	
LOGEC50	-8.711
HILLSLOPE	-0.9756
Std. Error	
LOGEC50	0.03692
HILLSLOPE	0.04359
95% Confidence Intervals	
LOGEC50	-8.788 to -8.635
HILLSLOPE	-1.066 to -0.8852
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9983
Weighted Sum of Squares (1/Y)	6.973
Absolute Sum of Squares	47.94
Sy.x	1.476
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

log [Econazole]	Percent of Control Activity		
	Y1	Y2	Y3
-3.00	1.78	0.50	0.15
-4.00	1.77	0.50	0.50
-5.00	0.50	0.02	0.50
-6.00	0.23	0.16	0.11
-7.00	2.26	2.61	2.15
-8.00	17.05	15.69	16.95
-9.00	67.38	69.44	62.56
-10.00	94.05	93.09	91.82

Figure 9a. Average Aromatase Inhibition by Chrysin

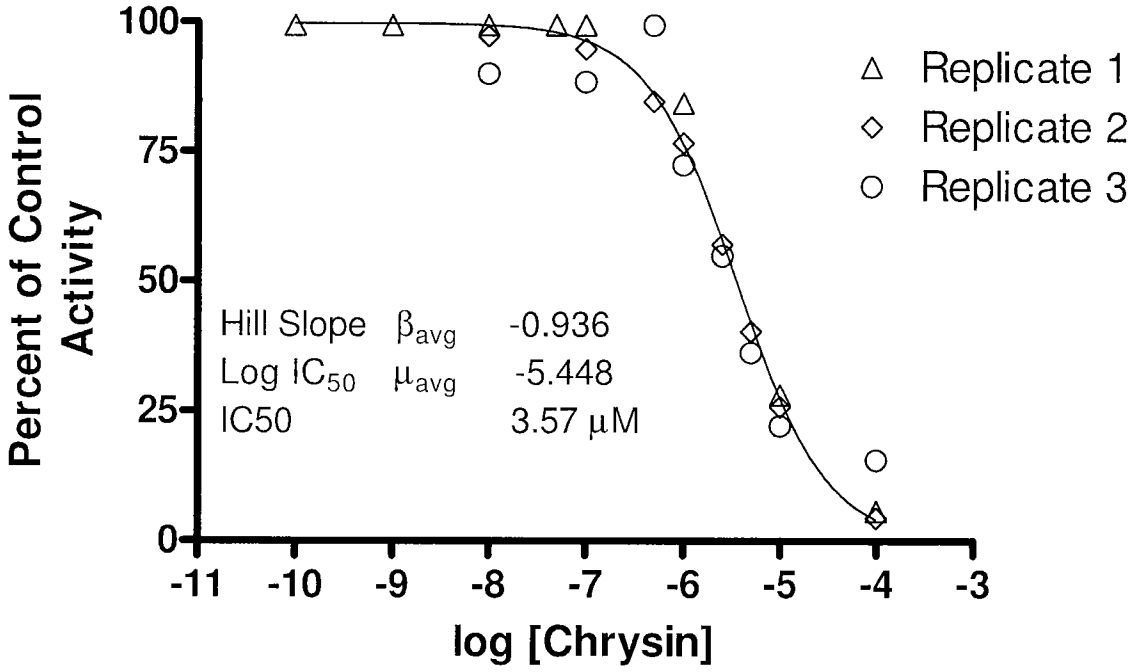


Figure 9b. Average Aromatase Inhibition by Aminoglutethimide

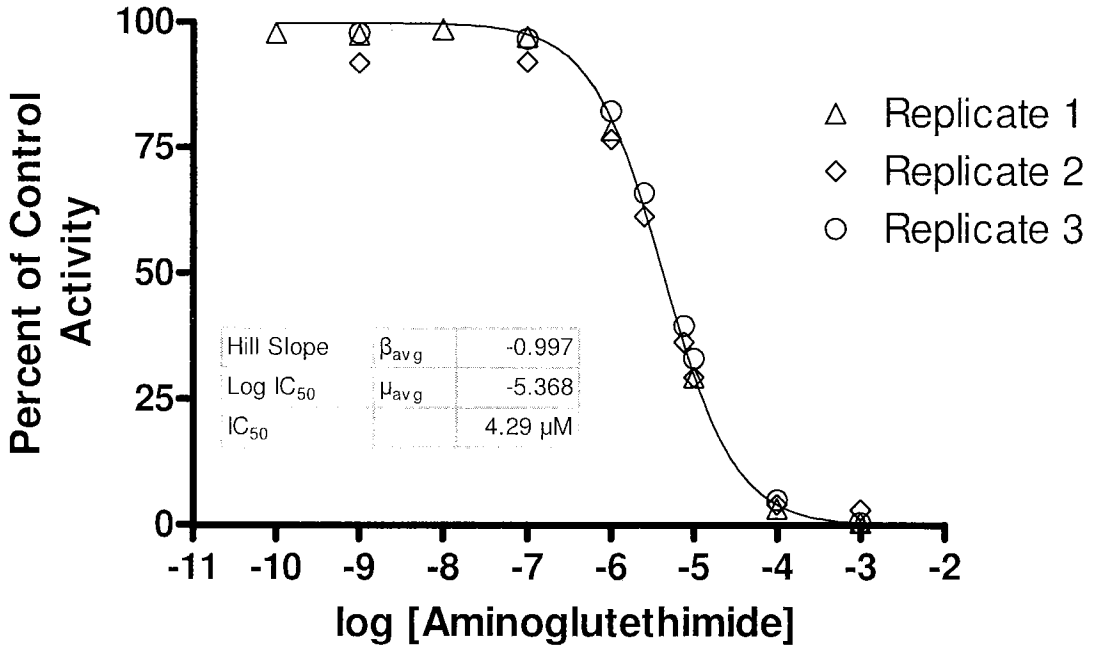


Figure 9c. Average Aromatase Inhibition by Ketoconazole

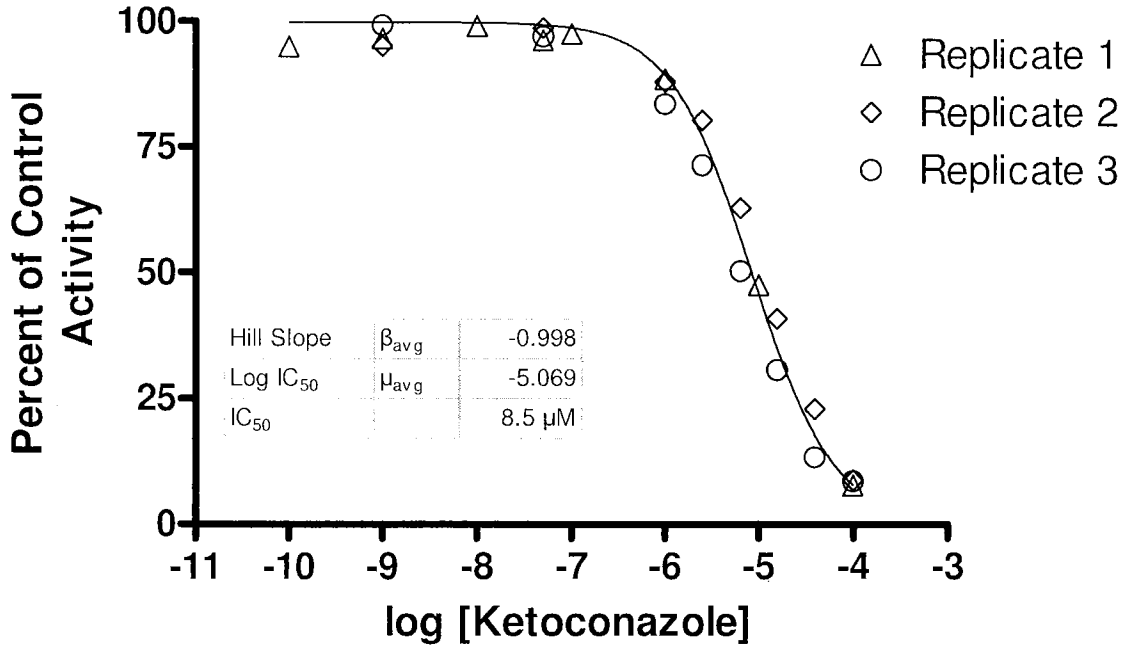


Figure 10a. Chrysin Response Curve
Summary Table - Hill Slope

		Replicate	Status	Values	Std. Error	95% Confidence Level	
β	HILLSLOPE	1	C	-0.97	0.03	-1.03	-0.8998
β	HILLSLOPE	2	C	-0.92	0.02	-0.9624	-0.8814
β	HILLSLOPE	3	C	-0.93	0.11	-1.168	-0.6938
β (avg)	AVG HILLSLOPE			-0.936	0.019	-1.017	-0.854

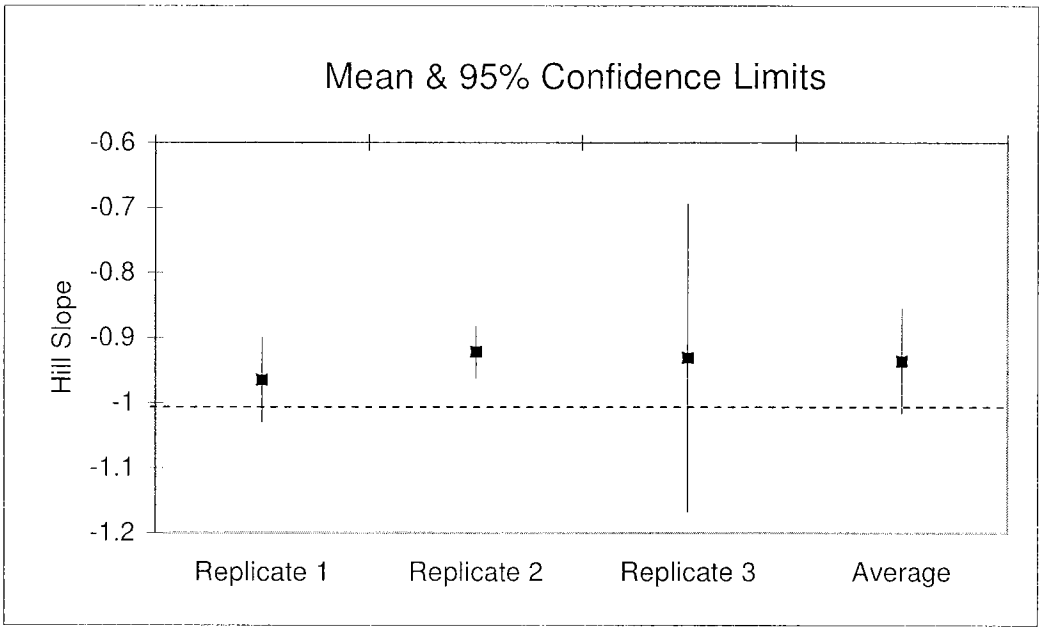


Figure 10b. Aminoglutethimide Response Curve Summary Table - Hill Slope

		Replicate	Status	Values	Std. Error	95% Confidence Level	
β	HILLSLOPE	1	C	-1.01	0.02	-1.059	-0.9643
β	HILLSLOPE	2	C	-0.92	0.05	-1.018	-0.823
β	HILLSLOPE	3	C	-1.00	0.02	-1.03	-0.9663
β (avg)	AVG HILLSLOPE			-0.997	0.012	-1.05	-0.944

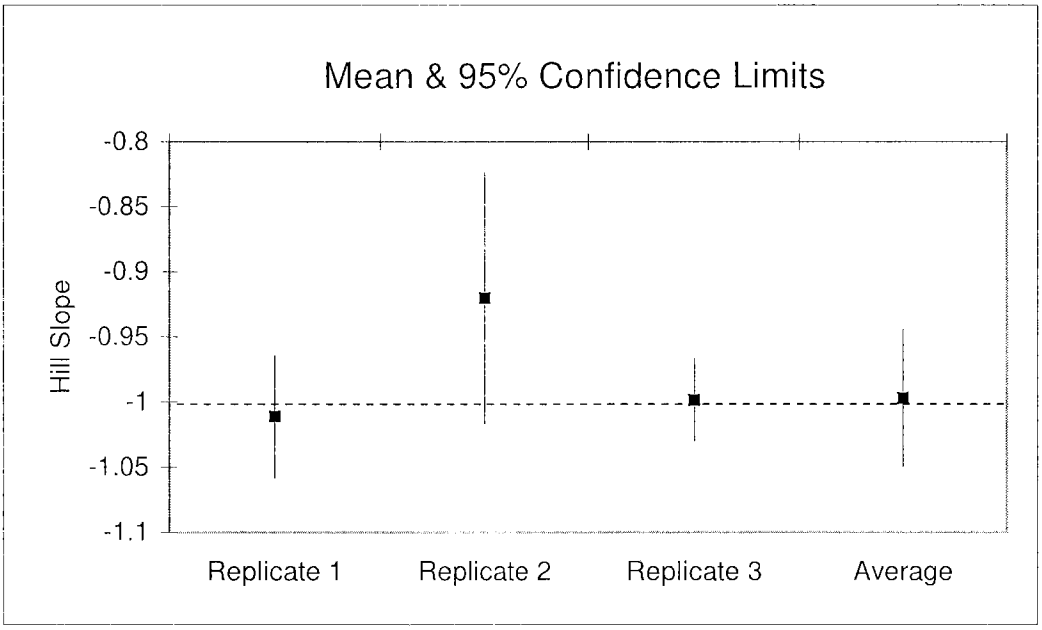


Figure 10c. Ketoconazole Response Curve Summary Table - Hill Slope

		Replicate	Status	Values	Std. Error	95% Confidence Level	
β	HILLSLOPE	1	C	-1.00	0.04	-1.079	-0.9233
β	HILLSLOPE	2	C	-0.99	0.02	-1.046	-0.9437
β	HILLSLOPE	3	C	-1.01	0.05	-1.112	-0.9072
β (avg)	AVG HILLSLOPE			-0.998	0.019	-1.08	-0.917

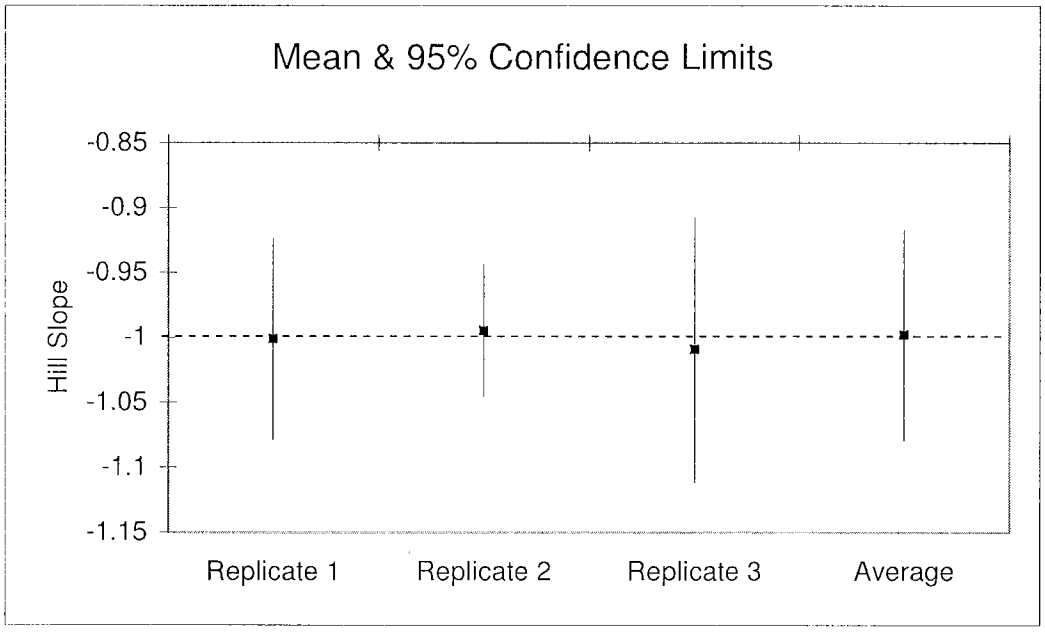


Figure 11a. Chrysin Response Curve
Summary Table - LOG IC50

		Replicate	Status	Values	Std. Error	95% Confidence Level	
μ	LOG IC50	1	C	-5.37	0.02	-5.414	-5.325
μ	LOG IC50	2	C	-5.48	0.01	-5.503	-5.456
μ	LOG IC50	3	C	-5.52	0.06	-5.653	-5.391
μ (avg)	AVG LOG IC50			-5.45	0.05	-5.641	-5.255

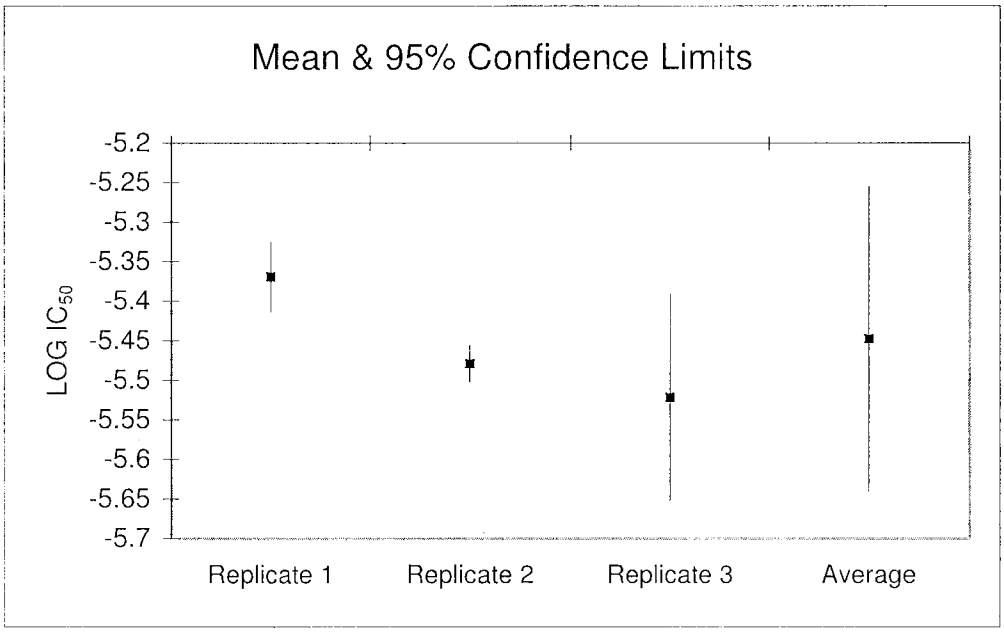


Figure 11b. Aminoglutethimide Response Curve Summary Table - LOG IC50

		Replicate	Status	Values	Std. Error	95% Confidence Level	
μ	LOG IC50	1	C	-5.40	0.02	-5.436	-5.364
μ	LOG IC50	2	C	-5.41	0.03	-5.471	-5.343
μ	LOG IC50	3	C	-5.31	0.01	-5.326	-5.289
μ (avg)	AVG LOG IC50			-5.37	0.03	-5.513	-5.223

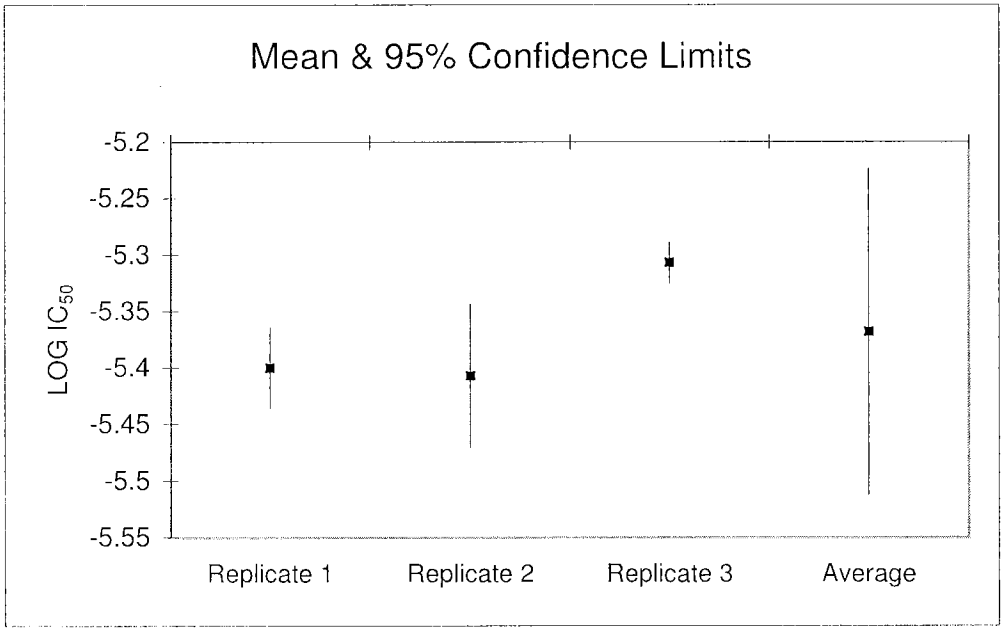


Figure 11c. Ketoconazole Response Curve Summary Table - LOG IC50

		Replicate	Status	Values	Std. Error	95% Confidence Level	
μ	LOG IC50	1	C	-5.06	0.02	-5.099	-5.01
μ	LOG IC50	2	C	-4.97	0.01	-4.997	-4.939
μ	LOG IC50	3	C	-5.19	0.03	-5.254	-5.129
μ (avg)	AVG LOG IC50			-5.07	0.06	-5.347	-4.792

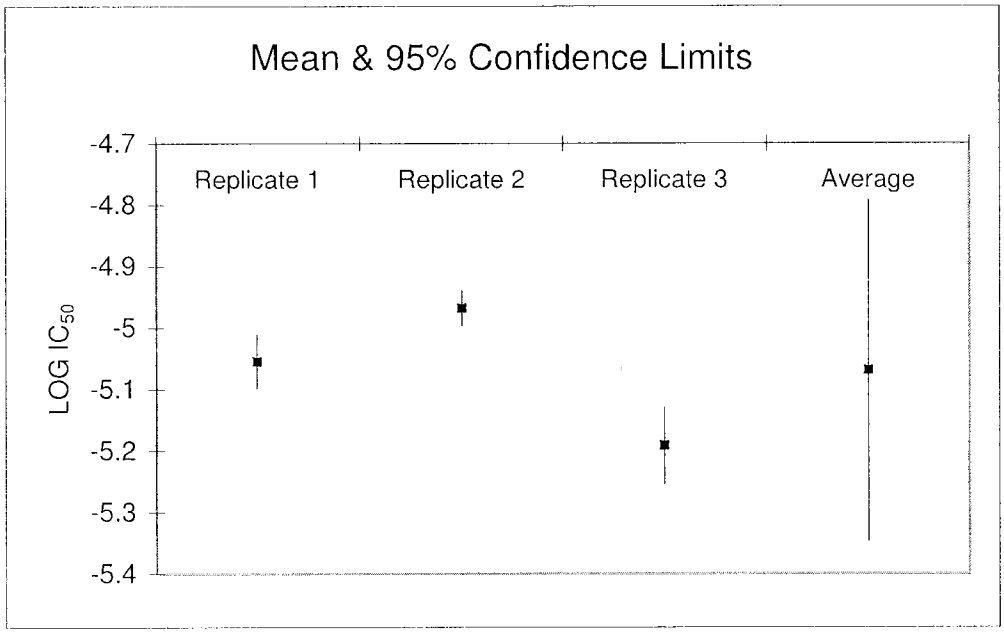


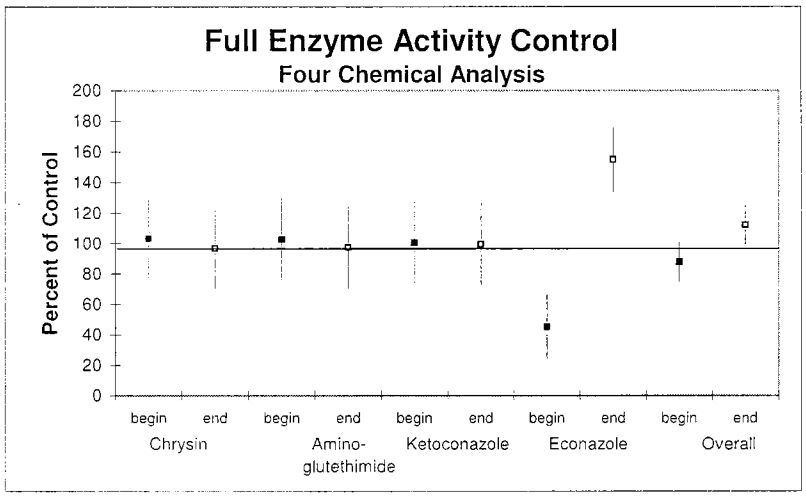
Figure 12a. Results of three-factor mixed effects analysis of variance across chemicals - Full Enzyme Activity

Portion Beginning	Statistic	CHEMICAL 1	2	3	4	Overall
	LSM	103.307	102.698	100.569	45.129	87.926
	LSM s.e.	12.16	12.16	12.16	9.419	5.768
	95% CI - lower	76.213	75.604	73.475	24.142	75.074
	95% CI - upper	130.401	129.793	127.663	66.116	100.778
End	LSM	96.693	97.302	99.431	154.869	112.074
	LSM s.e.	12.16	12.16	12.16	9.419	5.768
	95% CI - lower	69.599	70.207	72.337	133.882	99.222
	95% CI - upper	123.787	124.396	126.525	175.856	124.926
Overall	LSM	100	100	100	99.999	
	LSM s.e.	8.598	8.598	8.598	6.66	
	95% CI - lower	80.841	80.841	80.841	85.159	
	95% CI - upper	119.159	119.159	119.159	114.839	

ANOVA P-Values
Chemical = 1.0000
Portion = 0.0143
Chemical*Portion = 0.0004
Replicate(Chemical) = .
Portion*Replicate(Chemical) = 0.0579

Portion Beginning	Statistic	CHEMICAL 1	2	3	4	Overall
	LSM	103.307	102.698	100.569		102.191
	LSM s.e.	2.187	2.187	2.187		1.263
	95% CI - lower	97.955	97.346	95.217		99.101
	95% CI - upper	108.66	108.051	105.921		105.282
End	LSM	96.693	97.302	99.431		97.809
	LSM s.e.	2.187	2.187	2.187		1.263
	95% CI - lower	91.34	91.949	94.079		94.718
	95% CI - upper	102.045	102.654	104.783		100.899
Overall	LSM	100	100	100		
	LSM s.e.	1.547	1.547	1.547		
	95% CI - lower	96.215	96.215	96.215		
	95% CI - upper	103.785	103.785	103.785		

ANOVA P-ValuesChemical = 1.0000
Portion = 0.0495
Chemical*Portion = 0.4679
Replicate(Chemical) = .
Portion*Replicate(Chemical) = 0.4606



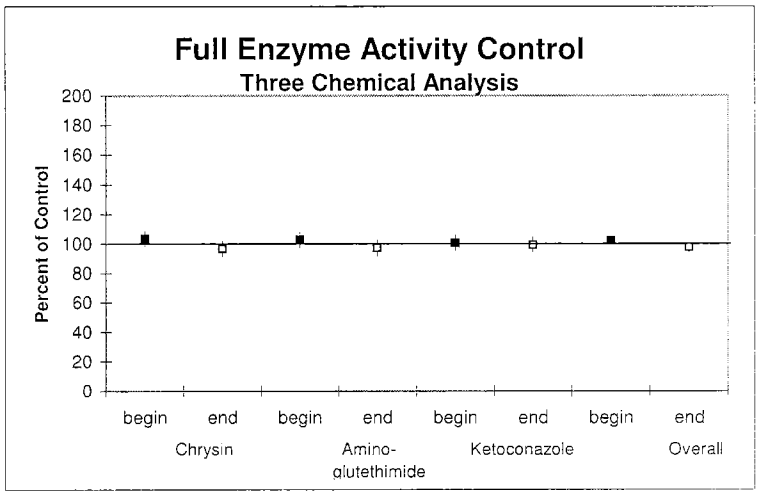
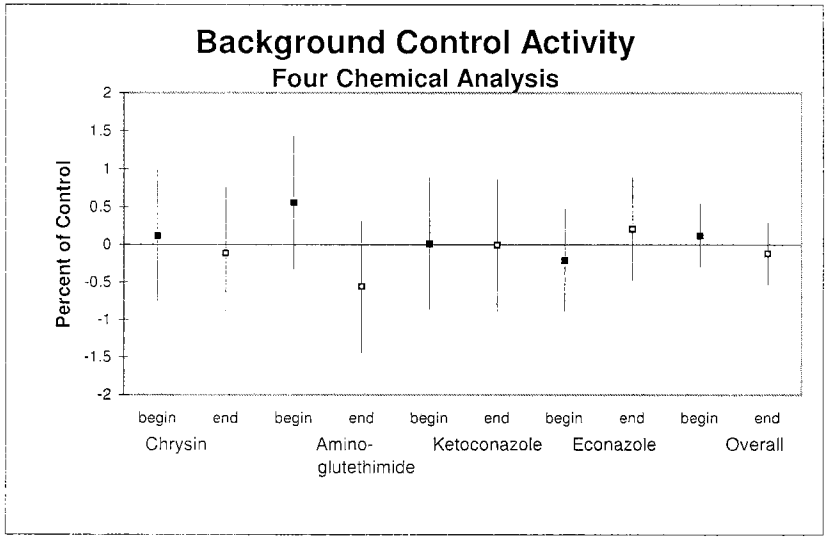


Figure 12b. Results of three-factor mixed effects analysis of variance across chemicals - Background Control Activity

Portion	Statistic	CHEMICAL 1	2	3	4	Overall
Beginning	LSM	0.113	0.558	0.009	-0.206	0.119
	LSM s.e.	0.394	0.394	0.394	0.306	0.187
	95% CI - lower	-0.766	-0.321	-0.87	-0.887	-0.298
	95% CI - upper	0.992	1.437	0.888	0.475	0.535
End	LSM	-0.113	-0.558	-0.009	0.206	-0.119
	LSM s.e.	0.394	0.394	0.394	0.306	0.187
	95% CI - lower	-0.992	-1.437	-0.888	-0.475	-0.535
	95% CI - upper	0.766	0.321	0.87	0.887	0.298
Overall	LSM	0	0	0	0	
	LSM s.e.	0.279	0.279	0.279	0.216	
	95% CI - lower	-0.622	-0.622	-0.622	-0.481	
	95% CI - upper	0.622	0.622	0.622	0.481	

ANOVA P-Values
Chemical = 1.0000
Portion = 0.3912
Chemical*Portion = 0.2529
Replicate(Chemical) = .
Portion*Replicate(Chemical) = .



Portion	Statistic	CHEMICAL 1	2	3	4	Overall
Beginning	LSM	0.113	0.558	0.009		0.227
	LSM s.e.	0.333	0.333	0.333		0.192
	95% CI - lower	-0.702	-0.257	-0.807		-0.244
	95% CI - upper	0.929	1.374	0.824		0.698
End	LSM	-0.113	-0.558	-0.009		-0.227
	LSM s.e.	0.333	0.333	0.333		0.192
	95% CI - lower	-0.929	-1.374	-0.824		-0.698
	95% CI - upper	0.702	0.257	0.807		0.244
Overall	LSM	0	0	0		
	LSM s.e.	0.236	0.236	0.236		
	95% CI - lower	-0.577	-0.577	-0.577		
	95% CI - upper	0.577	0.577	0.577		

ANOVA P-Values
Chemical = 1.0000
Portion = 0.1467
Chemical*Portion = 0.2897
Replicate(Chemical) = .
Portion*Replicate(Chemical) = .

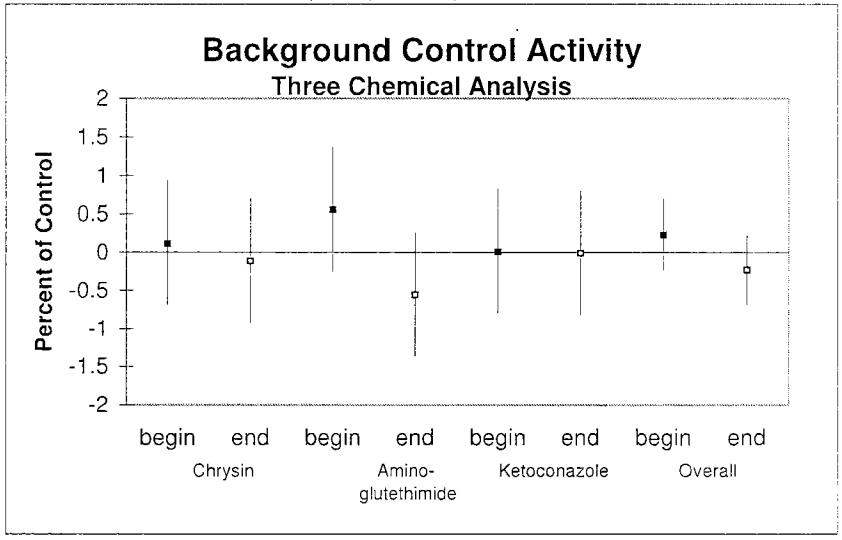
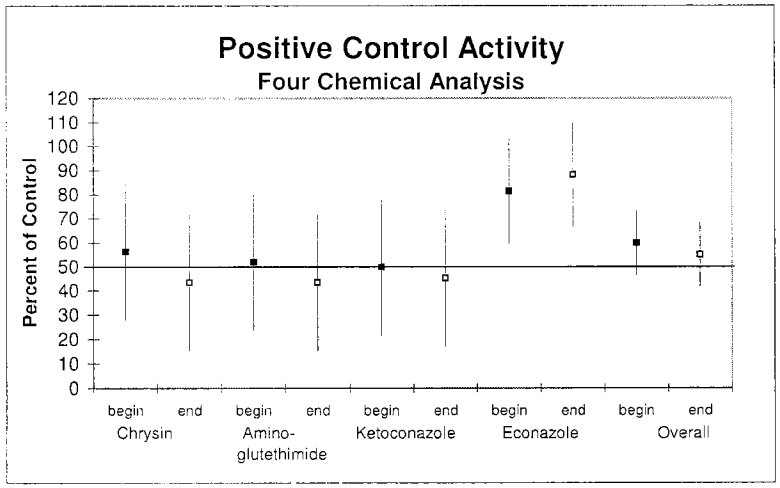


Figure 12c. Results of three-factor mixed effects analysis of variance across chemicals - Positive Control Activity

Portion	Statistic	CHEMICAL 1	2	3	4	Overall
Beginning	LSM	56.311	51.944	49.89	81.299	59.861
	LSM s.e.	12.684	12.684	12.684	9.825	6.016
	95% CI - lower	28.05	23.684	21.629	59.408	46.456
	95% CI - upper	84.572	80.205	78.15	103.19	73.266
End	LSM	43.485	43.428	45.237	88.234	55.096
	LSM s.e.	12.684	12.684	12.684	9.825	6.016
	95% CI - lower	15.224	15.168	16.976	66.343	41.691
	95% CI - upper	71.745	71.689	73.498	110.125	68.501
Overall	LSM	49.898	47.686	47.563	84.766	
	LSM s.e.	11.988	11.988	11.988	9.286	
	95% CI - lower	23.187	20.976	20.852	64.076	
	95% CI - upper	76.609	74.397	74.274	105.457	

ANOVA P-Values
Chemical = 0.0661
Portion = 0.2532
Chemical*Portion = 0.2918
Replicate(Chemical) = 0.0253
Portion*Replicate(Chemical) = 0.2223



Portion	Statistic	CHEMICAL 1	2	3	Overall
Beginning	LSM	56.311	51.944	49.89	52.715
	LSM s.e.	4.657	4.657	4.657	2.688
	95% CI - lower	44.917	40.55	38.495	46.137
	95% CI - upper	67.705	63.339	61.284	59.293
End	LSM	43.485	43.428	45.237	44.05
	LSM s.e.	4.657	4.657	4.657	2.688
	95% CI - lower	32.09	32.034	33.843	37.471
	95% CI - upper	54.879	54.823	56.631	50.628
Overall	LSM	49.898	47.686	47.563	
	LSM s.e.	3.56	3.56	3.56	
	95% CI - lower	41.186	38.975	38.852	
	95% CI - upper	58.609	56.398	56.274	

ANOVA P-Values
Chemical = 0.8753
Portion = 0.0465
Chemical*Portion = 0.6496
Replicate(Chemical) = 0.3415
Portion*Replicate(Chemical) = 0.3127

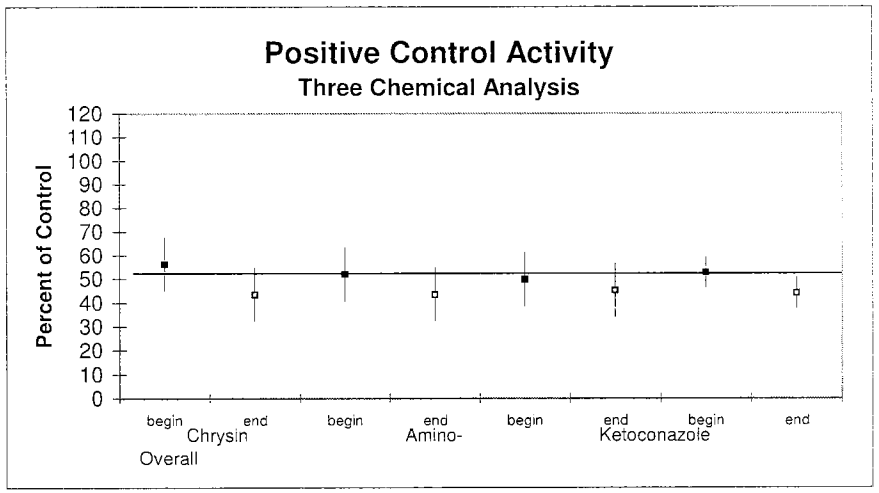


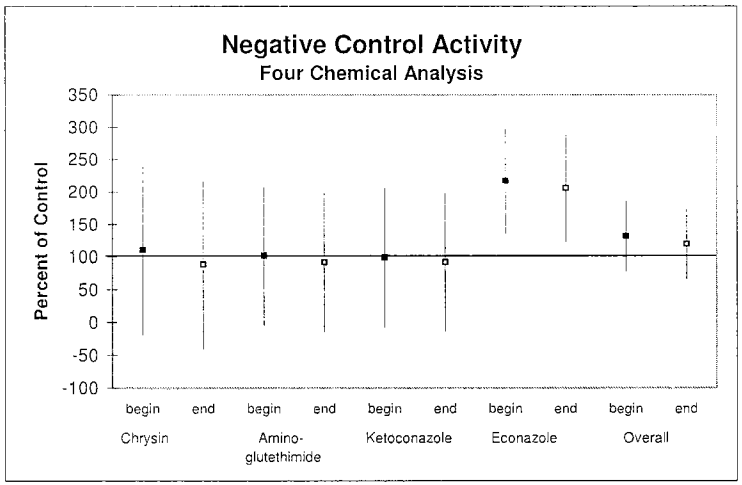
Figure 12d. Results of three-factor mixed effects analysis of variance across chemicals - Negative Control Activity

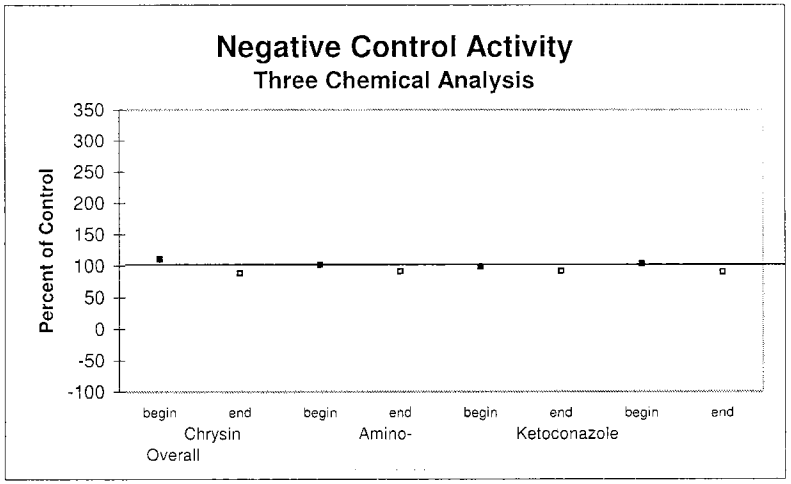
Portion	Statistic	CHEMICAL 1	2	3	4	Overall
Beginning	LSM	110.559	101.723	98.887	217.294	132.116
	LSM s.e.	57.595	47.026	47.026	36.426	23.805
	95% CI - lower	-19.73	-4.658	-7.494	134.892	78.265
	95% CI - upper	240.849	208.104	205.268	299.696	185.967
End	LSM	88.923	91.833	92.327	205.804	119.722
	LSM s.e.	57.595	47.026	47.026	36.426	23.805
	95% CI - lower	-41.367	-14.548	-14.054	123.402	65.87
	95% CI - upper	219.212	198.214	198.708	288.206	173.573
Overall	LSM	99.741	96.778	95.607	211.549	
	LSM s.e.	56.978	46.523	46.523	36.036	
	95% CI - lower	-29.153	-8.464	-9.634	130.029	
	95% CI - upper	228.635	202.02	200.849	293.069	

ANOVA P-Values
Chemical = 0.1745
Portion = 0.1082
Chemical*Portion = 0.9147
Replicate(Chemical) = 0.0190
Portion*Replicate(Chemical) = .

Portion	Statistic	CHEMICAL 1	2	3	Overall
Beginning	LSM	110.559	101.723	98.887	103.723
	LSM s.e.	2.386	1.948	1.948	1.215
	95% CI - lower	104.426	96.715	93.879	100.6
	95% CI - upper	116.693	106.731	103.895	106.846
End	LSM	88.923	91.833	92.327	91.028
	LSM s.e.	2.386	1.948	1.948	1.215
	95% CI - lower	82.789	86.825	87.319	87.904
	95% CI - upper	95.056	96.841	97.335	94.151
Overall	LSM	99.741	96.778	95.607	
	LSM s.e.	1.687	1.378	1.378	
	95% CI - lower	95.404	93.237	92.066	
	95% CI - upper	104.078	100.319	99.149	

ANOVA P-Values
Chemical = 0.2520
Portion = 0.0007
Chemical*Portion = 0.0432
Replicate(Chemical) = .
Portion*Replicate(Chemical) = 0.4810





Appendix A

Study Protocol

Study Number: WIL-431007

PROTOCOL AMENDMENT I

Sponsor: Battelle Memorial Institute

EPA Contract No.: 68-W-01-023

A. Title of Study:

Validation of the Placental Aromatase Assay for Endocrine Disruptor Screening (WA 4-16, Task 5)

B. Protocol Additions/Modifications

1) **4.2** **Control Chemicals:**

Table 2. Control Substances, the Basis for Selection of Lindane should read: "Affects StAR and cholesterol metabolism; no aromatase activity."

2) **5.2** **Microsomes:**

The entire Section has been replaced with (see Form A-198 dated February 14, 2005):

Human placental microsomes will be supplied to each laboratory by the lead laboratory. These samples should be treated as potentially infectious and appropriate precautions must be employed. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes is 14 mg/mL.

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. that is used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

Microsomes to be used for Task 5 must be stored in single-use vials in order to control the number of freeze-thaw cycles. RTI provided each laboratory ~10 vials with 0.5 mL/vial. For the remaining vials, thaw quickly in a 37 ± 1°C water bath, pool and homogenize the contents of the vials that have not been used or only used once using a Potter-Elvejhem homogenizer (about 5-10 passes). Do NOT pool the

microsomes if they are in vials that have been thawed and refrozen more than once. After homogenizing the pooled microsomes, aliquot 100 µL into individual single-use vials, flash freeze, and store at -70 to -80 °C for future use. By doing so, a single vial will be removed and used each time a replicate is performed (one daily replicate of the assay per vial). The residual microsomes from a used vial will not be retained and should be disposed of.

On the day of use, microsomes are thawed quickly in a 37°C water bath and then are immediately transferred to an ice bath. The microsomes will be rehomogenized using a short burst of vortex mixing prior to use. Vortex the sample about 5 seconds in the microcentrifuge tubes in which they are stored and then remove an aliquot directly from that tube for dilution for use in the assay. The microsomes are then diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. Microsomes are not to be left on ice for longer than approximately 2 h before proceeding with the assay. Appropriate documentation of the time of thaw and time of use must be maintained.

Diluted microsomes must be used only on the day of preparation. Under no conditions should diluted microsomes be refrozen for later use in the assay.

3) **6** **PROTEIN ASSAY:**

The following paragraph is added:

Quality control samples will be included in each replicate beginning February 23, 2005 per e-mail from the Sponsor dated February 23, 2005, a copy of which is in the study records. These quality control samples will be supplied by Pierce in a kit containing diluted BSA standards from 0.125 to 2.0 mg/mL. The standards containing 0.125, 0.5 and 1.0 mg/mL BSA will be analyzed in conjunction with the analysis of the microsome preparation. The experimental BSA concentration will be determined by the extrapolation of the

absorbance value using the curve developed using the protein standards, just as is done for the microsome preparation.

4) **8 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO REFERENCE CHEMICALS:**

The final two bulleted points should include the reference chemical concentrations and thus read:

- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at 5×10^{-8} M)
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at 1×10^{-6} M)

5) **9 DATA ANALYSIS:**

The notation FEAC and BAC will be discontinued at the request of a reviewer in all future reports and protocols and will be replaced with Full Enzyme Activity Control and Background Control, respectively.

6) **9.2.1 Concentration Response Fits for the Test Substance:**

The 'status' of the inhibition curve will now be characterized as:

- Complete curve – 0-100% Inhibition
- Incomplete curve – data points to at least 50% inhibition
- No Inhibition – no data points below 80%

C. Reasons for Protocol Additions/Modifications:

1) Table 2 Lindane Basis for Selection is changed due to a typographical error.

2) Microsomes were re-aliquoted into volumes sufficient to complete one assay in order to eliminate further freeze/thaw cycles and ensure that all labs completing this task use microsomes that have been treated similarly.

The thawing time of the microsomes on the day of the assay and the time of use must be documented to confirm that the microsomes were thawed for no more than 2 h before the initiation of the assay. The microsomal activity can decrease if the microsomes are kept in a thawed state for greater than 2 h.

The protein stock is viscous, and material readily adheres to the homogenizer and plunger. After the smaller aliquot of protein stock has been homogenized, the requisite volume cannot be collected from the vessel. This issue has resulted in a decision to omit the homogenization step and just use vortexing to disperse the microsomes prior to removing an aliquot for use on the assay.

In conjunction with this change, the protein concentration results and full enzyme activity values must be monitored from day-to-day and within a day where relevant to evaluate increases in variability. This decision is also documented on Form A-198.

3) Questions regarding the protein concentration of the microsomal preparation among laboratories participating in WA 4-16 necessitated the inclusion of externally prepared quality control samples.

4) The control chemical concentrations are included in Section 8 for clarity.

5) The notation FEAC and BAC for Full Enzyme Activity Control and Background Activity Control are not in favor with the reviewer and will no longer be used.

6) The status of the curves (C, II, IX) is not in favor with the reviewer and has been replaced with the descriptions complete, incomplete, and no inhibition.

Approved By:

Battelle Memorial Institute

Jerry D. Johnson

Jerry D. Johnson, Ph.D., D.A.B.T.
Sponsor Representative

3-10-05
Date

Prepared By:

WIL Research Laboratories, LLC

Jennifer Thomas-Wohlever

Jennifer Thomas-Wohlever, Ph.D.
Study Director

3/4/05
Date

Daniel W. Syed

Daniel W. Syed, Ph.D.
Director, Metabolism and
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3/15/05
Date

PROTOCOL

VALIDATION OF THE PLACENTAL AROMATASE ASSAY FOR ENDOCRINE DISRUPTOR SCREENING (WA 4-16, TASK 5)

EPA Contract No.: 68-W-01-023

Submitted To:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

WIL Research Laboratories, LLC
1407 George Road
Ashland, OH 44805-9281

1 OBJECTIVE:

Task 5: Conduct Studies with Centrally Prepared Microsomes

The objective of this protocol is to describe procedures for conduct of the aromatase assay using human placental microsomes. In this task, WIL (as one of the participating laboratories) will conduct studies with four potential aromatase inhibitors (reference chemicals) to demonstrate the responsiveness of the assay. Microsomes will be prepared by the lead laboratory (RTI International) and supplied to WIL, as one of the participating laboratories.

The test system for this study is human placental microsomes. This test system was selected because it provides a biological source of the aromatase enzyme and, since the assay is being evaluated for its potential to serve as a screening assay, the use of human tissue enhances its predictive potential.

There is no applicable route of administration in the sense of a dose administration route for this in vitro test. This in vitro test method involves combining microsomes, substrate, appropriate co-factors and test substances in a common reaction vessel. The effect of the reference and control chemicals on microsomal enzyme activity is evaluated by measuring the amount of the product of the enzyme-catalyzed substrate oxidation that is formed.

2 PERSONNEL INVOLVED IN THE STUDY:

2.1 Sponsor Representatives:

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2.2 U.S. EPA Representatives:

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U.S. EPA

Linda Phillips, Ph.D.
Project Officer
Endocrine Disruptor Screening Program
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Staff Toxicologist, Developmental
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2.5 WIL Staff Involved with Study:

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President, Director

Daniel W. Sved, Ph.D.
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Terry L. Johnson, Ph.D.
Associate Director, Metabolism

Donald G. Stump, Ph.D., D.A.B.T.
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Justin Godsey, B.S.
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Pete Resnis, B.S.
Senior Research Chemist, Metabolism

Aimee Mahoney, B.S.
Group Supervisor, Metabolism

2.6 Statistical Analysis:

Les Freshwater, M.S.
BioSTAT Consultants, Inc.

3 STUDY SCHEDULE:

Proposed First Replicate Assay Date:	January 31, 2005
Proposed Last Replicate Assay Date:	February 16, 2005
Proposed Unaudited Data Submission Date:	February 18, 2005
Proposed Audited Report Date:	March 18, 2005

4 REFERENCE & CONTROL CHEMICAL DATA:

Reserve samples of the reference and control chemicals used in this study will be collected by the Sponsor and will be stored at the Sponsor's facility. Therefore, no reserve samples for this study will be collected by WIL Research Laboratories, LLC.

4.1 Reference Chemicals:

The reference chemicals for this task are aminoglutethimide, chrysin, econazole and ketoconazole (see Table 1).

Table 1. Reference Chemicals for Task 5

Reference Chemical	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Basis for Selection
aminoglutethimide	125-84-8	$C_{13}H_{16}N_2O_2$	232.3	Non-steroidal aromatase inhibitor
chrysin	480-40-0	$C_{15}H_{10}O_4$	254.2	Potent flavonoid
econazole (nitrate)	24169-02-6	$C_{18}H_{15}Cl_3N_2O\text{-}HNO_3$	444.7	Potent imidazole anti-fungal
ketaconazole	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	531.43	Weak imidazole anti-fungal

Reference chemical stock solutions will be prepared and analyzed by Battelle's Chemical Repository (CR) and distributed to the laboratories. Therefore, documentation that the specified reference chemicals, CAS numbers, lot numbers, etc. were used and stored according to the Sponsor's standard operating procedures will be maintained by the Sponsor and stored at the Sponsor's facility. All four reference chemicals will be formulated in dimethylsulfoxide (DMSO). The total volume of reference chemical formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use such that the target concentration of reference chemical can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Information on storage conditions for reference chemical stock solutions will be provided by the CR. Stability of the reference chemical stock solutions will also be conducted by the Sponsor. Personal safety data are to be provided by the Sponsor. It is the responsibility of the Sponsor to notify the testing facility of any special handling requirements of the reference chemical stock solution. A material safety data sheet (MSDS) will be provided by the Sponsor by the time the reference chemicals arrive at the laboratory.

4.2 Control Chemicals:

The known aromatase inhibitor, 4-hydroxyandrostendione (4-OH ASDN), is used as the positive control substance. A known aromatase non-inhibitor, lindane, will be used as the negative control substance. Table 2 contains identity and property information for these substances.

Table 2. Control Substances

Control Chemical	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Target Concentration in Assay (M)	Basis for Selection
4-OH ASDN	566-48-3	$C_{19}H_{26}O_3$	302.4	5×10^{-8}	Known aromatase inhibitor
Lindane	58-89-9	$C_6H_6Cl_6$	290.8	1×10^{-6}	Affects and cholesterol metabolism; no aromatase activity

Control chemical stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. Therefore, documentation that the specified control chemicals and lot numbers were used and stored according to the Sponsor's standard operating procedures will be maintained by the Sponsor and stored at the Sponsor's facility. The first control chemical, 4-OH ASDN, will be formulated in ethanol. Lindane will be formulated in DMSO. The total volume of control substance formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to

minimize the potential of the solvent to inhibit the enzyme. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dilutions will be prepared such that the target concentration of control chemical (Table 2) can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume. Information on storage conditions for control chemical stock solutions will be provided by the CR. Stability of the control chemical stock solutions will also be conducted by the Sponsor. Personal safety data are to be provided by the Sponsor. It is the responsibility of the Sponsor to notify the testing facility of any special handling requirements of the control chemical stock solution. A material safety data sheet (MSDS) will be provided by the Sponsor by the time the control chemicals arrive at the laboratory.

5 ASSAY MATERIALS RECEIPT AND/OR PREPARATION:

A sufficient supply of chemical reagents, radiolabeled androstenedione, and placental microsomes will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies. The detailed procedures for preparation of the assay substrate, assay buffer, microsomes and NADPH solution will be documented in the study records.

The procedure for identification of the test system will be that each test tube used in the conduct of the aromatase assay will be uniquely identified by applying a label or writing directly on the test tube.

5.1 Assay Substrate, [^{3}H]ASDN + ASDN:

5.1.1 Substrate Name/Supplier:

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN will be used. The non-radiolabeled ASDN and the radiolabeled androstenedione ([1□-^{3}H]-androstenedione, [^{3}H]ASDN) will be provided to the laboratories by Perkin Elmer. The CR will forward all applicable information regarding supplier, lot numbers and reported/measured purity for the substrate to the laboratories and this information will be included in study reports. The radiochemical purity of the [^{3}H]ASDN will be assessed by the lead laboratory and this information will be included in the study reports. The radiochemical purity of the [^{3}H]ASDN will be greater than approximately 95 percent.

5.1.2 Preparation of Substrate Solution for use in Aromatase Assay:

Since the specific activity of the stock [^{3}H]ASDN is too high for use directly in the assay, a solution containing a mixture of nonradiolabeled and radiolabeled [^{3}H]ASDN is prepared such that the final concentration

of ASDN in the assay is 100 nM and the amount of tritium added to each incubation is about 0.1 µCi. This substrate solution should have a concentration of 2 µM with a radiochemical content of about 1 µCi/mL.

The following illustrates the preparation of a substrate solution using a stock of [^{3}H]ASDN with a specific activity of 25.3 Ci/mmol and a concentration of 1 mCi/mL. Prepare a 1:100 dilution of the radiolabeled stock in buffer. Prepare a 1 mg/mL solution of ASDN in ethanol and then prepare dilutions in buffer to a final concentration of 1 µg/mL. Combine 4.5 mL of the 1 µg/mL solution of ASDN, 800 µL of the [^{3}H]ASDN dilution and 2.7 mL buffer to make 8 mL of substrate solution (enough for 80 tubes). Record the weight of each component added to the substrate solution. After mixing the solution well, weigh aliquots (ca 20 µL) and combine with scintillation cocktail for radiochemical content analysis. The addition of 100 µL of the substrate solution to each 2 mL assay volume yields a final [^{3}H]ASDN concentration of 100 nM with 0.1 µCi/tube.

5.2 Microsomes:

Human placental microsomes will be supplied to each laboratory by the lead laboratory. These samples should be treated as potentially infectious and appropriate precautions must be employed. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes is 14 mg/mL.

Caution: Microsomes can be denatured by detergents. Therefore, it is important to ensure that all glassware, etc. that is used in the preparation or usage of microsomes is free of detergent residue. New disposable test tubes, bottles, vials, pipets and pipet tips may be used directly in the assay. Durable labware that may have been exposed to detergents should be rinsed with water and/or buffer prior to use in the assay.

On the day of use, microsomes are thawed quickly in a 37 ± 1°C water bath and then are immediately transferred to an ice bath. The microsomes will be rehomogenized using a Potter-Elvejhem homogenizer (chilled in ice bath, about 5-10 passes) prior to use. The microsomes are diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. Microsomes will not be left on ice for longer than approximately 2 h before proceeding with the assay or microsomal enzyme activity may be decreased.

Excess undiluted stock microsomes may be flash frozen in dry ice/ethanol and returned to -70 to -80°C storage for future use. If possible, stock microsomes should be refrozen and divided into aliquots appropriate for use prior to refreezing in order to minimize the number of freeze/thaw cycles.

Diluted microsomes must be used only on the day of preparation. Under no conditions should diluted microsomes be refrozen for later use in the assay.

5.3 Other Assay Components:

5.3.1 Buffer:

The assay buffer is 0.1 M sodium phosphate buffer, pH 7.4. Sodium phosphate monobasic and sodium phosphate dibasic are used in the preparation of the buffer. Solutions of each reagent at 0.1 M are prepared in distilled, deionized water and then the solutions are combined to a final pH of 7.4. The assay buffer may be stored refrigerated (2-8°C) for up to one month.

5.3.2 Propylene Glycol:

Propylene glycol is added directly to the assay as described in Section 7.

5.3.3 NADPH:

NADPH (β-nicotinamide adenine dinucleotide phosphate, reduced form, tetrasodium salt) is the required co-factor for CYP19 (aromatase enzyme). The Sponsor will provide the NADPH to be used in the assay. The final concentration in the assay will be 0.3 mM. Typically, a 6 mM stock solution is prepared in assay buffer and then 100 µL of the stock is added to the 2 mL assay volume. NADPH must be prepared fresh each day and is kept on ice.

6 PROTEIN ASSAY:

The protein concentration of the microsome preparation will be determined on each day of use of the microsomes in the aromatase assay. A 6-point standard curve will be prepared, ranging from approximately 0.13 to 1.5 mg protein/mL. The protein standards will be made from bovine serum albumin (BSA). Protein will be determined by using a DC Protein Assay kit purchased from Bio-Rad (Hercules, CA). To a 25 µL aliquot of unknown or standard, 125 µL of BioRad DC Protein Kit Reagent A will be added and mixed. Next, 1 mL of BioRad DC Protein Kit Reagent B will be added to each standard or unknown and the samples will be vortex mixed. The samples will be allowed to sit at room temperature for at least 15 minutes to allow for color development. The absorbances are stable for about 1 hour. Each

sample (unknown and standards) will be transferred to appropriate cuvettes and the absorbance (750 nm) will be measured using a spectrophotometer. The protein concentration of the microsomal sample will be determined by extrapolation of the absorbance value using the curve developed using the protein standards.

7 AROMATASE ASSAY (SEE APPENDIX A):

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN + ASDN substrate, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The final concentrations for the assay components are presented in the table below.

Human Placental Microsomal Aromatase Assay-Optimized Conditions	
Microsomal Protein	0.0125 mg/mL[a]
NADPH	0.3 mM[a]
[^{3}H]ASDN + ASDN	100 nM[a]
Incubation Time	15 minutes

[a] Final concentrations

The tubes and the microsomal suspension will be placed in a 37 ± 1°C water bath for at least five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension to the reaction mixture in the labeled test tube. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 minutes. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for approximately 5 seconds and placed on ice. The tubes are then vortex-mixed an additional 20-25 seconds. The tubes will then be centrifuged for 10 minutes at approximately 162 x g. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, approximately 10 mL) will be added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

Analysis of the samples will be performed using a liquid scintillation counter (LSC). Radiolabel found in the aqueous fractions represents ^3H_2O formed. One ^3H_2O molecule is released per molecule of ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of estrogen product formed is determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate (expressed in dpm/nmol). Results will be presented as the activity (velocity) of the enzyme reaction. The activity of the enzyme reaction is expressed in nmol

(mg protein)$^{-1}$min^{-1} and is calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 15 minutes.

8 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO REFERENCE CHEMICALS

Each replicate will test the response of aromatase activity to the presence of eight concentrations of a reference chemical. The reference chemicals must be coded prior to distribution to the assaying technicians to ensure that the replicates are conducted blind for reference chemical identity. This task will be conducted in three independent replicates by each laboratory. All three replicates for a given reference chemical must be conducted by the same technician. However, the same technician is not required to perform the three replicates for all four reference chemicals. Multiple reference chemicals may be conducted by a single technician in a given day. Each replicate for a given reference chemical must be conducted entirely independently of the other replicates for that reference chemical. Thus, it is recommended that if multiple replicates are conducted on a given day by a single technician, those replicates should use different reference chemicals. Each reference chemical will be tested at eight concentrations and there will be three (triplicate) repetitions for each concentration of a given replicate. A single replicate study of a given reference chemical is described in Table 4.

Four types of control samples will be included for each replicate. These include:

- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)
- background activity controls (all components that are in the full aromatase activity controls, except NADPH)
- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration)
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a single concentration)

Four test tubes of each type of control are included with each replicate and are treated the same as the other samples. The controls sets will be split so that two tubes (of each control type) are run at the beginning and two at the end of each replicate set.

The assay will be conducted as described in Section 7 with the following modification. Reference chemical solution (or vehicle) will be added to the mixture of propylene glycol, substrate, NADPH and buffer in a volume not to exceed 20 µL prior to preincubation of that mixture. The volume of buffer used will be adjusted so the total incubation volume remains at 2 mL.

After completion of the first replicate, the data will be reviewed and, if necessary, the concentration of reference chemical used in the second and third replicates can be adjusted. The decision whether to adjust test concentrations rests with the Study Director. The decision should be based on the results from the first replicate with the following guidelines in mind:

- If insolubility is observed at the high concentration (10^{-3} M), then set the highest concentration for the second and third replicates at the highest concentration that appeared to be soluble (limited to 10^{-4} or 10^{-5} M). Do not use a concentration lower than 10^{-5} M for the highest concentration tested.
- If the highest concentration to be tested is lowered to 10^{-4} or 10^{-5} M, then add mid-log concentration(s) near the estimated IC_{50} based on the replicate one results in order to keep eight concentrations in the test set.
- The lowest concentration to be tested is 10^{-10} M.

Table 4. Reference Chemical Study Design

Sample Type	Repetitions (test tubes)	Description	Test Chemical Concentration (M final)
Full Enzyme Activity Control	4	Complete assay[a] with reference chemical vehicle control	N/A
Background Activity Control	4	Complete assay with reference chemical vehicle control omitting NADPH	N/A
Positive Control	4	Complete assay[a] with positive control chemical (4-OH ASDN) added	5×10^{-8}
Negative Control	4	Complete assay[a] with negative control chemical (lindane) added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}

[a] The Complete Assay contains buffer, propylene glycol, microsomal protein, [^{3}H]ASDN+ASDN and NADPH

9 DATA ANALYSIS:

9.1 Aromatase Activity and Percent of Control Calculations:

Relevant data are entered into the latest version of the Excel spreadsheet Aromatase_Master_Versionx.y.xls (where x and y denote version number designation) for calculation of aromatase activity and percent of control. The version of the spreadsheet used will be included in the reports. A working guidance document detailing the use of this spreadsheet is included as Appendix B.

9.2 Statistical Analysis:

9.2.1 Concentration Response Fits for the Test Substance:

For the reference chemicals multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three.

For each replicate two repeat tubes of the full enzyme activity controls (FEAC), the background activity controls (BAC) and the positive and negative controls will be run prior to the to repetitions of the graded concentrations of the reference chemical and two repeat tubes of each control will be run following the repetition of the reference chemical. Three repetitions will be prepared for each concentration of the reference chemical.

For each repeat tube (FEAC, BAC, positive, and negative controls and each reference chemical concentration) the Excel database spreadsheet will include total observed (uncorrected) disintegrations per minute (DPMs) per tube and total aromatase activity per tube. The DPM and aromatase activity values are corrected for the background DPMs, as measured by the average of the BAC control tubes. The aromatase activity is calculated as the corrected DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The average (corrected) DPMs and aromatase activity across the four BAC control repeat tubes must necessarily be equal to 0 within each replicate.

For each tube percent of control is determined by dividing the background corrected aromatase activity for that tube by the average background corrected aromatase activity for the four FEAC tubes and multiplying by 100. Nominally one might expect for an inhibitor the percent of control activity values to vary between approximately 0% near the high inhibition concentrations and approximately 100% near the

low inhibition concentrations. However due to experimental variation individual observed percent of control values will sometimes extend below 0% or above 100%.

Concentration response trend curves will be fitted to the percent of control activity values within each replicate at each reference chemical concentration. Concentration is expressed on the log scale. In agreement with past convention, logarithms will be common logarithms (i.e. base 10). Let X denote the logarithm of the concentration of reference chemical (e.g. if concentration = 10^{-5} then X = -5). Let:

Y ≡ percent of control activity in the inhibitor tube
X ≡ logarithm (base 10) of the concentration
DAVG ≡ average DPMs across the repeat tubes with the same reference chemical concentration
β ≡ slope of the concentration response curve (β will be negative)
μ ≡ $\log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to percent of control activity equal to 50%)

The following concentration response curve will be fitted to relate percent of control activity to logarithm of concentration within each replicate:

$$Y = 100/[1 + 10^{(\mu-X)\beta}] + \varepsilon$$

where ε is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The variance is approximated by Y.

The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits will be carried out using Prism software (Version 3 or higher). Observed individual percent activity values above 100% will be set to 99.5%. Observed individual percent activity values below 0% will be set to 0.5%.

Concentration response models will be fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate the extent of aromatase inhibition will be summarized as IC_{50} (10 μ) and slope (β). The estimated IC_{50} for a reference chemical will be a (weighted) geometric mean across the replicates. The estimated overall standard error will be based on the standard errors within each replicate and the replicate-to-replicate variability. The average value and standard error of $\log_{10}IC_{50}$ or β and the replicate-to-replicate component of variation will be calculated based on a one-way

random effects analysis of variance model fit. For each reference chemical and replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve is indicated as:

- "C" Complete. i.e. ranging from essentially 0 percent to 100 percent of control.
- "II" Incomplete. But can interpolate to $\log_{10}IC_{50}$.
- "IX" Incomplete. But must extrapolate to $\log_{10}IC_{50}$.

Replicates for which a concentration response curve cannot be fitted (and so an IC_{50} cannot be estimated) will be referred to as "noninhibitors".

9.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits:

For each replicate the individual percent of control values will be plotted versus logarithm of the reference chemical concentration. The fitted concentration response curve will be superimposed on the plot. Individual plots will be prepared for each replicate.

Additional plots will be prepared to compare the percent of control activity values across replicates. For each replicate the average percent of control values will be plotted versus logarithm of reference chemical concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curves for each replicate will be superimposed on the plots. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of reference chemical concentration. The average concentration response curve across replicates will be superimposed on the same plot.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}). Let X and Y ($0< Y <100$) denote logarithm of concentration and percent of control, as defined above. The average response curve is

$$Y_{avg} = 100/[1 + 10^{\beta avg(\mu avg - X)}].$$

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on one-way random effects analysis of variance, treating the replicates as random effects. For each of β and μ, plots will be prepared that display the parameters within each replicate with associated 95%

confidence intervals based on the within replicate standard error and the average across replicates with associated 95% confidence interval incorporating replicate-to-replicate variation.

9.2.3 Graphical and Analysis of Variance Comparisons of FEAC, BAC, and Positive and Negative Control Percent of Control Across Reference Chemicals and Replicates:

Within each replicate of each reference chemical quadruplicate repetitions will be made of the FEAC control, BAC control, and negative and positive control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are consistent throughout the replicate test, the control tubes at the beginning should be equivalent to those at the end.

To assess whether this is the case the control responses will be adjusted for background DPMs, divided by the average of the (background adjusted) FEAC control values, and expressed as percent of control. The average of the four BAC controls within a replicate must necessarily be 0 percent and the average of the four FEAC controls within a replicate must necessarily be 100 percent. The FEAC controls percent of control, the BAC controls percent of control, and the negative and positive controls percent of control values will be plotted across reference chemical and replicate within reference chemical, with plotting symbol distinguishing between beginning and end, and with reference line 0% (BAC control) or 100% (FEAC control) respectively. These plots will display the extent of consistency across reference chemicals and replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Additional plots will be prepared displaying the difference of the average of the first two percent of control values (i.e., those based on the "beginning" tubes) and the average of the last two percent of control values (i.e., those based on the "end" tubes) across reference chemicals and replicates within reference chemicals. Each plot will have a reference line of 0.

Three-factor mixed effects analysis of variance models will be fitted, separately for the FEAC control, the BAC control, and the positive and negative control tubes. The fixed effect factors in the analysis of variance will be

- reference chemical
- portion (beginning or end)
- portion by reference chemical interaction.

The random effects will be

- replicate nested within reference chemical
- portion by replicate within reference chemical interaction.

The residual error variation corresponds to repetition within reference chemical, replicate, and portion. The response will be percent of control. Since for the BAC and FEAC controls the average of the repetitions within a reference chemical and replicate are constrained to be 0 and 100 respectively, by the way in which "percent of control" is defined, the variation associated with the reference chemical effect and the replication within reference chemical effect are both necessarily constrained to be 0.

If the daily replicates are in control the portion main effect and the portion by replicate within reference chemical interaction should be nonsignificant. If the portion by replicate within reference chemical interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate within reference chemical to the portion effect averaged across replicates within reference chemical, adjusting for simultaneity by Bonferroni's method.

9.2.4 Statistical Software:

Concentration response curves will be fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 3 or higher. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using PRISM, Excel, the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS), as convenient.

9.2.5 Interlaboratory Statistical Analysis:

The lead laboratory and each of the participating laboratories will carry out "intra-laboratory" statistical analyses based on their test data, according to this common statistical analysis plan, developed by the Data Coordination Center (Battelle). The Data Coordination Center will carry out the "inter-laboratory" statistical analysis. It will combine summary values developed in each of the intra-laboratory analyses to assess relationships among the laboratory results, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

10 QUALITY ASSURANCE:

The study will be audited by the WIL Quality Assurance Unit with in-phase inspections to assure compliance with the study protocol and protocol amendments, WIL standard operating procedures and the appropriate provisions of the EPA TSCA and FIFRA Good Laboratory Practice Standards published in the Federal Register (40 CFR Part 792 and 40 CFR Part 160). The raw data and draft report will be audited by the WIL Quality Assurance Unit prior to submission to the Sponsor to assure that the Final Report accurately describes the conduct and the findings of the study. Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that will be prepared for this study.

Data requiring statistical analysis will be analyzed by BioSTAT Consultants, Inc. following the current procedural guidelines of BioSTAT Consultants Inc. BioSTAT Consultants Inc. will provide a statistical analysis report, which will be included as an appendix to the final report. Quality Assurance auditing of the statistical report (for internal consistency with the study report) will be conducted under the direction of the Quality Assurance Unit of WIL Research Laboratories, LLC.

Formulation of the reference and control chemical stock solutions will be conducted by the Sponsor following the standard operating procedures of the Sponsor and in accordance with GLPs. Quality assurance monitoring of these activities for SOP and GLP compliance is the responsibility of the Sponsor. Upon completion of the prescribed activities the Sponsor will provide a signed Quality Assurance statement that will be included in the Battelle Chemical Repository Chemistry Report and included in the final report as an appendix.

This study will be included on the WIL master list of regulated studies.

11 RECORDS TO BE MAINTAINED:

All specimens and original raw data records, as defined by WIL SOPs and the applicable GLPs, will be stored as described in Section 12 in the Archives at WIL Research Laboratories, LLC.

Raw data records generated by the Sponsor will be stored as defined by the Sponsor's applicable standard operating procedures.

12 WORK PRODUCT:

The Sponsor will have title to all documentation records, raw data, specimens and other work product generated during the performance of the study. All work product, including raw paper data, pertinent electronic storage media and specimens, will be retained at no charge for a period of six months following issuance of the final report in the Archives at WIL Research Laboratories, LLC. Thereafter, WIL Research

Laboratories, LLC will charge a monthly archiving fee for retention of all work product. Appropriate supporting documentation for statistical analyses conducted and reported by BioSTAT Consultants, Inc. will be maintained in the Archives at WIL Research Laboratories, LLC. All work product will be stored in compliance with regulatory requirements.

Any work product, including documents, specimens, and samples, that are required by this protocol, its amendments, or other written instructions of the Sponsor, to be shipped by WIL Research Laboratories, LLC to another location will be appropriately packaged and labeled as defined by WIL's SOPs and delivered to a common carrier for shipment. WIL Research Laboratories, LLC will not be responsible for shipment following delivery to the common carrier.

13 REPORTS:

An interim data set, in the form of a spreadsheet and data summary, will be submitted to the Sponsor. The spreadsheets will be submitted within 14 calendar days of completing the incubations/analyses. This interim data submission will not be audited by the Quality Assurance Unit and will be identified as "unaudited preliminary data." The data will be checked for accuracy by the technical staff.

Interim data summaries, draft and final reports will be submitted as described in Section 9.5 of the QAPP.

The data to be reported in the interim data summaries will include (but is not limited to) the following information: assay date and run number, technician code, chemical code and log chemical concentration, background corrected aromatase activity (for each control and test chemical repetition), percent of control activity, IC_{50}, slope and graphs of activity versus log chemical concentration.

In addition, draft and final reports will contain tables and graphs, as appropriate, containing the results of the intra-laboratory statistical analyses described in Section 9 of this document.

Draft final and final reports will be written. The format for the draft final report will be provided by the Sponsor. The draft final report will be submitted to the Sponsor. One revision of the full report will be permitted as part of the cost of the study, from which the Sponsor's reasonable revisions and suggestions will be incorporated into the final report, as appropriate. Additional changes or revisions, requiring a new report, may be made, at extra cost. It is expected that the Sponsor will review the draft report and provide comments to WIL within a two-month time frame following submission. WIL will submit the final report in a timely manner following receipt of comments. If the Sponsor's comments and/or authorization to finalize the report have not been received by WIL within one year following submission of the draft report, WIL may elect to finalize the report following appropriate written notification to the

Sponsor. One electronic copy (PDF format) will be provided; requests for paper copies of the final report may result in additional charges.

14 PROTOCOL MODIFICATION:

Modification of the protocol may be accomplished during the course of this investigation. However, no changes will be made in the study design without the written permission (electronic email or paper document) of the Sponsor. In the event that the Sponsor requests or approves a change in the protocol, such changes will be made by appropriate documentation in the form of a protocol amendment. All alterations of the protocol and reasons for the modification(s) will be signed by the Study Director and the Sponsor Representative.

15 PROTOCOL APPROVAL:

Sponsor approval received via email on 1/28/05
Date

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Jennifer Thomas-Wohlever, Ph.D. Date
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WIL Research Laboratories, LLC

Daniel W. Sved 2/7/05
Daniel W. Sved, Ph.D. Date
Director, Metabolism and
Analytical Chemistry
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Jerry D. Johnson 2-1-05
Jerry D. Johnson, Ph.D., D.A.B.T. Date
Work Assignment Leader/Study Monitor
Endocrine Disruptor Screening Program
Battelle Memorial Institute

David P. Houchens 2/4/05
David P. Houchens, Ph.D. Date
Program Manager
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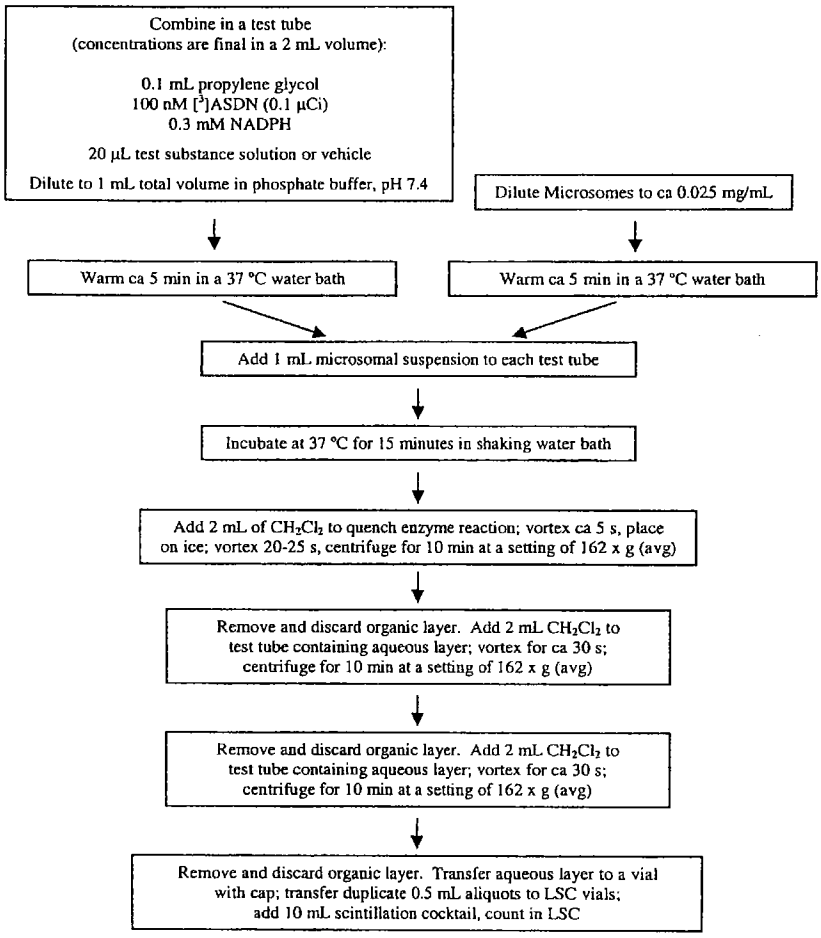
Heather L. Osborn, B.S., RQAP-GLP Date
Manager, Quality Assurance
WIL Research Laboratories, LLC

Terri L Pollock 2-1-05
Terri L. Pollock, B.A. Date
Quality Assurance Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

Appendix A


Combine in a test tube
(concentrations are final in a 2 mL volume):
0.1 mL propylene glycol
100 nM [3]ASDN (0.1 µCi)
0.3 mM NADPH
20 µL test substance solution or vehicle
Dilute to 1 mL total volume in phosphate buffer, pH 7.4
Dilute Microsomes to ca 0.025 mg/mL
Warm ca 5 min in a 37 °C water bath
Warm ca 5 min in a 37 °C water bath
Add 1 mL microsomal suspension to each test tube
Incubate at 37 °C for 15 minutes in shaking water bath
Add 2 mL of CH2Cl2 to quench enzyme reaction; vortex ca 5 s, place on ice; vortex 20-25 s, centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH2Cl2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH2Cl2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Transfer aqueous layer to a vial with cap; transfer duplicate 0.5 mL aliquots to LSC vials; add 10 mL scintillation cocktail, count in LSC

Appendix B

Notes for use of the spreadsheet:
Aromatase_Master_Version1.1.xls

Substrate Specific Activity Worksheet

This worksheet calculates:
1. The radiochemical content (DPM/mL) of the substrate solution
2. The new specific activity of the [^{3}H]ASDN in the substrate solution

The first item is based on the results of LSC analysis of weighed aliquots of the substrate solution

The second item is calculated by:
1. determining the mass of ASDN (both radiolabeled and nonradiolabeled)/g of solution. This calculation uses both the measured mass of nonradiolabeled ASDN used in the solution preparation and also the specific activity of the stock [^{3}H]ASDN.

2. the radiochemical content (mCi/g) of the solution is then divided by the mass of ASDN/g solution to arrive at the new specific activity for [^{3}H]ASDN in the substrate solution.

Data to be input include
Substrate solution aliquot weights (g) and DPM results
Weight (mg) of ASDN used in original stock and volume (mL) of the original stock
All dilution factors for the dilution of ASDN stock to the solution that was finally used in substrate preparation.
Weight (g) of ASDN dilution used to prepare substrate solution and total weight (g) of substrate solution
Specific activity of the stock [^{3}H]ASDN (μCi/mmol)

Protein Worksheet

This worksheet calculates protein content based on absorbance data of standards and unknown samples obtained when samples are analyzed using a commercially available kit.

Data to be input include the concentration of protein standard stock solution (mg/10 mL), protein stock ID, Sample IDs, absorbance data (in triplicate) for standards and unknowns and appropriate dilution factors.

Absorbance values are corrected for blank absorbance. A calibration curve is prepared by linear regression of the standards data (corrected absorbance vs. mg protein

measured). The concentration of protein in the unknowns is calculated based on the standard curve.

Microsome and Chemical Dilutions Worksheet

This worksheet calculates the concentration of protein in the final microsome dilution. It also serves as the data input center for the test chemical concentrations used in the assay.

Data input include volumes used in the preparation of microsome dilutions. Also entered is the protein concentration of the stock microsomes. Normally, this value will be determined using the protein worksheet described above.

Test chemical concentrations are entered in molar units of the final concentrations used in the assay.

Activity Calculation Worksheet

The primary aim of this worksheet is to calculate aromatase activity for each sample in a set based on measured DPM, protein concentration and incubation time.

The function of each section is described below:

Section 1 (Columns A-B)
This section contains fields for sample identification

Section 2 (Columns C-I)
This section calculates the total DPM that remain in the incubation mixture after extraction (this is a measure of the ^3H_2O formed in the reaction).

Data input:
Aliquot volume
DPM measured for each aliquot of each sample.

Output:
The worksheet calculates the average DPM/mL for each repetition (test tube sample) and the total DPM contained in the sample (based on the aliquots and total sample volumes)

Section 3: (Columns J-L)
This section calculates the percent turnover of the substrate to product.

Data input:.Volume of substrate solution used in each assay tube

Linked Data: Column K links to radiochemical content value for the substrate that is calculated in the substrate specific activity worksheet

Output: Percent conversion to product

Section 4 (Columns M-N)
This section calculates the nmol ^3H_2O formed

Data input: None

Linked Data: Column N links to specific activity value for the substrate that is calculated in the substrate specific activity worksheet

Calculations: Column M corrects the total DPM in each tube for background DPM determined in negative control tubes

Column N Converts DPM data to nmol using the substrate specific activity

Data output: nmol ^3H_2O formed

Section 5 (Columns O-R)

This section calculates aromatase activity in each tube.

Data input: Volume of diluted microsomes used in assay tube and incubation time

Output: Aromatase activity (nmol/mg protein/min)

Results Summary Worksheet

This worksheet summarizes the results.

Section 1 (Columns A-D, Rows 3-15)
This section summarized control data.

Data input: none

Output: average and SD for control samples for beginning, end and overall portions

Section 2 (Columns A-F, Rows 18-42)

This section summarizes activity values according to inhibitor level and replicate

Data input: None

Output: Log[test inhibitor]

Section 3 (Columns H-L, Rows 18-28)

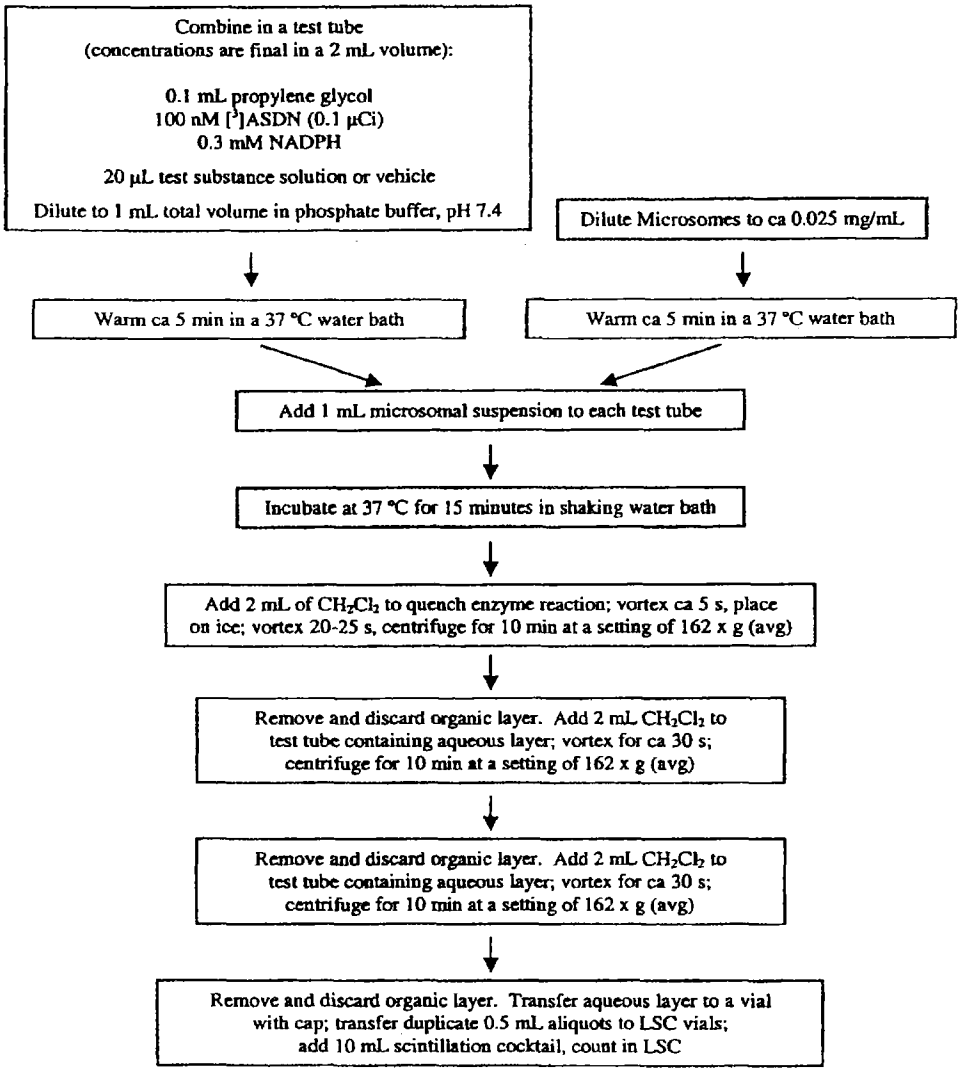
This section calculates percent of control values for each test chemical concentration and replicate and organizes the data in a format suitable for importation into Prism Software.

Data input: None

Output: Percent of control values with data arranged in a format suitable for importation into Prism Software.

Appendix B

Assay Procedure


Combine in a test tube
(concentrations are final in a 2 mL volume):
0.1 mL propylene glycol
100 nM [3]ASDN (0.1 µCi)
0.3 mM NADPH
20 µL test substance solution or vehicle
Dilute to 1 mL total volume in phosphate buffer, pH 7.4
Dilute Microsomes to ca 0.025 mg/mL
Warm ca 5 min in a 37 °C water bath
Warm ca 5 min in a 37 °C water bath
Add 1 mL microsomal suspension to each test tube
Incubate at 37 °C for 15 minutes in shaking water bath
Add 2 mL of CH2Cl2 to quench enzyme reaction; vortex ca 5 s, place on ice; vortex 20-25 s, centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH2Cl2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Add 2 mL CH2Cl2 to test tube containing aqueous layer; vortex for ca 30 s; centrifuge for 10 min at a setting of 162 x g (avg)
Remove and discard organic layer. Transfer aqueous layer to a vial with cap; transfer duplicate 0.5 mL aliquots to LSC vials; add 10 mL scintillation cocktail, count in LSC

Appendix C

Quality Assurance Project Plan

1.0 TITLE AND APPROVAL

Quality Assurance Project Plan (QAPP)
For Work Assignment 4-16
Placental Aromatase Validation Study

Task 5 -Conduct Multiple Chemical Studies
with Centrally Prepared Microsomes

for

EPA CONTRACT NUMBER 68-W-01-023

January 24, 2005

SIGNATURE PAGE

Quality Assurance Project Plan for WA 4-16, Task 5
Placental Aromatase Validation Study
EPA CONTRACT NUMBER 68-W-01-023

Concurrences and Approvals

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Appendix A: Template Protocol for Task 5

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4.0 PROJECT ORGANIZATION

The U.S. Environmental Protection Agency (EPA) is implementing the Endocrine Disruptor Screening Program (EDSP). To support this program, the EPA has contracted with Battelle to provide comprehensive toxicological and ecotoxicological testing services, including chemical, analytical, statistical, and quality assurance (QA)/quality control (QC) support, to assist EPA in developing, standardizing, and validating a suite of *in vitro*, mammalian, and ecotoxicological screens and tests for identifying and characterizing endocrine effects through exposure to pesticides, industrial chemicals, and environmental contaminants. The studies conducted will be used to develop, standardize and validate methods, prepare appropriate guidance documents for peer review of the methods, and develop technical guidance and test guidelines in support of the Office of Prevention, Pesticides and Toxic Substances regulatory programs. The validation studies will be conducted under the EDSP Quality Management Plan (QMP), study protocols, applicable Quality Assurance Project Plans (QAPPs), relevant program and facility Standard Operating Procedures (SOPs), guidance documents, and Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice Standards (GLPs).

One of the assays recommended for validation and consideration for inclusion in the screening program is the aromatase assay. A Detailed Review Paper (DRP) was prepared for the U.S. EPA in 2002 to review the scientific basis of the aromatase assay and examine assays reported in the literature used to measure the effect of chemical substances on aromatase.

Prevalidation studies on the aromatase assay (Work Assignment [WA] 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placental microsomes, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10).

The objectives of this work assignment are to use the now optimized assay: (1) to obtain intra- and interlaboratory assay variability estimates by conducting experiments at multiple laboratories, (2) to conduct microsome preparation and analysis experiments at multiple laboratories, and (3) to test up to 10 reference chemicals with different modes of action in order to evaluate assay relevance.

This work assignment is composed of multiple studies that are to be conducted by the lead laboratory (Research Triangle Institute International [RTI], Research Triangle Park, NC) and three participating laboratories (Battelle, Columbus, OH; In Vitro Technologies, Baltimore, MD; WIL Research Laboratories, LLC, Ashland, OH). This QAPP will address the work to be conducted in Task 5 of the work assignment.

A summary of the work assignment organization is shown in Figure 4-1.

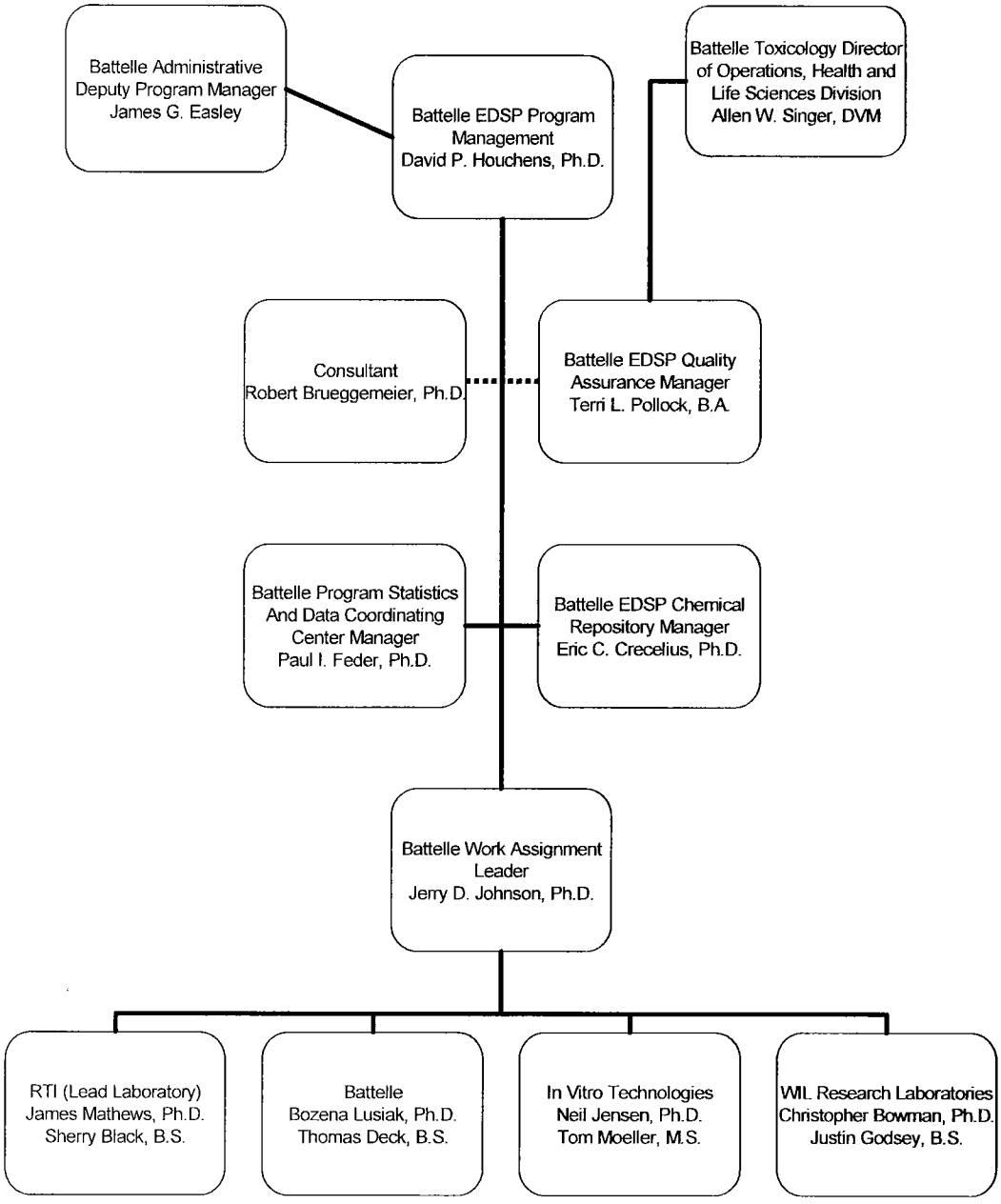


Figure 1. WA 4-16 Project Organization Overview

Portions of this work assignment will be managed at RTI, Battelle, WIL, and In Vitro. At each of these laboratories, there will be a person responsible for preparing the protocol, assigning appropriate staff to complete specified tasks within the protocol, and monitoring the progress of both technical and fiscal milestones as outlined in the technical work plan. A study director from each laboratory will report on the progress of the work assignment to Drs. David Houchens and Jerry D. Johnson at Battelle through a series of planned conference calls and through the use of written monthly reports.

General scientific direction and supervision of the work performed under this work assignment will be provided by Dr. Jerry D. Johnson, Battelle and Dr. James Mathews, RTI International. Dr. Johnson will serve as the Work Assignment Leader (WAL) for the participating laboratories and Dr. Mathews for the lead laboratory (RTI).

Each laboratory will have a study director in charge of overseeing the daily operation and conduct of the study. The individual laboratory teams will execute the necessary tasks required in the study protocols and ensure the data are collected and handled appropriately. All of these tasks are clearly defined in the study protocol.

The Quality Assurance Unit (QAU) representative for each laboratory will administer the QAPP for the EDSP facility QA team members. The specific responsibilities include:

- Interact with the Study Director to ensure that QA and QC procedures are understood by WA personnel.

- Conduct technical systems audits (TSAs) and audits of data quality (ADQs) to evaluate the implementation of the program WAs with respect to the EDSP QMP, the WA QAPPs and/or GLP protocol, and applicable program and facility SOPs.

- Prepare and track reports of deficiencies and submit them to both line and program management.

- Consult with the Study Director and, as necessary, the EDSP Battelle QA Manager and Program Manager on actions required to correct deficiencies noted during the conduct of the WA.

- Ensure that all data produced as part of the EDSP WAs are maintained in a secure, environmentally-protected archive.

- Ensure, during the conduct of TSAs, that all staff participating on the EDSP are adequately trained.

- Maintain complete facility-specific QA records related to the program.
- Submit copies of resolved audits to the EDSP Battelle QA Manager.
- Submit a QA Statement to the EDSP Battelle QA Manager and Program Manager with each written deliverable that describes the audit and review activities completed and any outstanding issues that could affect data quality or interpretation of the results discussed in the report.
- Maintain effective communication with the EDSP QA Manager.
- Act as the facility's EDSP SOP Custodian for all SOPs received from the SOP Administrator.

As EDSP manager, Dr. David Houchens will have ultimate responsibility for quality, timeliness, and budget adherence for all activities on the contract. He also will serve as the principal interface with the EPA's project officer on all contract-level administrative and technical issues. Because of the high level of subcontracting and purchases required by the program, such as test laboratory subcontracts and purchases of chemical supplies, Dr. Houchens will be assisted by an administrative deputy manager, Mr. James Easley. Mr. Easley will manage the procurement of all subcontracts, consultants, and purchased materials and services, and will facilitate schedule and cost control. He has played a similar role on ten other large, multi-year, level-of-effort task-order contracts for EPA. Thus, he will be able to assure that all purchases are compliant with government regulations and that EPA is provided timely, accurate accounting of these substantial costs in our monthly progress reports.

Ms. Terri Pollock, the EDSP QA manager at Battelle, will direct a team of QA specialists to monitor the technical activities on the chemical repository program, and provide oversight to all associated QA functions. Ms. Pollock will be responsible for reporting her findings and any quality concerns to Dr. Houchens. Ms. Pollock reports, for the purposes of this program, to Dr. Allen W. Singer, Director of Operations in the Toxicology Product Line in Battelle's Health and Life Sciences Division. This reporting relationship assures that the QA function is independent of the technical activities on the program.

5.0 PROBLEM DEFINITION/BACKGROUND

5.1 Problem Definition

Prevalidation studies on the placental aromatase assay (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of

the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10).

With the prevalidation studies successfully completed, this work assignment directs Battelle to conduct the interlaboratory studies to determine the performance of several laboratories in conducting the assay and should complete the validation of the placental aromatase assay. A companion work assignment (WA 4-17) has been issued for the conduct of the recombinant aromatase assay.

The work assignment is comprised of 9 tasks of which five tasks involve experimentation. Task 3 is a training task. The work in Tasks 4 through 7, is described in this QAPP. Table 1 summarizes the validation tasks and the laboratory(ies) involved for each experimental task.

Table 1. Validation Study Plan Experiments

Task Number	Description of Experimental Task	Experimental Task Assignment
1	Not applicable (Develop work plan, study plan, and identify/select participating laboratories)	Not an experimental task
2	Not applicable (Develop QAPP and protocols)	Not an experimental task
3	Training Participating Laboratories in the Conduct of the Assay	Lead Laboratory + 3 Participating Laboratories
4	Conduct Positive Control Studies in the Participating Laboratories	3 Participating Laboratories
5	Conduct Multiple Chemical Studies with Centrally Prepared Microsomes (RTI/Participating Laboratories)	Lead Laboratory + 3 Participating Laboratories
6	Prepare/Analyze Microsomes and Conduct Positive Control Study at Two Participating Laboratories; Analyze Microsomes at Lead and One Participating Laboratory	Lead Laboratory + 3 Participating Laboratories
7	Conduct Multiple Chemical Studies with Microsomes Prepared in Participating Laboratories (RTI/Participating Laboratories)	Lead Laboratory + 3 Participating Laboratories
8	Prepare Study Reports (RTI/Participating Laboratories)	Not an experimental task
9	Prepare Presentation for EDMVAC*	Not an experimental task

*EDMVAC = Endodrine Disruptor Method Validation Committee

5.2 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the U.S. EPA is implementing an EDSP. In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the EDMVAC will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) evaluating the literature and personal communications.

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome P450arom and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome P450arom and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The *in vitro* subcellular assay using human placental microsomes, is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these natural plant products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system, with IC_{50} values for aromatase inhibition ranging from 0.04 mM to greater than 50 mM.

The human placental microsomal aromatase assay was recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. Prevalidation studies on recombinant aromatase (WA 2-24) were conducted to optimize the microsomal aromatase assay protocol for human placenta, demonstrate the utility of the microsomal assay to detect known aromatase inhibitors, and compare the performance of a recombinant assay system and the placental microsomal assays. Concerns with this initial work involving high variability in some runs and partial inhibition curves were addressed in a supplemental prevalidation study (WA 4-10). The objective of the current work assignment is to use the now optimized assay to obtain intra- and interlaboratory assay variability estimates to complete the validation of the human placental microsome aromatase assay.

6.0 PROJECT/TASK DESCRIPTION

Only Task 5 is under the control of this QAPP. However, this QAPP also addresses the other three experimental tasks in this work assignment and will be reissued prior to the start of each new task together with a finalized task-specific template protocol included as an attachment. The Task 5 template protocol is attached to the present QAPP. The task numbering scheme for the original work assignment is employed in this document for ease of cross-referencing.

Task 4: Conduct Positive Control Studies in the Participating Laboratories

This task was completed by staff at Battelle, WIL and In Vitro. RTI staff did not conduct any experiments on this task but were involved in the review of the data produced by the other laboratories. RTI provided human placental microsomes to the other laboratories for use in this task. Battelle/RTI provided a boilerplate protocol for this Task to the participating laboratories which they used to prepare their laboratory-specific protocols. These protocols contained all necessary technical detail for the conduct of this Task. Briefly, the Task required that each laboratory conduct three independent replicates of a Positive Control Study. In this Study, 4-OH androstenedione (4-OH ASDN, a known aromatase inhibitor) was tested in the aromatase assay at 6 concentrations to construct a dose/response curve from which an IC_{50} may be calculated. Control runs also were included in the assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). Battelle's Chemical Repository (CR) supplied 4-OH ASDN to each laboratory as a stock solution and conducted all necessary pre-assay chemistry activities for 4-OH ASDN.

Each laboratory presented its results in a separate spreadsheet for each of the three replicates and the results were compared both within and between laboratories.

The results of this experiment require technical review and approval prior to proceeding to Task 5.

Task 5: Conduct Studies with Centrally Prepared Microsomes

This Task will be completed by staff at RTI, Battelle, WIL and In Vitro. RTI will provide human placental microsomes to the other laboratories for use in this task. Battelle/RTI will provide a boilerplate protocol for this Task to the participating laboratories which they will use to prepare their laboratory-specific protocols. These protocols will contain all necessary technical detail for the conduct of this Task. Briefly, the Task requires that each laboratory conduct three independent replicate studies on each of four test chemicals. All three replicates for a given chemical will be conducted by the same technician within a laboratory. Control runs are also included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). In additional positive control samples (containing a known aromatase inhibitor) and negative control samples (containing a known aromatase non-inhibitor) will be included in each assay set. Battelle's CR will supply the test and control chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test and control chemicals.

Each laboratory will present their results in a separate spreadsheet for each of the three replicates and the results will be compared both within and between laboratories.

The results of this experiment would require technical review and approval prior to proceeding to Task 7.

Task 6: Prepare Microsomes in Two Participating Laboratories

There are two activities in this Task. The first, to be conducted by Battelle and In Vitro, requires those laboratories to obtain a human placenta, prepare microsomes and then to analyze their microsome preparations for protein content and (uninhibited) aromatase activity. In addition, those laboratories will conduct two independent replicates of the Positive Control Study (as used in Task 4) using their microsomal preparations. RTI/Battelle will supply a template protocol that includes all technical detail required for the conduct of these experiments. Battelle's CR will supply 4-OH ASDN to each laboratory as a stock solution. The laboratories will submit the results of these studies to Battelle and the data will be reviewed by Battelle and RTI prior to submission to EPA. After EPA approves the results, the second portion of the Task can be initiated.

For the second activity in this Task, Battelle and In Vitro will each ship portions of their placental microsomes preparations to the other three participating laboratories. Each laboratory will measure the protein content and (uninhibited) aromatase activity of the microsomal preparations from both laboratories.

Each laboratory will present their results in a separate spreadsheet for each replicate and the results will be compared both within and between laboratories.

Task 7: Conduct Studies with Microsomes Prepared in Participating Laboratories

Battelle and In Vitro will conduct the studies in this task with microsomes prepared in their laboratory in Task 6. RTI and WIL will receive microsomes from Battelle and In Vitro, respectively, for use on this task.

RTI/Battelle will supply a template protocol describing all technical details for this task to the participating laboratories from which they will prepare their laboratory-specific protocols. Each laboratory will conduct three independent replicate studies with each of 10 chemicals. All three replicates for a given chemical will be conducted by the same technician within a laboratory. Control runs also will be included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). In addition, positive control samples (containing a known aromatase inhibitor) and negative control samples (containing a known aromatase non-inhibitor) will be included in each assay set. Battelle's CR will supply the test and control chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test and control chemicals.

7.0 QUALITY OBJECTIVES AND CRITERIA

The endpoints for WA 4-16 include the aromatase activity measured in the control and inhibitor samples, the inter- and intralaboratory variance, and the IC_{50} and slope values for each inhibitor tested.

7.1 Data Quality Indicators

7.1.1 Precision

The activities of replicate tubes should be within the mean activity ± 15%. Each control activity for each assay/laboratory should be within the overall mean ± 15% activity for that control type for that laboratory.

Variance between laboratories and within laboratories will be assessed for an appropriate level of precision as part of this WA. It is anticipated that full aromatase control activity between and within laboratories should be statistically equivalent at the $p > 0.1$ level. Any modifications to this criterion will be discussed with the sponsor and added to the QAPP by amendment.

IC_{50} and slope values calculated for each inhibitor should be statistically equivalent at the $p>0.1$ level both between and within laboratories. If data from an assay are statistical outliers, the assay may be repeated.

7.1.2 Bias

The control samples that are run with each assay will be used to control for bias. If the control samples for any assay do not meet the precision criteria described above, the assay may be rerun. Assays will be conducted blind at the technician level for test chemical identity.

7.1.3 Accuracy

Accuracy of the liquid scintillation spectrometry (LSS) data (from which is derived the aromatase activity) will be assessed by analysis of a sealed standard of known radioactive content. If the radioactivity in the sealed standard is more than 5% different from the known value, the data will not be used. Samples may be recounted on another LSS or on the same LSS after any problems with the instrument are corrected.

8.0 SPECIAL TRAINING/CERTIFICATION

All personnel involved in handling radiolabeled materials will have completed a Radiation Safety Training course. Training documentation will be maintained in the individual training files. Each laboratory will be licensed to receive radiolabeled materials.

All personnel involved in handling human placental microsomes will have appropriate training in the handling and disposition of biohazards. Training documentation will be maintained in the individual training files.

Staff from the participating laboratories have been trained on the performance of the aromatase assay at RTI International as part of Task 3 of this work assignment. Personnel participating in this training conducted the aromatase assay including full aromatase control and background control samples and a series of samples containing varying amounts of a known aromatase inhibitor (4-OH ASDN). The resultant data were evaluated by Battelle and RTI International and then submitted to EPA for review.

9.0 DOCUMENTS AND RECORDS

9.1 Retention of Specimens and Records

Archiving procedures will be specified in the individual protocols.

9.2 Quality Assurance Project Plan

This QAPP will be distributed to project participants initially, and whenever revised. Previous versions will be marked as "obsolete" when newer versions are distributed, or collected and destroyed so that there is no confusion regarding the version in effect. The right-justified document control header example shown here

Version 1
Month, Year
Page 1 of 1

is used to ensure that revision numbers and dates are obvious to document users. The QAPP will be reviewed annually and a determination made to either modify the document based on new or modified project requirements, or leave as is.

Controlled copies of the QAPP will be maintained, tracked, and managed by the laboratories' QAU through the use of a master distribution list.

9.3 Data Forms

All data forms will include a title identifying the type of data to be recorded, a unique study code or protocol number, and the initials and date of the data recorder(s) to authenticate the records.

Corrections to data entries will be made by drawing a single line through the error, recording the correct entry, initials, date, and error code that explains the reason for the correction.

9.4 Microsome Storage Conditions

Microsomes must be stored at -70 to -80°C and the freezer temperature records must be maintained.

9.5 Reports

9.5.1 Interim Data Summary, and Draft and Final Reports

An interim data summary from each laboratory will be submitted to the EPA after completion of each task. These data summaries will not be audited by Quality Assurance but will be checked for accuracy by technical staff. This procedure is necessary to provide a rapid turn around of the data so that approval to proceed can be given by EPA.

Each laboratory will prepare an individual report for each task to be based on a template provided by Battelle and will submit these reports to Battelle. The purpose of these reports is to provide a complete description about how the experiments were performed, present the results that were obtained (including tables and graphs), and state the conclusions that were made for each applicable WA task. RTI/Battelle will prepare a report for each task that summarizes all work on the particular task and incorporates the reports from the participating laboratories as Appendices for submission to EPA. After EPA comments have been received on each task report and, if applicable, incorporated into a new version of the draft task report, then it will be issued as a final report.

Each final task report will include:

- Abstract
- Objectives
- Materials and Methods
- Results
- Discussion
- Conclusions

- References
- Summary data with statistical analyses
- Appendices which will include final reports with compliance statements for each participating laboratory
- Protocol, any amendments, or any deviations from the protocol
- QAPP, any amendments, or any deviations from the QAPP.

RTI/Battelle will prepare a final Work Assignment report that summarizes the results of the entire Work Assignment. This report will consist of a statement of the objectives of the work assignment, a summary of the results and a statement of conclusions for the Work Assignment. The individual task reports will be referenced within this final report.

9.5.2 QA Assessment Reports

QA assessment reports are maintained as confidential files in the QAU.

9.5.3 Status Reports

Status/progress reports will be submitted to the EPA Project Officer by Battelle on a monthly basis as stipulated in the contract.

10.0 SAMPLING PROCESS DESIGN (EXPERIMENTAL DESIGN

The details of the experimental design for the task subject to this QAPP will be contained in a GLP compliant protocol. A template protocol for this task is attached as an Appendix to this document.

11.0 SAMPLING METHODS

The entire aqueous portion of the incubation mixtures remaining after extraction with methylene chloride (CH_2Cl_2) will be placed in appropriate containers. The samples will be mixed well prior to the removal of aliquots for liquid scintillation counting (LSC). If there is insufficient time for preparing LSC samples on the day the assay is run, the samples will be refrigerated overnight. Samples remaining after preparation of LSC aliquots should be frozen and stored at about -20°C. These samples may be thawed, mixed and realiquoted, if necessary, due to problems with LSC samples.

Each test and standard chemical will be supplied to the participating laboratories by Battelle as a stock solution at the highest concentration necessary for use in the assay. These

solutions will be well-mixed prior to the preparation of dilutions of these stock solutions by the individual participating laboratories.

12.0 SAMPLE HANDLING AND CUSTODY

12.1 Test and Reference Chemical Solutions

The test and standard chemical stock solutions will be transferred to the Laboratories' Material Handling Facility with a study specific transfer of material form. The samples will be processed according to the SOPs for packing, shipment and documentation of shipment and receipt.

12.2 Sample Collection Documentation

All samples (or sample sets) will be labeled with enough information to allow for unequivocal identification of each sample along with suitable storage conditions in accordance with applicable regulations.

13.0 ANALYTICAL METHODS

Analytical methods are described in the study protocol (Appendix). Failures of analytical systems are addressed in the relevant SOPs.

14.0 QUALITY CONTROL

14.1 Methods

Control samples are run with each assay. These include 1) full aromatase enzyme activity controls (FEAC), 2) background controls (BAC), 3) positive controls and 4) negative controls. Acceptance criteria and corrective actions where acceptance criteria are not met are described in Section 7. Replicates are used as a means to monitor variability of the assay. Replicates will be assessed for variance and those that are outside the acceptable range (mean ± 15%) will be flagged as statistical outliers.

14.2 Data Collection

Data collection documentation will be as described in applicable SOPs or protocols.

Assay data, including weights and/or volumes of chemicals, solvents or other materials used to prepare necessary solutions or samples, will be recorded manually on data sheets. Protein assay absorbance data may also be recorded manually on data sheets. All data sheets include a title identifying the type of data to be recorded, the unique study code or protocol number, and the initials and date of the data recorder(s) to authenticate the records.

Scintillation counter data will be automatically saved to a data file that will automatically be assigned a unique filename. The data must be annotated to identify samples with the sequential vial number. Procedures for converting CPM data to DPM data must be documented.

Relevant data from the data sheets and scintillation counter output (as DPM) will be typed into a validated MS Excel spreadsheet for calculation of 1) substrate specific activity 2) protein content and/or 3) aromatase activity. All transcribed data will be verified (100% QC) before they are reported and this QC check will be documented on the spreadsheet printouts by technician initials and date.

Aromatase activity data will be entered automatically (through linked validated spreadsheets) or manually into Prism data files for calculation of IC_{50}. Data will be entered automatically (through linked validated spreadsheets) or manually into spreadsheets for import into SAS data files for statistical analysis. All manually entered data will undergo a 100% QC check.

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

The following types of equipment are required for this WA: temperature controlled shaking water bath, pH meter, analytical balances, centrifuges (low and high speed and ultracentrifuges), pipettors, scintillation counters, spectrophotometer, and high performance liquid chromatography (HPLC) equipment (injector, pumps, detectors [radiochemical and ultraviolet {UV}], data collection system). The equipment will be tested, inspected and maintained according to schedules contained in the relevant SOPs.

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

Balances used to obtain weight measurements, as well as the check weights that are used to verify a balance's calibration status will be calibrated and maintained according to the schedule specified in relevant SOPs. Balances that do not meet the criteria specified in the SOP will not be used for this work assignment.

Scintillation Counters will be calibrated using procedures described in the relevant SOPs. Calibration of pH meters occurs as specified in relevant SOPs. The water bath, pipettes,

spectrophotometer, and HPLC equipment will be calibrated using the procedures and schedule in applicable SOPs. Any equipment or instrument that does not meet acceptance criteria as described in the relevant SOP will not be used for this work assignment.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

Upon receipt, purchased items must be inspected for conformance to quality requirements prior to use. All use of the product must be prior to the expiration dates, if applicable. Chemicals will be received and stored in accordance with applicable SOPs.

18.0 NON-DIRECT MEASUREMENTS

No collection of any samples or sample data will be obtained from non-direct measures such as computer data bases or programs.

19.0 DATA MANAGEMENT

19.1 Data Management Overview

Data will be maintained in notebooks and/or files according to applicable facility SOPs. The records will be kept in the appropriate rooms until there is a signed final report at which time they will be inventoried and placed in the facility archives according to applicable facility SOPs, unless the sponsor requests that they be transferred to another archive location.

19.2 Data Transfer

Information will be sent to the Data Coordination Center in electronic format as specified in SOP EDSP.D-003-01. Specifically all raw data, all tables, graphs summarizing results of statistical analyses as presented in study reports, statistical analysis data files, statistical analysis programs, and all study documents will be sent to the EDSP Data Coordination Center in electronic format.

20.0 ASSESSMENTS AND RESPONSE ACTIONS

EDSP QA team members will perform assessments on WA activities and operations affecting data quality and the raw data and final report. They will report any findings to the Study Director and management to ensure that the requirements in relevant SOPs, study protocols and

WA QAPP, the QMP, and the FIFRA GLPs are met. The assessments for this study include TSAs and ADQs. Performance Evaluations do not apply to this QAPP.

20.1 Technical Systems Audits

A TSA is a process by which the quality of a study is assessed through evaluating a study activity's conformance with the protocols, applicable facility or program SOPs, QAPP, QMP, and GLPs. The acceptance criteria are that WA activities and operations must meet the requirements of these planning documents and the GLPs or be explained and evaluated in a deviation report. Deviations from the GLPs, QAPP, protocol, or SOPs will be properly documented and assessed by management and the study director as to their impact on the study.

20.2 Type, Scheduling, and Performance of Technical Systems Audits

The following paragraphs provide an example of how the laboratories may perform technical system audits.

Prior to the experimental start, the facility QA Team Member will convey a list of inspections targeted for the study to the study director. Whenever possible, TSAs should be done at the commencement of the WA critical phase to ensure WA integrity based on compliance with the protocol, QAPP, SOPs, and GLPs. Critical phases targeted for TSAs include, but are not limited to:

- Protocol review
- Placental collection and microsome preparation
- Aromatase assay sample preparation and analysis.

During the TSA, EDSP QA team members will record observations to be used later in preparing the audit report. EDSP QA team members will observe the procedure, data recording, and any equipment maintenance and calibration procedures and/or documentation, noting whether or not the activities adhered to the study protocols and QAPP, applicable SOPs, QMP, and the GLPs. Any findings will be communicated to the technical personnel at the completion of the procedure unless an error could compromise the study (e.g., misdiluting the stock solution). EDSP QA team members immediately notify the Study Director by telephone and/or e-mail of any adverse findings that could impact the conduct of the study. This direct communication will also be documented in the audit report.

20.3 Audits of Data Quality

An ADQ is a process by which the accuracy of data calculations and reporting will be assessed to ensure that the reported results are of high quality and accurately reflect the raw data and accurately describe the materials used in the study. The acceptance criteria for the ADQ are

that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA protocols and QAPP, QMP, and the FIFRA GLPs, or be explained and evaluated in a deviation report, as previously described.

20.4 Scheduling and Performance of Audits of Data Quality

Direct and frequent communication between the WA Leader/Study Director, laboratory supervisor, and the QA Manager will provide for sufficient time to perform an ADQ so that the submission date of the draft final report meets that specified in the study protocol. The scheduling process should also allow for a reasonable amount of time for corrections and subsequent verification of the corrections by QA.

EDSP QA team members will audit the study records at a frequency adequate to ensure that approved protocol requirements are met. The frequency required is specified by the type of data in the QMP, Section 2.4.1. Findings will be reported and corrective actions undertaken as described earlier. EDSP QA team members will review the final report using the audited data and corrected tables. The report text will be reviewed to ensure that every statement is supported by the data and any discussions or conclusions drawn from the study are supported by the data. Findings will then be reported and corrective actions undertaken as described earlier.

20.5 Audit Report Format

The following paragraphs provide an example of how the laboratories may format an audit report.

The audit report consists of a cover page for study information and additional page(s) with the audit findings. All pages have header information containing the study protocol number, audit report date, and audit type. The audit report date is the date on which the EDSP QA team member signs the audit report and sends it to the Study Director and management.

The cover page contains the study protocol title, number, and code; Sponsor; Study Director; audit type; audit date(s); EDSP QA team member; distribution list; the dated signature of the auditor; the date that the Study Director received the audit report; and the dated signatures of the Study Director and management. The distribution list may include additional names for individuals who have findings pertaining to their area of responsibility (e.g., the ARF Manager would address a finding pertaining to the ARF) and is used to ensure that the report is sent to all who need to respond. Subsequent page(s) contain the audit finding(s), any recommended remedial actions, and space for the Study Director to respond to the findings and document remedial actions taken or to be taken.

20.6 Response Actions and Resolution of Issues

The Study Director will respond to the TSA report within a specified number of working days of receipt of the report as required by the laboratory's SOPs. There is no deadline for the Study Director's response to an ADQ report except for the time constraint deriving from the submission date of the final WA report. The Study Director forwards the audit report to management for review. Management adds comments as necessary, signs and dates the report and returns it to the EDSP QA team member. The EDSP QA team member assesses the responses and verifies the corrective actions. If a disagreement between the Study Director and EDSP QA team member arises over a finding, it will be discussed among the other EDSP QA team members. The EDSP QA team member will then present the majority opinion to the Study Director for further consideration. If the disagreement remains, the issue will be reported to the Study Director's management. The action decided on by management will be documented in the QA files.

During an assessment, if the auditor determines that adverse health effects could result or WA objectives of acceptable quality cannot be achieved, the auditor follows the Stop Work Procedure specified in the EDSP QMP (Section 3.3).

20.7 Independent Assessments

The EDSP Battelle QA Manager (QAM), or designee, may conduct an independent TSA and ADQ during the conduct of this work assignment. Typically one independent audit may be conducted during the work assignment. If major deficiencies are uncovered, additional independent audits may be scheduled. The conduct and reporting of the audits will be consistent with the procedures described in the EDSP QMP (Section 3.3).

In addition, the EDSP EPA QAM, or designee, has the option of conducting external TSAs/ADQs.

21.0 REPORTS TO MANAGEMENT

The QA Manager will send periodic reports to the study director and management, which detail significant regulatory, protocol, and SOP issues. Also, the participating laboratories will report to the EDSP Program Manager and WAL.

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel for the validation process and by EDSP QA team members for the verification process (see

section 23). The criteria used for validation depend on the type of data. For dose solution sample data, information regarding the condition of the containers and whether or not samples were compromised is recorded in the sample chain-of-custody records. Compromised samples are not analyzed. The criteria for validating data are those found in Section 7 (Data Quality Objectives).

23.0 VERIFICATION AND VALIDATION METHODS

23.1 Chain of Custody for Data

Study data, records, and specimens will be maintained in a secure and designated location, e.g., in the respective laboratory offices until study completion. Chain-of-custody procedures will be implemented according to facility SOPs. Chain-of-custody information, including the date, study record(s) removed or returned, and the name of the person removing or returning the data will be documented. At study completion, the Study Director will follow the procedures specified in the facility SOP for archiving study materials.

23.2 Data Validation

Data validation is a process by which the WA Leader/Study Director and/or other technical personnel evaluate the data for conformance to the stated requirements for methodology and quality. These personnel are responsible for reviewing the data, evaluating any technical deviations or non-conformances, and then determining the degree to which the data meet the quality criteria stated in Section 7.

23.3 Data Verification

Data verification constitutes part of the ADQ process performed by EDSP QA team members and described earlier. Verification ensures that 1) the data are of high quality and were collected according to the planning documents' requirements, and 2) the reported results accurately reflect the raw data. Each data type will be evaluated against its collection and reduction requirements specified in the planning documents. Errors discovered during the data evaluation will be corrected. The reported conclusions drawn from the data are verified by EDSP QA team members during the report audit to confirm that they are true and accurate. The procedure for resolving issues of data verification has been detailed in prior sections of this document.

24.0 RECONCILIATION AND USER REQUIREMENTS

Proposed methods for data analysis, including a test for statistical outliers, are specified in the Study Plan and/or protocols.

25.0 REFERENCES

The following references were used to prepare the QAPP. Not all references are cited in the text.

Battelle (2003). Endocrine Disruptor Screening Program Quality Management Plan, Version 2. May 12, 2003.

Battelle (2004). Technical Work Plan on Microsomal Aromatase Validation Study, EPA Contract Number 68-W-01-023, Work Assignment 4-16. September 8, 2004.

FQPA (1996). Food Quality Protection Act of 1996, U.S. Public Law 104-170, 21 U.S.C. 46a(p), Section 408(p), 110 STAT.1489. August 3, 1996.

Appendix D

[^{3}H]ASDN Purity Assessment Report (RTI International)

DRAFT ANALYSIS REPORT

PLACENTAL AROMATASE VALIDATION STUDY

[^{3}H]ASDN Radiochemical Purity Determination

EPA Contract Number 68-W-01-023
Work Assignment 4-16

Sponsor:

Battelle Memorial Institute
505 King Avenue
Columbus, OH 43201-2693

Performing Laboratory:
Drug Metabolism and Pharmacokinetics
RTI International
Post Office Box 12194
Research Triangle Park, NC 27709

DRAFT REPORT

Title:	PLACENTAL AROMATASE VALIDATION STUDY [^{3}H]ASDN Radiochemical Purity Determination
Author:	Sherry Black
Performing Laboratory:	Drug Metabolism and Pharmacokinetics RTI International Post Office Box 12194 Research Triangle Park, NC 27709
Sponsor:	Battelle Memorial Institute 505 King Avenue Columbus, OH 43201-2693
Sponsor=s Representatives:	David Houchens, Ph.D. EDSP Program Manager Battelle Jerry D. Johnson, Ph.D. Diplomate, A.B.T. Work Assignment Leader Battelle
Study Initiation Date:	
Experimental Dates:	January 5, 2005
Draft Task Report Date	February 3, 2005

Author:		Approved:	
Sherry Black Research Chemist	Date	James Mathews, Ph. D, DABT Study Director	Date

Introduction

The objective of this work is to determine the radiochemical purity of the [3H]ASDN to be used in the conduct of WA 4-16 and WA 4-17. The criteria for acceptance of the material for this use is 95% radiochemical purity as determined by high performance liquid chromatography (HPLC) and liquid scintillation counting.

Materials and Methods

[^{3}H]Androstenedione ([^{3}H]ASDN) of lot number 3538496 was received from Perkin Elmer Life Science (Boston, MA).

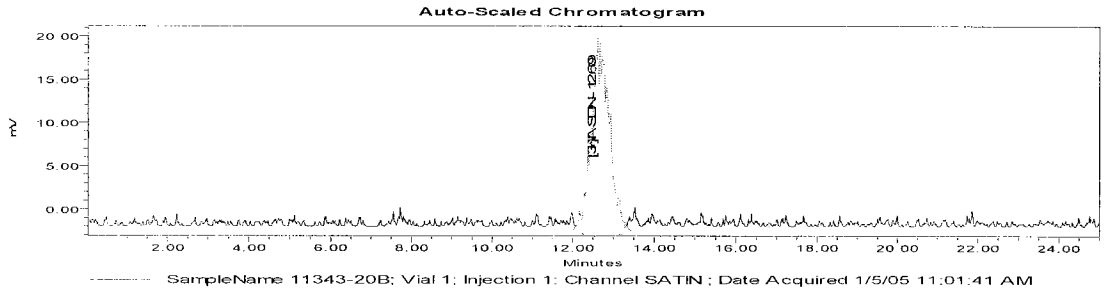
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through radioactivity detector (IN/US, Inc., Tampa, FL) with a 250 μL glass scintillant cell. Data was collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method used a Zorbax Rx-C_{18} column (4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant was monitored by ultraviolet (UV) absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions were collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS)

Results

The HPLC radiochromatogram of the [^{3}H]ASDN, lot number 3538496, is presented in Figure 1. The measured radiochemical purity of the [^{3}H]ASDN was 97%.

Figure 1. HPLC Radiochromatogram of [3H]ASDN



Conclusion

[^{3}H]ASDN, lot number 3538496, is acceptable for use on WA 4-16 and WA 4-17.

Appendix E

Chemistry Reports (Sponsor-Provided Data)

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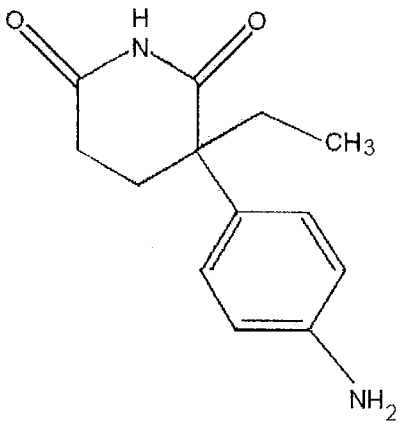
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

AMINOGLUTETHIMIDE

CAS No.: 125-84-8	Lot No.: 043K0939 (Sigma Aldrich)
Receipt Date: 10/27/04	Amount Received: 2.40 g
Appearance: Solid	Vendor Purity: >99% by TLC
Receipt Date: 6/24/05	Lot No.: 06016JS (Sigma Aldrich)
Appearance: Solid	Amount Received: 3.0 g
Storage Conditions (@ Battelle): Room Temperature (~25°C)	Vendor Purity: 99% by TLC

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	232.28 g/mol	$C_{13}H_{16}N_2O_2$

Prepared By:

Denise A. Contos, M.S.

Approved By:

10-18-05

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and Management as follows:

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Test substance receipt*	10/26/04	10/26/04
Formulation preparation*	12/2/04	12/2/04
Dispensing*	12/2/04	12/2/04
Formulation analysis*	12/2/04	12/2/04
Audit study file	7/26/05	7/26/05
Audit analytical report	7/26/05	7/26/05
Audit study file	10/5/05	10/5/05
Audit analytical report	10/5/05	10/5/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Hillary Flory 10-14-05
Quality Assurance Unit Date

EXECUTIVE SUMMARY

The title compound, aminoglutethimide (AG), was analyzed in support of the Environment Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

Solubility of aminoglutethimide was determined to be acceptable in dimethylsulfoxide (DMSO) at a concentration of 23.2 mg/mL (0.1 M).

An aminoglutethimide formulation analysis method was validated on the previous EPA WA 3-10 study. This method was used without technical modification for analysis of formulation and stability samples on the current study.

Storage stability was previously determined (EPA WA 3-10 study) as 39 days when stored at approximately 5°C and protected from light at a target formulation concentration of 27.6 mg/mL in DMSO. In the current study, a formulation sample at a target concentration of 23.2 mg/mL in DMSO was stable when stored refrigerated and protected from light for 59 days.

The stock formulation prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for aminoglutethimide on EPA Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO)
- Preparing and analyzing a stock formulation and a formulation stability sample.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle containing 2.40 grams of aminoglutethimide, Lot No. 043K0939, and one 30-mL clear glass bottle containing 3.0 grams of aminoglutethimide, Lot No. 06016JS was received on October 27, 2004 and on June 24, 2005, respectively, from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA. The chemicals were received and subsequently stored at room temperature.

A copy of the manufacturer's Certificates of Analysis for these lots are shown in Figures 1 and 2. The purity of the chemicals were > 99% and 99%, respectively, based on thin layer chromatography.

SIGMA-ALDRICH

Certificate of Analysis

Product Name	DL-Aminoglutethimide
Product Number	A9657
Product Brand	SIGMA
CAS Number	125-84-8
Molecular Formula	$C_{13}H_{16}N_2O_2$
Molecular Weight	232.28

TEST	SPECIFICATION	LOT 043K0939 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML OF ACETIC ACID:METHANOL (1:1)	CONFORMS
IDENTITY	CONSISTENT WITH STRUCTURE BY IR OR NMR	IR SPECTRUM CONFORMS
CARBON	66.0 TO 68.5%	67.2% *
NITROGEN	11.8 TO 12.3%	12.1% *
PURITY BY THIN LAYER CHROMATOGRAPHY	98% MINIMUM	> 99%
		* SUPPLIER'S INFORMATION
SHELF LIFE SOP QC-12-006	7 YEARS	OCTOBER 2007
QC ACCEPTANCE DATE		APRIL 2003
PRODUCT CROSS REFERENCE INFORMATION		REPLACEMENT FOR ALDRICH #259195

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis, Lot No. 043K0939

3050 Spruce Street
Saint Louis, Missouri 63105 USA
Telephone (800) 521-8956 • (314) 771-5765
Fax (800) 325-5052 • (314) 77-5757
Visit Us At www.sigma-aldrich.com

Certificate of Analysis

11236693MEC
BATTELLE NORTHWEST
1529 W SEQUIM BAY RD
SEQUIM WA 98382

PO NBR: 11236693MEC

PRODUCT NUMBER: 259195-500MG

LOT NUMBER: 06016JS

PRODUCT NAME: AMINOGLUTETHIMIDE, 99%

FORMULA: C13H16N2O2

FORMULA WEIGHT: 232.29

APPEARANCE	WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD AS ILLUSTRATED ON PAGE 3072B OF EDITION I, VOLUME 2 OF "THE ALDRICH LIBRARY OF FT-IR SPECTRA".
ELEMENTAL ANALYSIS	CARBON 66.60% HYDROGEN 6.93% NITROGEN 11.94%
THIN-LAYER CHROMATOGRAPHY	CONSISTENT WITH 99% PURITY
SOLUBILITY	5% HOAC:MEOH 1:1; SLIGHTLY HAZY, FAINT YELLOW SOLUTION.
QUALITY CONTROL ACCEPTANCE DATE	JULY 1998

ALDRICH CHEMICAL COMPANY
RONNIE MARTIN
NOVEMBER 5, 2003

We are Committed to the success of our Customers, Employees and Shareholders through leadership in Life Science, High Technology and Service.

Figure 2 – Certificate of Analysis, Lot No. 06016JS

3 SOLUBILITY STUDY

A solubility study was conducted to determine the solubility of aminoglutethimide in 100% dimethylsulfoxide (DMSO) at a target concentration of 23.2 mg/mL (0.1 M). The solution was prepared by weighing 0.23228 ± 0.02322 g of aminoglutethimide into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and the contents were mixed. The flask was diluted to volume with DMSO, sealed and mixed. The aminoglutethimide easily went into solution with shaking. This experiment showed that DMSO was an acceptable solvent for a 23.2 mg/mL (0.1 M) formulation.

4 FORMULATION PREPARATION AND ANALYSIS

A formulation was prepared and analyzed on January 24, 2005 according to SOP No. COMSPEC.II-007-01, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Aminoglutethimide (AG) in 100% DMSO." In addition, the January 24, 2005 formulation was re-analyzed to determine stability on March 24, 2005, 59 days of storage at approximately 5°C and protection from light. A second formulation was prepared and analyzed on June 30, 2005 according to SOP No. COMSPEC.II-007-01. The following sections describe the method, results, and conclusions.

4.1 Preparation of Formulation

Aminoglutethimide formulations with a target concentration of 23.2 mg/mL (0.1M) in DMSO were prepared on January 24, 2005 (Batch 1-AG) and on June 30, 2005 (Batch 2-AG) by accurately weighing 1160.00 ± 46 mg of aminoglutethimide into a tarred 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and mixed well until the aminoglutethimide dissolved. The content of the flask was diluted to volume with DMSO, sealed, and mixed well.

4.2 Preparation of Standards and Blanks

4.2.1 Internal Standard (IS)

An internal standard (IS) solution was prepared by pipetting 100 µL of octanophenone into a 100-mL volumetric flask. The content of the flask was diluted to volume with acetone, sealed, and mixed well.

4.2.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 35 ± 1 mg and 30 ± 1 mg of aminoglutethimide into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with acetone. This produced stocks A and B with target concentrations of 1.4 and 1.2 mg/mL, respectively.

4.2.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 1. The contents of the flasks were diluted to volume with acetone, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 1 – Preparation of Vehicle/Calibration Standards

Vehicle/ Calibration Std	Target Conc (µg/mL)	Source	Source Volume (mL)	IS Volume (mL)	DMSO Volume (mL)	Final Volume (mL)
VS1	56	A	2	1	0.10	50
VS2	48	B	2	1	0.10	50
VS3	28	A	1	1	0.10	50
VS4	24	B	1	1	0.10	50

4.2.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.10 mL of DMSO into three individual 50-mL volumetric flasks. The contents of the flasks were diluted to volume with acetone, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.10 mL of DMSO into three individual 50-mL volumetric flasks. The contents of the flasks were diluted to volume with acetone, sealed, and mixed well.

4.3 Preparation of Formulation and Formulation Stability Samples

In triplicate, 0.10 mL of the formulation and 1 mL of the IS were pipetted into individual 50-mL volumetric flasks, diluted to volume with acetone, sealed, and mixed well.

4.4 Analysis

A portion of each vehicle/calibration standard, blank, and sample was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the GC parameters for aminoglutethimide shown in Table 2.

Table 2 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-1, 30 m × 0.53 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at 10 mL/minute
Oven Temperature	160°C, hold for 1 minute, increase at 10°C/minute to 300°C
Detector Type	FID
Detector Flow Rates	Hydrogen at 30 mL/minute; Air at 300 mL/minute
Detector Temperature	300°C
Injector Temperature	260°C
Injection Volume	1 µL using a split ratio of 2 and a flow of 20 mL/minute
Run Time	15 minutes

4.5 Calculations

The integration of the aminoglutethimide and the IS peaks by the chromatography data system were evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation, un-weighted, was calculated relating the response ratio, aminoglutethimide/IS, (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each vehicle/calibration standard and formulation sample. The percent relative error (%RE) for each vehicle/calibration standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent relative error for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.6 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank as presented in Figure 3. The blank and blank with IS exhibited no peaks that would significantly interfere with the aminoglutethimide or IS peaks.

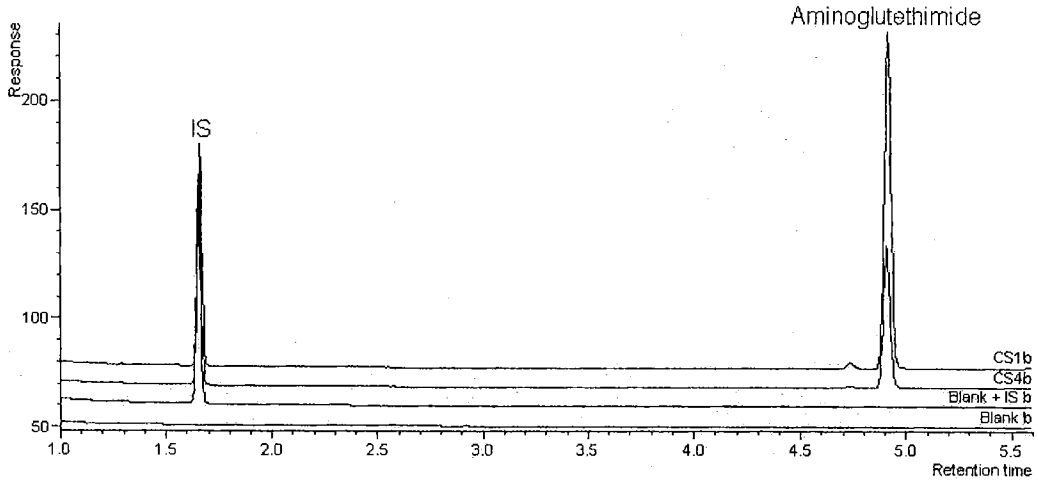

Figure 3 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from 1/24/2005 Formulation Analysis (Shown Top to Bottom)

The regression analysis results from the vehicle/calibration standard curves for the analyses indicated linearity and are shown in Figures 4 and 5.

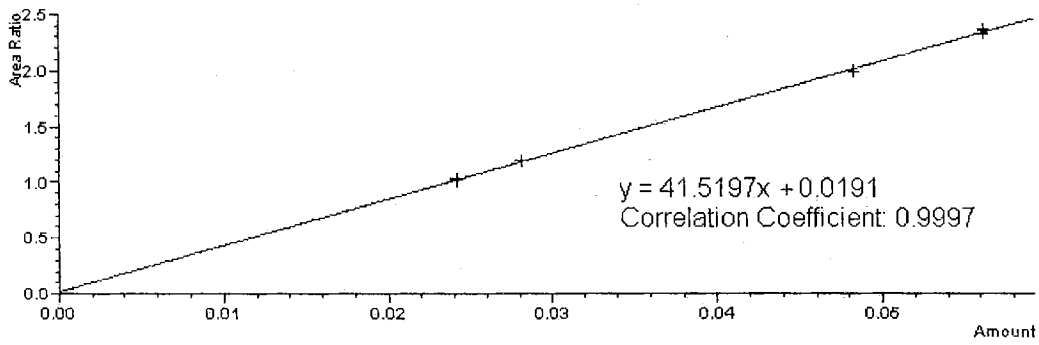

Figure 4 – Vehicle/Calibration Standard Curve for 1/24/2005 Analysis

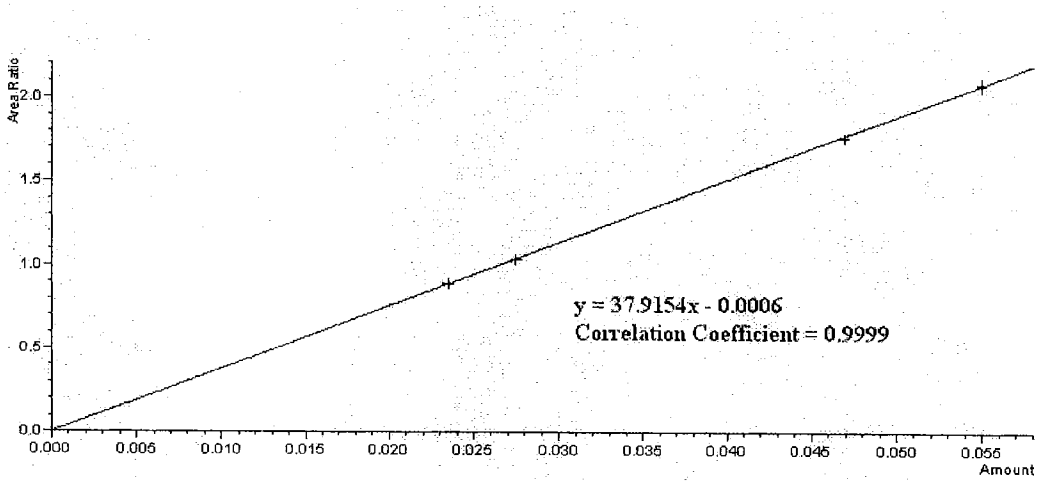

Figure 5 – Vehicle/Calibration Standard Curve for 6/30/2005 Analysis

The precision and accuracy results from a representative vehicle/calibration standard of January 24, 2005 analysis is shown in Table 3.

Table 3 –Vehicle/Calibration Standard 1/24/2005 Analysis Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	%RSD	%RE	Avg %RE
56.13	55.75	56.28	0.00047	0.8	-0.7	0.3
	56.44				0.6	
	56.66				0.9	
48.32	47.71	NA	NA	NA	-1.3	NA
28.06	28.08	NA	NA	NA	0.1	NA
24.16	24.26	24.20	0.000067	0.3	0.4	0.2
	24.13				-0.1	
	24.22				0.2	

The results of the formulation and formulation stability sample analysis are shown in Table 4 and 5. The formulation stability sample was the same formulation sample prepared and analyzed on January 24, 2005 that had been stored refrigerated for 59 days, protected from light in an amber glass bottle.

The results of the formulation analysis met all acceptance criteria (RE within 10% of target and RSD of $\leq$ 10%).

Table 4 – Formulation Analysis Results

Batch	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1-AG-1	23.03	22.99	22.81	22.94	-1.1	0.5
2-AG-1	22.48	22.00	22.34	22.27	-4.0	1.1

The formulation stability sample analyzed on 3/24/2005 was within -7.8% of the Day 0 value (1/24/2005 analysis value) and met acceptance criteria ± 10 %.

Table 5 –Formulation Stability Analysis 3/24/2005 Results

Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % of Day 0 Conc ± s
21.14	21.14	21.16	21.15	92.2 ± 0.1

4.7 Conclusions

The average concentration of the stock formulation and its percent relative standard deviation were within acceptance criteria. Therefore, the formulation was suitable for use.

The aminoglutethimide formulation at a target concentration of 23.2 mg/mL (0.1M) in DMSO was stable for 59 days when stored refrigerated and protected from light.

5 ACKNOWLEDGMENTS

Analytical support for this work was provided by Sandy Runyon. The report was written by Louise Baughman and Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3

Lot No.: 063K4069 (Sigma Aldrich)

Receipt Date: 10/22/04

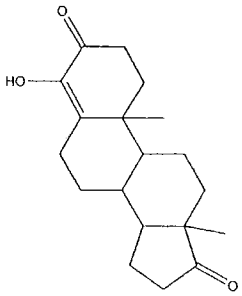
Amount Received: 3.1 g

Appearance: Solid

Vendor Purity: 99% by TLC

Storage Conditions (@ Battelle): Refrigerated (~5°C)

STRUCTURE:



Mol. Wt.: 302.41 g/mol

Mol. Formula: $C_{19}H_{26}O_3$

Prepared By:

Denise A. Contos 12/19/05

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/04	10/26/04
Formulation preparation	12/2/04	12/2/04
Dispensing	12/2/04	12/2/04
Formulation analysis	12/2/04	12/2/04
Audit analytical report	10/20/05	10/20/05
Audit study file	10/20/05	10/20/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 12-14-05 Date

EXECUTIVE SUMMARY

The title compound, 4-hydroxyandrostenedione (4-OH ASDN), was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation Work, Work Assignment 4-16/17.

The solubility of 4-hydroxyandrostenedione was determined to be acceptable in 95% ethanol for preparing formulations.

A formulation analysis method was developed and validated to analyze 4-hydroxyandrostenedione in 95% ethanol at a concentration of 3.02 mg/mL (0.01M). This method was used to analyze samples from both formulation and formulation storage stability studies at 3.02 mg/mL.

The storage stability study indicated that a 3.02 mg/mL formulation, stored in sealed amber glass bottles and protected from light, was stable for 173 days at approximately 5°C.

The stock formulation prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for 4-hydroxyandrostenedione on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in 95% ethanol.
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle of 4-hydroxyandrostenedione, 063K4069, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 22, 2004. The label amount indicated 3.1 grams was sent. The chemical was received and subsequently stored at approximately 5°C.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99% based on thin layer chromatography (TLC).

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of 4-hydroxyandrostenedione (4-OH ASDN) in 95% ethanol, at a concentration of at least 30.2 mg/mL. The 4-OH ASDN (0.30200 ± 0.03020 g) was weighed into a 10-mL volumetric flask, diluted to approximately 80% volume with 95% ethanol, sealed and shaken to mix. The flask was diluted to volume with 95% ethanol, sealed, shaken, sonicated for approximately 50 minutes and stirred. The 4-OH ASDN did not go into solution.

A second solubility study was conducted to determine the solubility of 4-OH ASDN in 95% ethanol, with a solubility of at least 3.02 mg/mL being required for acceptability. The 4-OH ASDN (0.03020 ± 0.00302 g) was weighed into a 10-mL volumetric flask, diluted to approximately 80% volume with 95% ethanol, sealed and shaken to mix. The flask was diluted to volume with 95% ethanol, sealed, shaken and sonicated for approximately 2 minutes. The 4-OH ASDN went into solution. This experiment showed that 95% ethanol was an acceptable solvent for the 3.02 mg/mL formulation (0.01M).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	4-Androsten-4-ol-3,17-dione
Product Number	A5791
Product Brand	SIGMA
CAS Number	566-48-3
Molecular Formula	$C_{19}H_{26}O_3$
Molecular Weight	302.41

TEST	SPECIFICATION LOT 063K4069 RESULTS
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 10 MG/ML OF METHANOL
ELEMENTAL ANALYSIS	75.45% CARBON
PROTON NMR SPECTRUM	CONSISTENT WITH STRUCTURE
PURITY BY THIN LAYER CHROMATOGRAPHY	99%
QC ACCEPTANCE DATE	JUNE 2003

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of 4-hydroxyandrostenedione in 95% ethanol at a target concentration of 3.02 mg/mL (0.01 M) for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic columns and conditions. The selected method was one which produced acceptable retention time for the major peak, apparent resolution of significant impurities and acceptable peak shape. The detection method chosen was gas chromatography with flame ionization detection (GC/FID).

4.2 Method

The GC parameters for 4-hydroxyandrostenedione are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5 MS, 15 m × 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at 2 mL/minute
Oven Temperature	150°C, hold for 1 minute, increase at 15°C/minute to 320°C
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at 30 mL/minute; Air at 380 mL/minute
Detector Temperature	320°C
Injector Temperature	250°C
Injection Volume	1 µL
Injection Mode	Split 1:10
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Fifty (50) milligrams ± 4 mg of benzophenone was added to a 25-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 50 ± 1 mg of 4-OH ASDN each into individual 50-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 1000 µg/mL each.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with methanol, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	IS (mL)	95% Ethanol (mL)	Final Volume (mL)
VS1	500	A	5	1	1	10
VS2	300	B	3	1	1	10
VS3	200	A	2	1	1	10
VS4	100	B	1	1	1	10

4.3.1.4 Blanks

Triplicate blanks without IS were prepared by pipetting 1 mL of 95% ethanol into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 1 mL of 95% ethanol into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

4.3.2 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.3 Calculations

The integration of the 4-OH ASDN and IS peaks by the chromatography data system was evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation weighted 1/x was calculated relating the response ratio of 4-OH ASDN divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standard at each concentration.

4.3.4 Results

Specificity is shown by representative overlaid chromatograms from low and high vehicle/calibration standards, blank with IS, and a blank from the validation data as presented in Figure 2.

The blank and blank with IS exhibited no peaks that would significantly interfere with the 4-OH ASDN or IS peaks.

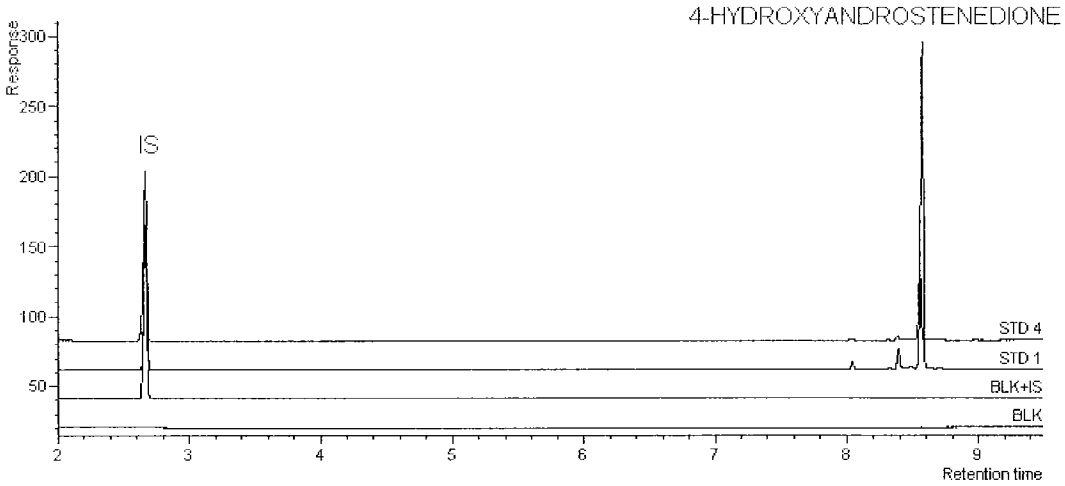


Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

The regression analysis results from the validation standard curve indicate linearity and are shown in Table 3.

Table 3 – Regression Analysis Validation Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0038	-0.0272	0.9975	0.0565

The vehicle/calibration standard validation results are shown in Table 4.

Table 4 –Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	%RE	Avg %RE
	496.8				-1.9	
506.4	494.5	509.6	24.2	4.7	-2.3	0.6
	537.5				6.1	
298.1	289.4	NA	NA	NA	-2.9	NA
202.6	198.8	NA	NA	NA	-1.9	NA
	100.7				1.3	
99.38	99.89	100.4	0.4	0.4	0.5	1.0
	100.5				1.1	

The method validation sensitivity was 1.266 µg/mL, the LOD, which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 13 µg/mL when a formulation is diluted 1 to 10 for analysis. The LOQ was 4.219 µg/mL, defined as ten times the standard deviation of the lowest standard because there was no blank response. This is equivalent to a formulation concentration of 42 µg/mL when a formulation is diluted 1 to 10 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 99.38 µg/mL.

4.3.5 Conclusions

The method met all acceptance criteria for precision, accuracy, linearity, sensitivity and specificity. The method was suitable for the stability study and subsequent formulation analyses for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a concentration of 3.02 mg/mL (0.01 M) in 95% ethanol for 173 days in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A sample was analyzed on the day of preparation (Day 0) and Day 14. A second sample was analyzed on the day of preparation Day 0, Days 27, 54, 83 and 173. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on November 10, 2004 (Day 0) for the storage stability study at a target concentration of 3.02 mg/mL (0.01 M) in 95% ethanol by accurately weighing 75.50 ± 0.75 mg of 4-OH ASDN into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with 95% ethanol. The flask was sealed, sonicated for 10 minutes and allowed to cool to room temperature. The contents of the flask was diluted to volume with 95% ethanol, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four, 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After 14 days of storage, a vial was removed from the refrigerator, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation was prepared on December 2, 2004 (Day 0) at a target concentration of 3.02 mg/mL (0.01 M) in 95% ethanol by accurately weighing 151.00 ± 0.50 mg into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with 95% ethanol, sealed and mixed well. The contents of the flask were diluted to volume with 95% ethanol and mixed well. Approximately 18 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0, 27, 54, 83 and 173 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards, blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report with the exception that the standard stocks were prepared by accurately weighing 25 ± 1 mg of 4-OH ASDN into 25-mL volumetric flasks.

In triplicate, 1 mL of the formulation and 1 mL of IS were pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (3.02 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
11/10/04	11/10/04	0	2.871	2.873	2.928	2.891 ± 0.032	100.0 ± 1.1
11/10/04	11/24/04	14	3.006	3.085	3.149	3.080 ± 0.072	106.5 ± 2.5
12/2/04	12/2/04	0	3.005	3.022	3.005	3.011 ± 0.010	100.0 ± 0.3
12/2/04	12/29/04	27	3.168	3.123	3.117	3.136 ± 0.028	104.2 ± 0.9
12/2/04	1/25/05	54	3.008	3.126	3.110	3.081 ± 0.064	102.3 ± 2.1
12/2/04	2/23/05	83	3.027	3.131	3.217	3.125 ± 0.095	103.8 ± 3.2
12/2/04	5/24/05	173	3.126	3.142	3.129	3.133 ± 0.008	104.1 ± 0.3

For the sample prepared November 10, 2004, the pooled RSD of the analytical method was 1.9%. This means that there would have to be a difference of more than 4.4% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the sample prepared December 2, 2004, the pooled RSD of the analytical method was 1.8%. This means that there would have to be a difference of more than 4.0% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

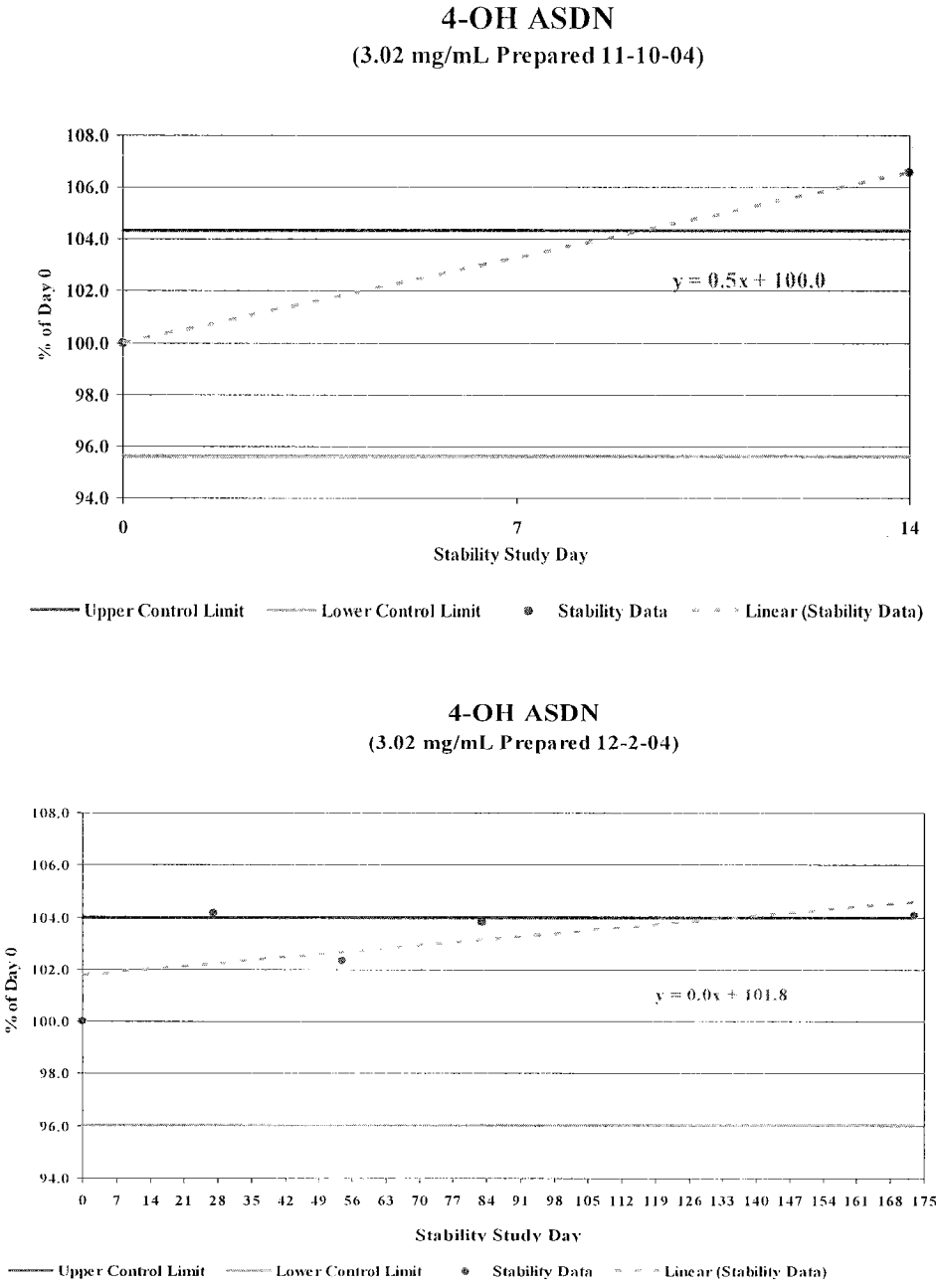


Figure 3 – Control Charts for the Storage Stability Studies

5.5 Discussion and Conclusions

The concentration of the samples stored at approximately 5°C protected from light in amber glass vials for Day 14 was above the upper significance level, but was within 6.5% of the Day 0 value (prepared November 10, 2004). Concentrations for Days 54 and 83 samples were within the upper and lower significance levels and Days 27 and 173 were just above the upper significant level. A linear trend analysis indicated there was no significant trend to changing concentration over time for the samples. These data indicate the formulation was stable when stored protected from light at approximately 5°C for 173 days.

6 FORMULATION PREPARATIONS AND ANALYSES

Formulations were prepared and analyzed on December 2, 2004, January 25, 2005, March 21, 2005, and June 27, 2005, according to SOP No. COMSPEC.II-027, "Standard Operating Procedure (SOP) for the Formulation and Analysis of 4-Hydroxyandrostenedione (4-OH ASDN) in 95% Ethanol." This section describes the method, results, and conclusions.

6.1 Preparation of Formulations

An accurate weight of 151.00 ± 0.50 mg of 4-OH ASDN was added to a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with 95% ethanol, sealed and mixed well. The contents of the flask were diluted to volume with 95% ethanol and mixed well. This produced a target concentration of 3.02 mg/mL (0.01 M) 4-OH ASDN in 95% ethanol.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the method validation, Section 4.3.1 of this report.

6.3 Preparation of Formulation Samples

One (1) mL of the formulation and 1-mL of IS were pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well.

6.4 Analysis

Autoinjector vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the conditions from the method validation (Table 1).

6.5 Calculations

The peaks for 4-hydroxyandrostenedione and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation weighted 1/x was calculated relating the response ratio (4-hydroxyandrostenedione/IS) (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios

were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/ calibration standards and formulation samples when applicable.

6.6 Results

Specificity is shown by the representative overlaid chromatograms of the high and low standards, blank with IS and a blank presented in Figure 4.

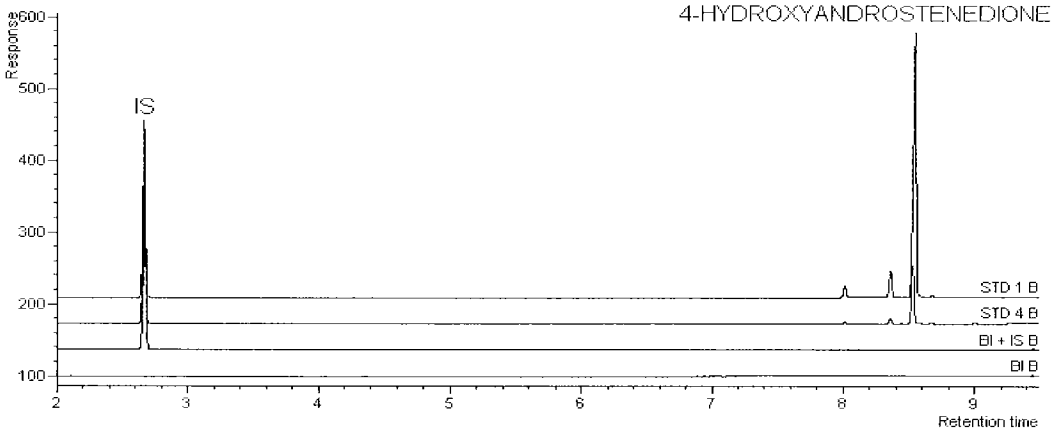


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

The regression analysis results of the vehicle/calibration standard curves indicated linearity and are shown in Table 6.

Table 6 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0038	-0.0140	0.9999	0.0117
0.0035	-0.0037	1.000	0.0061
0.0036	-0.0251	0.9999	0.0100
0.0038	-0.0218	0.9999	0.0104

The results of the formulation analyses are shown in Table 7.

Table 7 – Formulation Analysis Results

Batch	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	% RSD
1-ASDN	3.005	3.022	3.005	3.011	-0.3	0.3
2-ASDN	3.056	3.089	3.049	3.065	1.4	0.7
3-ASDN	3.112	3.053	3.063	3.076	1.9	1.0
4-ASDN	2.943	2.945	2.950	2.946	-2.5	0.1

The formulations met acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

6.7 Conclusions

The average concentration of the stock formulations and their percent RSD were within acceptance criteria. Therefore, the formulations were suitable for use.

7 ACKNOWLEDGMENTS

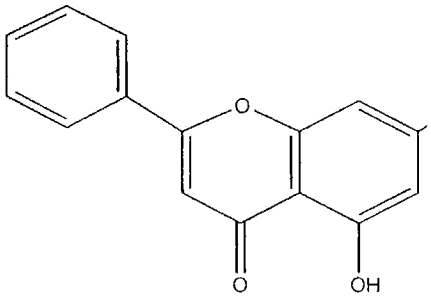
Analytical support for this work was provided by Sandy Runyon, Christina Zielinski, Tudor Fernando, Kevin Carrico, and Darren Brown. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

Battelle
The Business of Innovation

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

CHRYSIN

CAS No.: 480-40-0	Lot No.: 10101DC (Sigma Aldrich)
Receipt Date: 10/26/04	Amount Received: 25 g
Appearance: Solid	Vendor Purity: 98.20% by HPLC
Storage Conditions (@ Battelle): Room temperature (~25°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	254.24 g/mol	$C_{15}H_{10}O_4$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Test substance receipt	10/26/04	10/26/04
Dispensing*	12/2/04	12/2/04
Formulation analysis*	12/2/04	12/2/04
Formulation preparation*	12/2/04	12/2/04
Audit analytical report	8/9/05	8/9/05
Audit study file	8/9/05	8/9/05
Audit analytical report	12/14/05	12/14/05
Audit study file	12/14/05	12/14/05

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — Date: 12.22.05

Battelle

EXECUTIVE SUMMARY

The title compound, chrysin (CHRY), was analyzed in support of the EPA Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

The solubility of CHRY was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze CHRY in DMSO at a target concentration of 2.54 mg/mL (0.01 M). This method was used to analyze samples from both the formulation and formulation storage stability studies.

The storage stability study indicated that a 2.54 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 100 days at approximately 5°C.

The stock formulations prepared for shipment to the testing laboratory was analyzed and met the established acceptance criteria.

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Figure 3 – Control Chart for Storage Stability Analysis 8

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom) 9

1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for Chrysin (CHRY) on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO)
- Developing and validating a formulation analysis method
- Conducting a storage stability study
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 60-mL amber glass bottle of CHRY, 10101DC, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 26, 2004. The label amount indicated 25 grams was sent. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1, which states that purity was 98.20% based on high performance liquid chromatography (HPLC).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Chrysin
Product Number	C8,010-5
Product Brand	ALDRICH
CAS Number	480-40-0
Molecular Formula	$C_{15}H_{10}O_4$
Molecular Weight	254.24

TEST	SPECIFICATION	LOT 1010DC RESULTS
APPEARANCE	YELLOW TO YELLOW GREEN TO TAN POWDER	YELLOW POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD.
UV-VISIBLE SPECTRUM	C IN 0.1N NAOH	0.01G/L,0.01N NAOH
	E(348 +/- 2NM) = > 8,000	E348= 8,500
	E(282 +/- 2NM) = >22,000	E282=23,400
	E(263 +/- 2NM) = >20,000	E264=20,400
	E(224 +/- 2NM) = >27,000	E224=28,000
MISCELLANEOUS ASSAYS	97.0% - 103.0% (WITH TBAH)	101.4 % (WITH TBAH)
TITRATION	97.0% - 103.0% (WITH NAOH)	99.3 % (WITH NAOH)
HIGH PRESSURE LIQUID CHROMATOGRAPHY	CONSISTENT WITH CONTROL	98.20 %
SOLUBILITY	50 MG/ML PYRIDINE; CLEAR TO SLIGHT HAZY.	5%, PYRIDINE; CLEAR, YELLOW SOLUTION
QUALITY CONTROL ACCEPTANCE DATE		APRIL, 2004

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of CHRY in 100% DMSO, at a concentration of at least 2.54 mg/mL. CHRY (0.50848 ± 0.05085 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the CHRY dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well. The CHRY went readily into solution. Although the solution was prepared at approximately 50 mg/mL, higher than the target concentration, CHRY was readily soluble and would therefore, be soluble at the target concentration 2.54 mg/mL. This experiment showed that DMSO was an acceptable solvent for the 2.54 mg/mL formulation (0.01M).

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of CHRY in DMSO at a target concentration of 2.54 mg/mL (0.01 M) for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time and peak shape. The detection method chosen was HPLC with ultraviolet (UV) detection with the wavelength set at the absorbance maximum above 270 nm.

4.2 Method

The HPLC parameters for CHRY are presented in Table 1.

Table 1 – HPLC System

Instrument System	Waters (Milford, MA) and Agilent (Palo Alto, CA)
Column	Supelcosil LC-ABZ, 5 µm particle size, 150 mm × 4.6 mm (ID) (Supelco, Bellefonte, PA)
Mobile Phase	70:30 (v/v) Methanol:0.15% Ammonium Acetate, Isocratic
Flow Rate	1.0 mL/minute
Injection Volume	10 µL
Detector Type	UV
Detector Wavelength	270 nm
Run Time	~15 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and quantitation (LOQ). Triplicate vehicle/calibration blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Mobile Phase

A 0.15% ammonium acetate solution was prepared by weighing approximately 1.5 grams of ammonium acetate into a 1-L volumetric flask. The content of the flask was diluted to volume with Milli-Q water, sealed, and mixed well.

The mobile phase was prepared by mixing 700 mL of methanol and 300 mL of 0.15% ammonium acetate.

4.3.2 Preparation of Standards and Blanks

4.3.2.1 Internal Standard (IS)

Fifty (50) ± 2 mg of terconazole was added to a 25-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.2.2 Stock Standards

Two stock standards (A, B) were prepared by accurately weighing 25 ± 1 mg of CHRY each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 1000 µg/mL each.

4.3.2.3 Working Standards

Working standards were prepared as shown in Table 2. The content of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. One standard was prepared for each concentration.

Table 2 – Preparation of Working Standards

Working Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Final Volume (mL)
WS1	500	A	5	10
WS2	400	B	4	10
WS3	200	A	2	10
WS4	100	B	1	10

4.3.2.4 Vehicle/Calibration Standards

These standards were prepared in as shown in Table 3. The content of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 3 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Internal Std (mL)	DMSO (mL)	Final Volume (mL)
VS1	50	WS1	0.5	1	0.1	5
VS2	40	WS2	0.5	1	0.1	5
VS3	20	WS3	0.5	1	0.1	5
VS4	10	WS4	0.5	1	0.1	5

4.3.2.5 Triplicate Blanks without and with IS

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 5-mL volumetric flasks and adding 1 mL of methanol to each. The content of the flasks was diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 5-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.3.3 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autosampler vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.4 Calculations

The integration of the CHRY and IS peaks by the chromatography data system were evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of chrysin divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.5 Results

Specificity is shown by representative overlaid chromatograms from high and low vehicle/calibration standards, blank with IS, and a blank from the method validation are presented in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the CHRY or IS peaks.

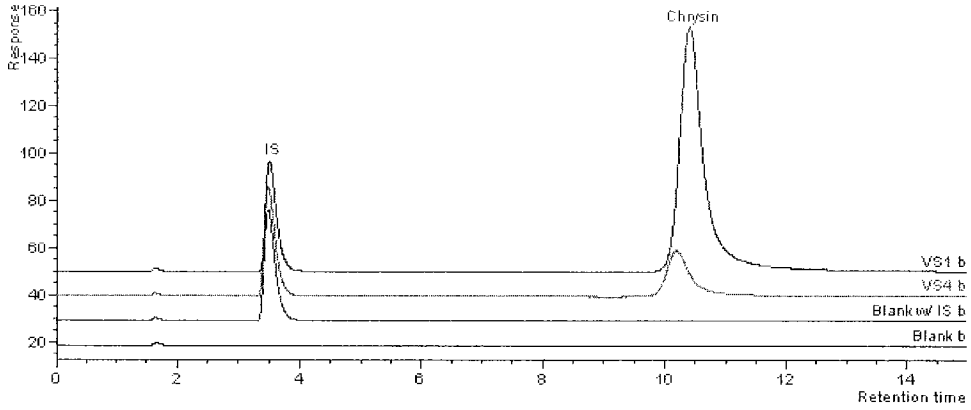

Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

The regression analysis results from the standard curve are linear and are shown in Table 4.

Table 4 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.1034	-0.0463	0.9999	0.0219

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 5.

Table 5 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	%RSD	Avg %RE
50.08	50.06	50.06	0.36	0.7	0.0	0.0
	49.70				-0.8	
	50.42				0.7	
40.14	40.20	NA	NA	NA	0.1	NA
20.03	20.08	NA	NA	NA	0.2	NA
10.04	10.09	10.02	0.06	0.6	0.5	-0.1
	10.00				-0.4	
	9.978				-0.6	

For the method validation the LOD is 0.18 µg/mL and is defined as three times the standard deviation of the lowest vehicle/calibration standard. This is equivalent to a formulation concentration of 18 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ is 0.59 µg/mL and is defined as ten times the standard deviation of the lowest vehicle/calibration standard because there was no blank response. This is equivalent to a

formulation concentration of 59 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest vehicle/calibration standard with acceptable accuracy and precision, was 10.04 µg/mL.

4.3.6 Conclusions

The method met all acceptance criteria for precision, accuracy, linearity, sensitivity, and specificity. The method was suitable for the stability study and subsequent formulation analyses for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a concentration of 2.54 mg/mL in DMSO for 100 days (approximately 14 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A single sample was analyzed on the day of preparation (Day 0), Days 14, 71, and 100. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on Day 0 of the storage stability study at a target concentration of 2.54 mg/mL in DMSO by accurately weighing 63.5 ± 6 mg into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with DMSO. The flask was sealed and manually shaken to mix the contents. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four 8-mL amber glass vials that were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After the desired storage period, a vial was removed from storage, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.2) of this report.

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One-half (0.5) mL of the diluted formulation and 1-mL of the IS were pipetted into individual 5-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. An appropriate volume of each was transferred to an autosampler vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 6 and presented in control chart format in Figure 3.

Table 6 – Formulation Storage Stability Results (2.54 mg/mL)

Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Avg Conc ± s
0	2.475	2.454	2.454	2.461 ± 0.012	100.0 ± 0.5
14	2.582	2.521	2.563	2.555 ± 0.031	103.8 ± 1.3
71	2.529	2.553	2.502	2.528 ± 0.025	102.7 ± 1.0
100	2.448	2.447	2.427	2.441 ± 0.012	99.2 ± 0.5

The pooled RSD of the analytical method was 0.987%. This means that there would have to be a difference of more than 2.24% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

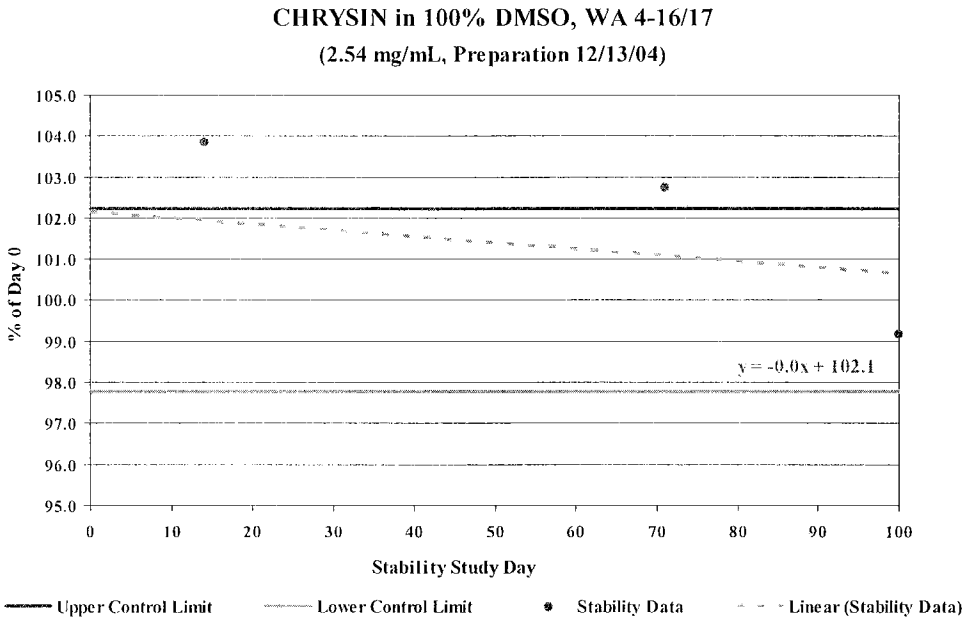

Figure 3 – Control Chart for Storage Stability Analysis

5.5 Discussion and Conclusions

The Day 0 determined value for the formulation was approximately 3.6% below nominal (the calculated concentration based on the weight of the chemical). There was no statistical difference between the Day 0 and Day 100 samples. However. Days 14 and 71 were above the upper significance level due to the tight precision of the assay but were within 3.8% and 2.7%. respectively, of the Day 0 value. These data indicate that the formulation was stable when protected from light at approximately 5°C for 100 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on January 25, 2005 and August 9, 2005 according to SOP COMSPEC.II-028-00, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Chrysin (CHRY) in 100% Dimethylsulfoxide (DMSO)." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

CHRY (127.00 ± 5.00 mg) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the CHRY dissolved. The content of the flask was diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.2 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One-half (0.5) mL of the diluted formulation and 0.5 mL of the IS were pipetted into individual 5-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

6.4 Analysis

Autosampler vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the HPLC conditions from the validation (Table 1). Representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with IS, and a blank are shown in Figure 4.

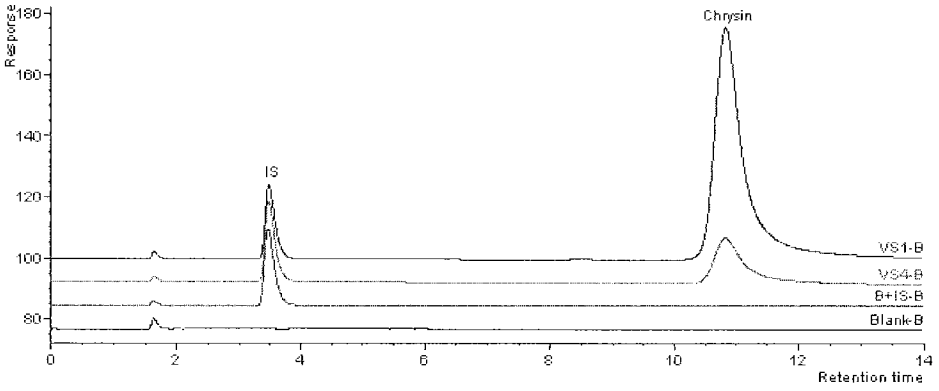

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

The peaks for CHRY and the IS were integrated for each injection by the chromatography data system. Any peak with inconsistent integration was manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio (CHRY/IS) (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation(s), and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

The results of the formulation analysis are shown in Table 7. The results of the standard curve regression analysis are shown in Table 8. The formulation met acceptance criteria (RE within 10% of target and RSD of $\leq$ 10%).

Table 7 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1-CHRY-1	1/25/05	2.592	2.409	2.395	2.466	-2.9	4.5
2-CHRY-1	8/9/05	2.479	2.531	2.487	2.499	-1.6	1.1

Table 8 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient
0.1852	-0.3622	0.9999
0.0970	-0.0921	0.9997

6.7 Conclusions

The average concentration of the stock formulations and its percent RSDs were within acceptance criteria. Therefore, the formulations were suitable for use.

7 ACKNOWLEDGMENTS.

Analytical support for this work was provided by Melinda Pauff, Sandy Runyon, Tudor Fernando, and Ron Haney. The report was written by Louise Glazier and Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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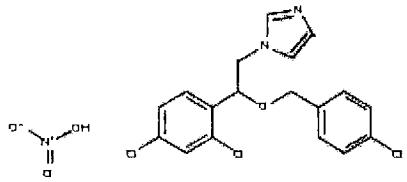
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

ECONAZOLE

CAS No.: 24169-02-6	Lot No.: 123K1220 (Sigma Aldrich)
Receipt Date: 10/26/04 and 12/4/04	Amount Received: 10 g
Appearance: Solid/White Powder	Vendor Purity: 98% by TLC
Storage Conditions (@ Battelle): Room temperature (~25°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	444.70 g/mol	$C_{18}H_{15}Cl_3N_2O \cdot HNO_3$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit study file	1/3/2006	1/3/2006
Audit analytical report	1/3/2006	1/3/2006

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 1-5-06 Date

EXECUTIVE SUMMARY

The title compound, econazole, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

The solubility of econazole was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze econazole in DMSO at a concentration of 44.47 mg/mL (0.1 M). This method was used to analyze samples from both formulation and formulation storage stability studies at 44.47 mg/mL.

The storage stability study indicated that a 44.47 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 56 days at approximately 5°C.

The stock formulations prepared for shipment to the testing laboratory were determined to meet the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for econazole on the Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

Two 15-mL amber glass bottles of econazole, Lot No. 123K1220, were received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA (one each on October 26, 2004 and December 4, 2004). The label amount indicated 5 grams was sent at each shipment. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 98% based on thin layer chromatography.

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Econazole nitrate salt,
Product Number	E4632
Product Brand	SIGMA
CAS Number	24169-02-6
Molecular Formula	$C_{18}H_{15}Cl_2N_2O \cdot HNO_3$
Molecular Weight	444.70

TEST	SPECIFICATION	LOT 123K1220 RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION WITH SOME INSOLUBLES AT 25MG/ML IN CHLOROFORM:METHANOL (1:1)	CLEAR COLORLESS
WATER BY KARL FISCHER	NMT 0.5%	0.0%
ELEMENTAL ANALYSIS	47.6 TO 49.6% CARBON	48.6%
	9.2 TO 9.8% NITROGEN	9.4%
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS (SUPPLIER DATA)
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 98%	98%
QC ACCEPTANCE DATE		JANUARY 2004

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of econazole in 100% DMSO, at a concentration of at least 44.47 mg/mL (0.1 M). Econazole (0.44470 ± 0.04447 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the econazole dissolved. The contents of flask were diluted to volume with DMSO, sealed, and mixed well. The econazole went readily into solution. This experiment showed that DMSO was an acceptable solvent for the 44.47 mg/mL formulation.

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of econazole in DMSO at a target concentration of 44.47 mg/mL for the stability study, the results and the conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time and peak shape. The detection method chosen was high pressure liquid chromatography with ultraviolet detection (HPLC/UV) with the wavelength set at the absorbance maximum of 271 nm.

4.2 Method

The HPLC parameters for econazole are shown in Table 1.

Table 1 – HPLC System

Instrument System	Agilent (Palo Alto, CA), Waters (Milford, MA)
Column	Supelcosil LC-ABZ, 150 mm × 4.6 mm (ID) (Supelco, Bellefonte, PA)
Guard Column	C-18 guard column
Mobile Phase	70:30 (v/v) Methanol:0.15% Ammonium Acetate, Isocratic
Flow Rate	1.0 mL/minute
Injection Volume	50 µL
Detector Type	UV
Detector Wavelength	271 nm
Run Time	~20 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of

detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle/calibration blanks with and without internal standard (IS) were used to assess the specificity of the method.

4.3.1 Preparation of Mobile Phase

A 0.15% ammonium acetate solution was prepared by weighing approximately 1.5 grams of ammonium acetate into a 1-L volumetric flask. The flask was diluted to volume with Milli-Q water, sealed, and mixed well.

The mobile phase was prepared by mixing 700 mL of methanol and 300 mL of 0.15% ammonium acetate.

4.3.2 Preparation of Standards and Blanks

4.3.2.1 Internal Standard (IS)

An IS solution was prepared by weighing 100 ± 4 mg of terconazole into a 50-mL volumetric flask. The content of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.2.2 Stock Standards

Two stock standards (A and B) were prepared by accurately weighing 25 ± 1 mg of econazole each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with HPLC mobile phase. This produced stocks A and B with target concentrations of 1 mg/mL each.

4.3.2.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/ Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	IS (mL)	DMSO (mL)	Final Volume (mL)
VS1	500	A	5	1	0.1	10
VS2	400	B	4	1	0.1	10
VS3	200	A	2	1	0.1	10
VS4	100	B	1	1	0.1	10

4.3.2.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.3.3 Analysis

A portion of each vehicle/calibration standard, blank and sample were transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development (Table 1).

4.3.4 Calculations

The integration of the econazole and IS peaks by the chromatography data system was evaluated to assure it was correct in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of econazole divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.5 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank from the validation as indicated in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the econazole or IS peaks. The regression analysis results from the standard curve indicate linearity and are shown in Table 3.

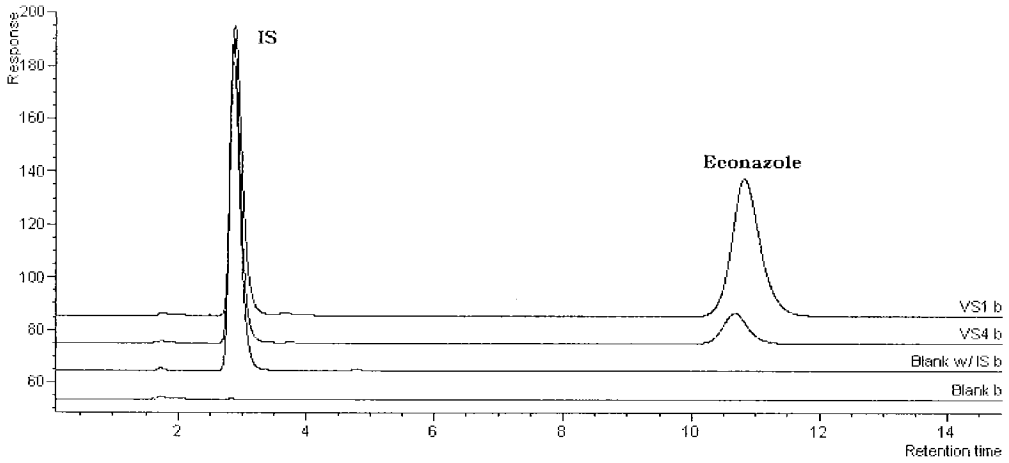


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0023	0.0088	1.000	0.0021

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	% RE	Avg % RE
512.6	513.2	512.5	0.8	0.1	0.1	0.0
	512.7				0.0	
	511.7				-0.2	
406.9	406.7	NA	NA	NA	0.0	NA
205.0	206.8	NA	NA	NA	0.9	NA
101.7	101.3	101.3	0.1	0.1	-0.4	-0.4
	101.2				-0.5	
	101.3				-0.4	

The method validation sensitivity was 0.1732 µg/mL, the LOD, which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 20 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 0.5774

µg/mL. This is equivalent to a formulation concentration of 60 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest vehicle/calibration standard with acceptable accuracy and precision, was 101.7 µg/mL.

4.3.6 Conclusions

The method met all acceptance criteria for specificity, linearity, precision, accuracy and sensitivity. The method was suitable for the stability study and subsequent formulation analysis for which it was used.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 44.47 mg/mL in DMSO for 56 days (8 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A formulation sample was analyzed on the day of preparation (Day 0) and Day 14. A second formulation sample was analyzed on the day of preparation (Day 0), Week 4 and Week 8. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on November 19, 2004, Day 0 of the storage stability study at a target concentration of 44.47 mg/mL in DMSO by accurately weighing 1110 ± 10 mg into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with DMSO. The flask was sealed and sonicated for approximately 5 minutes to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After 14 days of storage, a vial was removed from the refrigerator, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation was prepared on February 7, 2005, Day 0, at a target concentration of 44.47 mg/mL in DMSO by accurately weighing 2.22350 ± 0.08894 g into a 50-mL volumetric flask. The flask was diluted to approximately 80% volume with DMSO, sealed and sonicated for approximately 5 minutes. The content of the flask was inverted 10 times, and diluted to volume with DMSO and shaken to mix well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Day 0, Weeks 4 and 8 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.2) of this report.

In triplicate, 1 mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

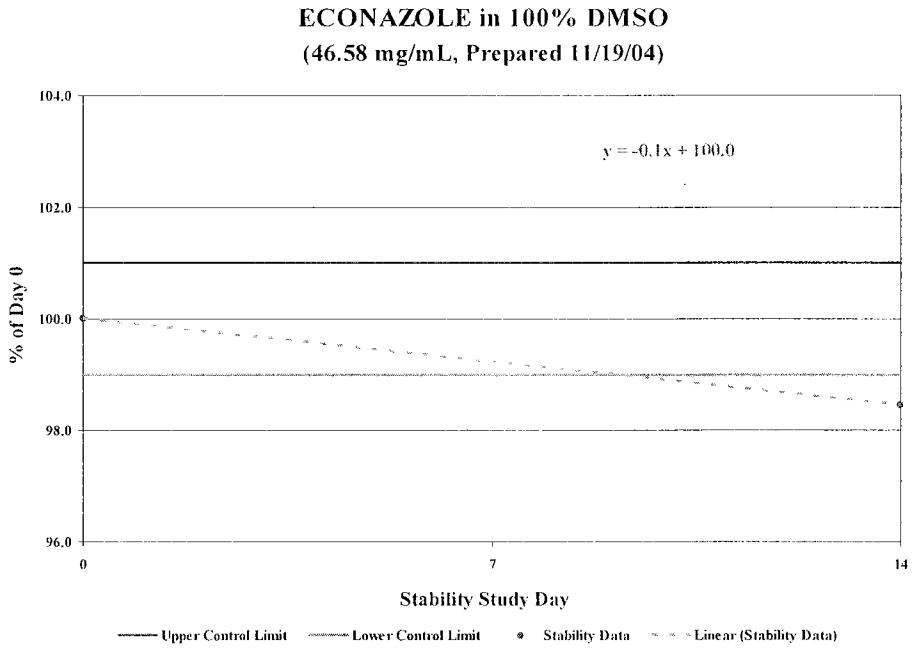
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (44.47 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
11/19/04	11/19/04	0	46.91	46.37	46.47	46.58 ± 0.29	100.0 ± 0.6
11/19/04	12/3/04	14	45.91	45.88	45.80	45.86 ± 0.06	98.5 ± 0.1
2/7/05	2/7/05	0	46.24	46.08	45.89	46.07 ± 0.17	100.0 ± 0.4
2/7/05	3/7/05	28	45.03	44.74	44.66	44.81 ± 0.19	97.3 ± 0.4
2/7/05	4/4/05	56	42.95	42.92	41.94	42.60 ± 0.58	92.5 ± 1.3

For the formulation stability sample prepared on November 19, 2004, the pooled RSD of the analytical method was 0.44%. This means that there would have to be a difference of more than 1.01% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation stability sample prepared on February 7, 2005, the pooled RSD of the analytical method was 0.79%. This means that there would have to be a difference of more than 1.80% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.



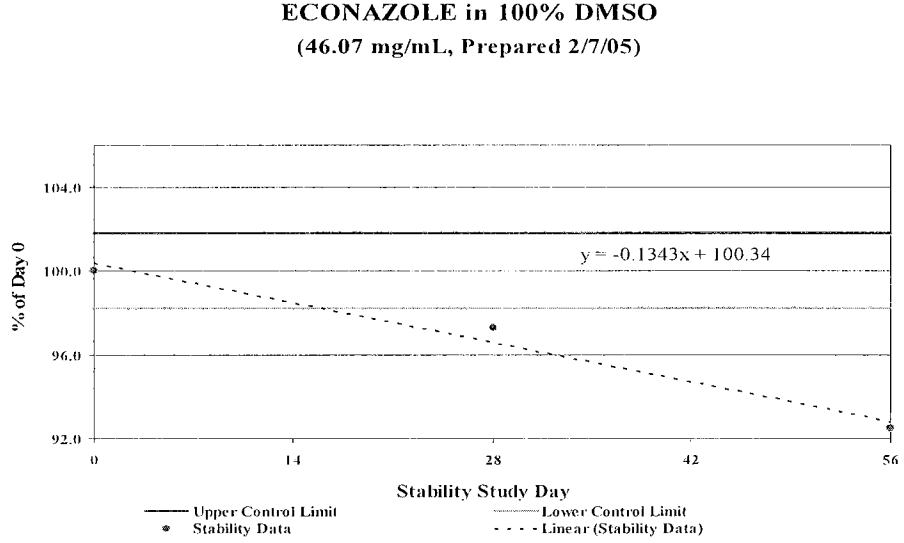


Figure 3 – Control Charts for the Storage Stability Analysis

5.5 Discussion and Conclusions

The Day 0 determined value for the November 19, 2004 formulation was approximately 4.7% above the nominal value (the calculated concentration based on the weight of the chemical). The concentration of the sample stored at approximately 5°C protected from light in amber glass vial for Day 14 was below the lower significance level due to the tight precision of the assay but was within 1.5% of the Day 0 value (November 19, 2004) and met acceptance criteria ± 10%.

The Day 0 determined value for the February 7, 2005 formulation was approximately 3.5% above the nominal value (the calculated concentration based on the weight of the chemical). The concentrations of the sample stored at approximately 5°C protected from light in an amber glass vial for Days 28 and 56 was below the lower significance level due to the tight precision of the assay but was within 2.7 and 7.5%, respectively of the Day 0 value (February 7, 2005) and met acceptance criteria ± 10%.

These data indicate the formulation was stable when protected from light at approximately 5°C for 56 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on February 7, 2005 and August 8, 2005 according to SOP COMSPEC.II-031, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Econazole in 100% DMSO." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Econazole (2.22350 ± 0.08894 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were sonicated for approximately 5 minutes until the econazole dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.2 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. One (1) mL of the diluted formulation and 1 mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

6.4 Analysis

Autoinjector vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the HPLC conditions from the validation (Table 1).

6.5 Calculations

The integration of the econazole and IS peaks by the chromatography data system was evaluated to assure it was correct in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of econazole divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. The percent RE for each vehicle/calibration standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation (s), and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

Specificity is shown by the representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with working IS and a blank are presented in Figure 4.

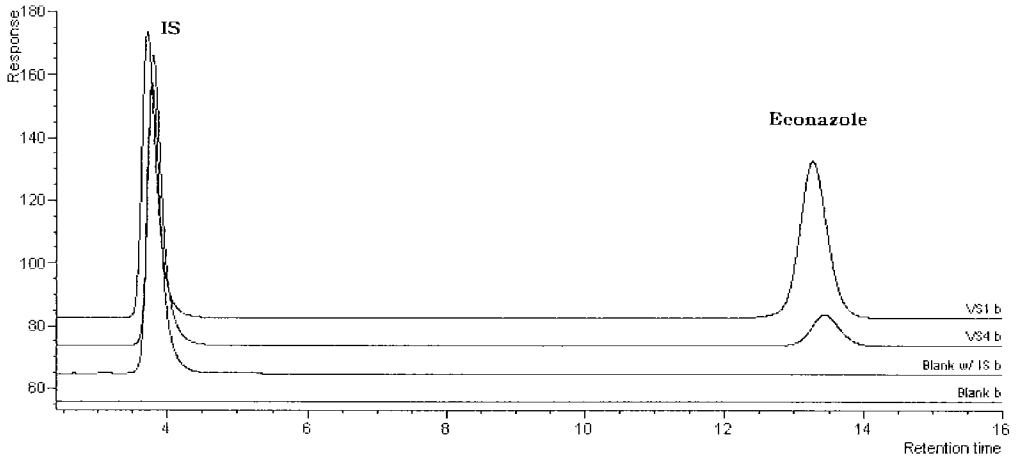


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

The vehicle/calibration standard curve and the results of the regression analysis indicated linearity and are shown in Figure 5.

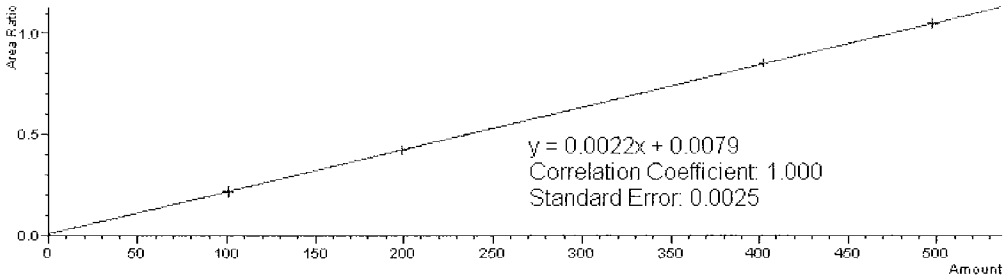


Figure 5 – Vehicle/Calibration Standard Curve and Regression Analysis Results

The results of the formulation analysis are shown in Table 6. The formulations met acceptance criteria (RE within 10% of target and RSD of $\leq$ 10%).

Table 6 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	RSD (%)
1-ECON-1	2/7/05	46.24	46.08	45.89	46.07	3.5	0.4
2-ECON-1	8/8/05	44.58	44.49	44.14	44.40	-0.2	0.5

6.7 Conclusions

The average concentration of the stock formulations and its percent RSDs were within acceptance criteria. Therefore, the formulations were suitable for use.

The econazole formulation at a target concentration of 44.47 mg/mL in DMSO was stable for 56 days when stored refrigerated and protected from light.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Christina Zielinski, Sandy Runyon, Tudor Fernando, Hans Whittenburg and Ron Haney. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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ANALYTICAL CHEMISTRY ACTIVITIES REPORT

KETOCONAZOLE

CAS No.: 65277-42-1

Receipt Date: 10/26/04

Appearance: Solid

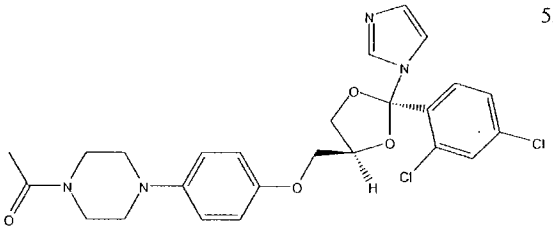
Storage Conditions (@ Battelle): Refrigerated (~5°C)

Lot No.: 121H0524 (Sigma Aldrich)

Amount Received: 2.7 g

Vendor Purity: > 99% by TLC

STRUCTURE:



Mol. Wt.: 531.43 g/mol

Mol. Formula: $C_{26}H_{28}Cl_2N_4O_4$

Prepared By:

Denise A. Contos 12/28/05

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit study file	8/23/2005	8/23/2005
Audit analytical report	8/23/2005	8/23/2005
Audit study file	12/16/2005	12/16/2005
Audit analytical report	12/16/2005	12/16/2005

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Quality Assurance Unit — 12.27.05 Date

EXECUTIVE SUMMARY

The title compound, ketoconazole, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation work, Work Assignment 4-16/17.

Solubility of ketoconazole was determined to be acceptable in dimethylsulfoxide (DMSO) at a concentration of 5.31 mg/mL (0.01 M).

A ketoconazole formulation analysis method was validated on the previous EPA WA 3-10 study. The method was modified by including an additional 1:10 dilution of the formulation which resulted in a 1:200 final dilution of the 5.31 mg/mL (0.01 M) formulation prior to analysis. This modified method was used to analyze both stability and formulation analysis samples.

Storage stability was previously determined (EPA WA 3-10 study, Analytical Chemistry Activities Report, Ketoconazole, 2004) as 28 days when stored at approximately 5°C and protected from light at a target formulation concentration of 0.532 mg/mL in DMSO. In the current study, a formulation sample with a target concentration of 5.31 mg/mL in DMSO was stable when stored refrigerated and protected from light for 60 days.

The stock formulations prepared for shipment to the testing laboratory were analyzed and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for ketoconazole on the Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Preparing and analyzing a stock formulation and a formulation stability sample.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle of ketoconazole, Lot No. 121H0524, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 26, 2004. The label amount indicated 2.7 grams was sent. The chemical was received and subsequently stored refrigerated.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was greater than 99% based on thin layer chromatography.

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Ketoconazole
Product Number	K1003
Product Brand	SIGMA
CAS Number	65277-42-1
Molecular Formula	$C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight	531.43

TEST	SPECIFICATION	LOT 121H0524 RESULTS
APPEARANCE	WHITE TO YELLOW WITH A LIGHT TAN CAST POWDER	WHITE POWDER WITH A LIGHT YELLOW CAST
SOLUBILITY	CLEAR FAINT YELLOW TO YELLOW SOLUTION AT 50MG/ML IN METHANOL	CLEAR FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF METHANOL
ELEMENTAL ANALYSIS	57.6 TO 59.9% CARBON	58.6% CARBON 10.5% NITROGEN
SPECIFIC ROTATION	+1 TO -1 DEG (C=4 IN METHANOL AT 20DEGCENTIGRADE)	+0.08 DEG (C = 3.8 IN METHANOL AT 20 DEG CENTIGRADE)
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 98%	GREATER THAN 99%
SHELF LIFE	3 YEARS	MARCH 2005
QC ACCEPTANCE DATE		MARCH 2004

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

Figure 1 - Certificate of Analysis

3 SOLUBILITY STUDY

A solubility study was conducted to determine the solubility of ketoconazole in either 95% ethanol or 100% DMSO. Initially, both 95% ethanol and DMSO were used to prepare a 53.14 mg/mL (0.1 M) ketoconazole solution. Neither of the solvents dissolved the ketoconazole at the 0.1 M concentration. At the direction of the Task Leader, a 5.31 mg/mL ketoconazole solution (0.01 M) in 100% DMSO was prepared by weighing 0.05314 ± 0.00531 g into a

10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was capped and contents were mixed. The content of the flask was diluted to volume with DMSO, sealed, mixed and sonicated. The ketoconazole went into solution with minimal shaking and sonication. This experiment showed that DMSO was an acceptable solvent for a 5.31 mg/mL (0.01 M) formulation.

4 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on February 4, 2005 and June 29, 2005 according to SOP COMSPEC.II-018-02, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Ketoconazole in 100% DMSO." In addition, the February 4, 2005 formulation was re-analyzed to determine stability on April 5, 2005, 60 days after storage at approximately 5°C and protected from light. This section describes the method, results, and conclusions.

4.1 Preparation of Formulation

A ketoconazole formulation with a target concentration of 5.31 mg/mL (0.01 M) in DMSO was prepared on February 4, 2005 by accurately weighing 265.5 ± 5 mg of ketoconazole into a tarred 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and sonicated for approximately 10 minutes then inverted ten times. The content of the flask was diluted to volume with DMSO, sealed, and mixed well by inverting at least ten times.

4.2 Preparation of High Performance Liquid Chromatography (HPLC) Mobile Phase

An accurate amount of ammonium acetate (3.0 g) was weighed into a 2000-mL HPLC mobile phase bottle. A 600 mL volume of Milli-Q water was added to the bottle and the contents were mixed well. A final volume of 1400 mL of methanol and 2.8 mL of diethanolamine were added to the bottle and the contents were mixed well. This produced a HPLC mobile phase containing 70:30:0.14 (v:v:v) of methanol:0.5% ammonium acetate: diethanolamine.

4.3 Preparation of Standards and Blanks

4.3.1 Internal Standard (IS)

Fifteen (15) ± 2 mg terconazole was added to a 50-mL volumetric flask. The content of the flask was diluted to volume with HPLC mobile phase, sealed, and mixed well. This produced a solution with a target concentration of 300 µg/mL.

4.3.2 Stock Standards

Two stock standards were prepared by accurately weighing 30 ± 3.0 mg of ketoconazole into two individual 200-mL volumetric flasks and dissolving in and diluting to volume with HPLC mobile phase. This produced stocks A and B with target concentrations of 150 µg/mL each.

4.3.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 1. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two intermediate concentrations.

Table 1 – Preparation of Vehicle/Calibration Standards

Vehicle Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Internal Std (mL)	DMSO (mL)	Final Volume (mL)
VS1	60	A	4	1	0.05	10
VS2	45	B	3	1	0.05	10
VS3	30	A	2	1	0.05	10
VS4	15	B	1	1	0.05	10

4.3.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.05 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.05 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.4 Preparation of Formulation and Formulation Stability Samples

Triplicate 1 mL aliquots of the formulation were pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. A 0.5 mL aliquot of the diluted formulation and 1 mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.5 Analysis

A portion of each vehicle/calibration standard, blank and sample were transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the HPLC parameters for ketoconazole which are presented in Table 2.

Table 2 – HPLC System

Instrument System	Agilent (Palo Alto, CA); Waters (Milford, MA)
Column	Supelcosil LC-ABZ, 5 µm particle size, 250 mm × 4.6 mm (ID) (Supelco, St. Louis, MO)
Guard	C_{18} guard cartridge
Mobile Phase	70:30:0.14 (v/v/v) Methanol:0.5% Ammonium Acetate:Diethanolamine, Isocratic
Flow Rate	1.5 mL/minute
Injection Volume	20 µL
Detector Type	Ultraviolet (UV)
Detector Wavelength	245 nm

4.6 Calculations

The integration of the ketoconazole and the IS peaks by the chromatography data system were evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation weighted 1/x was calculated relating the response ratio of ketoconazole/IS (y) to the concentration of the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each vehicle/calibration standard and formulation sample. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.7 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/ calibration standard, a blank with IS, and a blank as presented in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the ketoconazole or IS peaks.

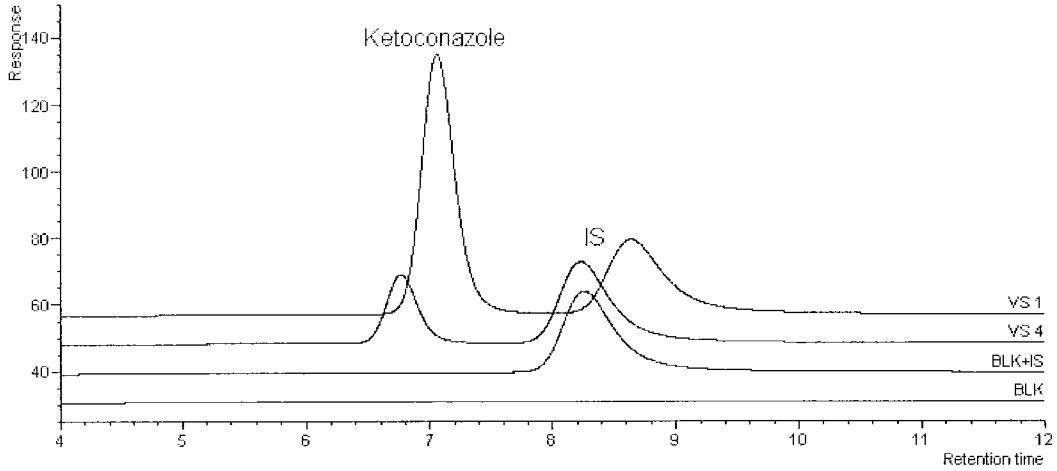

Figure 2 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from 2/4/2005 Analysis (Shown Top to Bottom)

The regression analysis results from the standard curve for February 4, 2005 analysis indicate linearity and are shown in Figure 3.

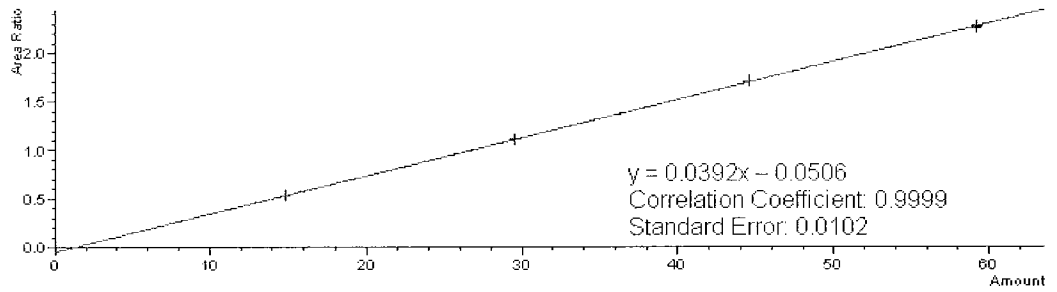

Figure 3 – Vehicle/Calibration Standard Curve for 2/4/2005 Analysis

The precision and accuracy of the vehicle/calibration standard results from February 4, 2005 analysis are shown in Table 3.

Table 3 –Vehicle/Calibration Standard Results for 2/4/2005 Analysis

Nominal Std Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s (µg/mL)	% RSD	% RE	Avg % RE
59.20	59.15	59.08	0.30	0.5	-0.1	-0.2
	59.33				0.2	
	58.75				-0.8	
44.60	44.98	NA	NA	NA	0.9	NA
29.60	29.64	NA	NA	NA	0.1	NA
14.87	14.69	14.85	0.14	0.9	-1.2	-0.1
	14.92				0.4	
	14.94				0.5	

The results of the formulation and formulation stability sample analysis are shown in Table 4 and 5. The formulation stability sample was the same formulation sample prepared and analyzed on February 4, 2005 that had been stored refrigerated for 60 days, protected from light in an amber glass bottle.

Table 4 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	RSD (%)
1-KET-1	2/4/2005	5.136	5.122	5.134	5.131	-3.4	0.1
2-KET-1	6/29/2005	5.458	5.487	5.464	5.470	3.0	0.3

The formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 5 –Formulation Stability Analysis Results

Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % of Day 0 Conc ± s
4/5/2005	5.316	5.359	5.369	5.348	104.2 ± 0.5

The formulation stability sample analyzed on April 5, 2005 was within 4.2% of the Day 0 value (February 4, 2005 analysis value) and met acceptance criteria + 10 %.

4.8 Conclusions

The average concentration of the stock formulation and its percent relative standard deviation were within acceptance criteria. Therefore the formulations were suitable for use.

The ketoconazole formulation at a target concentration of 5.31 mg/mL in DMSO was stable for 60 days when stored refrigerated and protected from light.

5 ACKNOWLEDGMENTS

Analytical support for this work was provided by Jim Hoskinson and Tudor Fernando. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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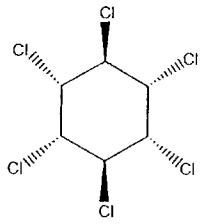
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

LINDANE

CAS No.: 58-89-9	Lot No.: 14419EB (Sigma Aldrich)
Receipt Date: 1/6/05	Amount Received: 10 g
Appearance: White Solid	Vendor Purity: 99.6% by GC
Storage Conditions (@ Battelle): Room temperature (~25°C)	

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	290.83 g/mol	$C_6H_6Cl_6$

Prepared By:

Denise A. Contos, M.S.

Approved By:

Steven W. Graves, B.S.
Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

This study was inspected by the Quality Assurance Unit (QAU) and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit analytical report	12/22/2005	12/22/2005
Audit study file	12/22/2005	12/22/2005

* These inspections are serving the purpose for all reference chemicals since QA was required to see only one phase inspection of a chemical.

Kathleen E. Reed 1-6-06
Quality Assurance Unit Date

EXECUTIVE SUMMARY

The title compound, lindane, was analyzed in support of the Environmental Protection Agency (EPA) Placental and Recombinant Aromatase Assay Prevalidation Work, Work Assignment 4-16/17.

Solubility of lindane was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze lindane in DMSO at a concentration of 29.08 mg/mL (0.1M). This method was used to analyze samples from both formulation and formulation storage stability studies at 29.08 mg/mL.

Storage stability study indicated that a 29.08 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 168 days at approximately 5°C.

The formulations prepared for shipment to the testing laboratory were determined and met the established acceptance criteria.

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1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for lindane on Environmental Protection Agency (EPA) Work Assignment 4-16/17, and consisted of:

- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

This work was done at Battelle, 505 King Avenue, Columbus, OH 43201.

2 CHEMICAL RECEIPT AND STORAGE

One 20-mL amber glass bottle of lindane, 14419EB, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on January 6, 2005. The label amount indicated 10 grams was sent. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99.6% based on gas chromatography (GC).

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Lindane
Product Number	23,339-0
Product Brand	ALDRICH
CAS Number	58-89-9
Molecular Formula	$C_6H_6Cl_6$
Molecular Weight	290.83

TEST	**SPECIFICATION**	**LOT 14419EB RESULTS**
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD
GAS LIQUID CHROMATOGRAPHY	96.5% (MINIMUM)	99.6%
QUALITY CONTROL ACCEPTANCE DATE		MAY, 2003

Ronnie J. Martin, Supervisor
Quality Control
Milwaukee, Wisconsin USA

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of lindane in 100% DMSO, at a concentration of at least 29.08 mg/mL. Lindane (0.29080 ± 0.02908 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well. The lindane went readily into solution. This experiment showed that DMSO was an acceptable solvent for the 29.08 mg/mL formulation.

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of lindane in DMSO at a target concentration of 29.08 mg/mL for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

Method development for this chemical involved the evaluation of various chromatographic conditions. The selected method was one which produced acceptable retention time for the major peak, apparent resolution of significant impurities and acceptable peak shape. The detection method chosen was gas chromatography with flame ionization detection (FID).

4.2 Method

The GC parameters for lindane are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5, 30 m x 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at ~2 mL/minute
Oven Temperature	150°C, hold for ~2 minutes, increase at 20°C/minute to 300°C; hold for 2 minutes
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at ~30 mL/minute; Air at ~380 mL/minute
Detector Temperature	320°C
Injector Temperature	285°C
Injection Volume	1 µL
Injection Mode	Split 5:1
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

Triplicate vehicle/calibration standards at the highest and lowest of four concentrations were prepared. A single standard was prepared at each intermediate concentration. The high and low concentrations were used to assess the precision of the method. The precision of the low concentration was used to calculate limits of detection (LOD) and limits of quantitation (LOQ). Triplicate vehicle/calibration blanks with and without working internal standard (WIS) were used to assess the specificity of the method.

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Approximately 25 ± 1 mg of phenanthrene was added to a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

The IS was prepared by pipetting 10 mL of stock IS into a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards were prepared by accurately weighing 50 ± 2 mg of lindane each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 2000 µg/mL each.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. The contents of the flasks were diluted to volume with methanol and mixed well. Triplicate vehicle/calibration standards were prepared at the low and high concentrations with single vehicle/calibration standards prepared at the two middle concentrations.

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	WIS (mL)	DMSO (mL)	Final Volume (mL)
VS1	800	A	4	1	0.1	10
VS2	600	B	3	1	0.1	10
VS3	400	A	2	1	0.1	10
VS4	200	B	1	1	0.1	10

4.3.1.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.1 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

4.3.2 Analysis

A portion of each vehicle/calibration standard and blank was transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the same chromatographic system and parameters determined during method development as shown in Table 1.

4.3.3 Calculations

The integration of the lindane and IS peaks by the chromatography data system was evaluated to assure it was consistent in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of lindane divided by the IS (y) to the concentration of

the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. These values were used to calculate the individual and average concentrations, percent relative errors (RE), standard deviation (s), and percent relative standard deviation (RSD) as appropriate for the vehicle/calibration standards at each concentration.

4.3.4 Results

Specificity is shown by the representative overlaid chromatograms from a high and low vehicle/calibration standard, a blank with IS, and a blank from the validation as indicated in Figure 2. The blank and blank with IS exhibited no peaks that would significantly interfere with the lindane or IS peaks. The regression analysis results from the standard curve indicate the linearity and are shown in Table 3.

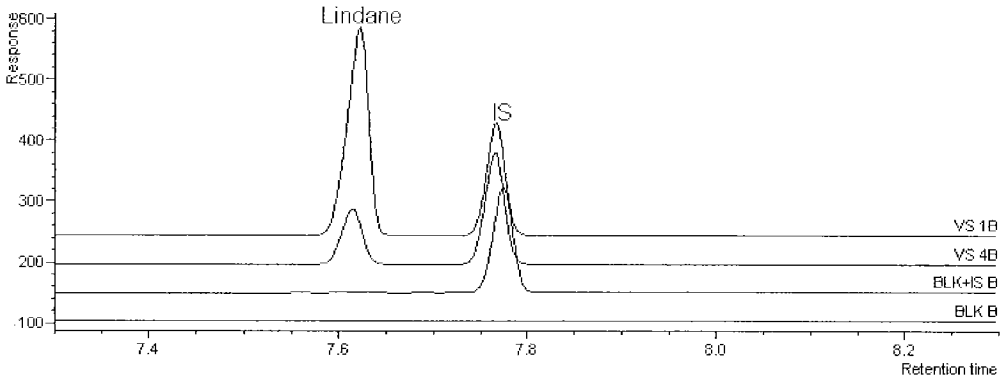

Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Method Validation Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0027	-0.0351	1.000	0.0047

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	%RSD	%RE	Avg %RE
	777.3				0.1	
776.3	777.6	776.8	1.1	0.1	0.2	0.1
	775.6				-0.1	
600.2	598.4	NA	NA	NA	-0.3	NA
388.2	387.0	NA	NA	NA	-0.3	NA
	202.8				1.4	
200.1	200.1	200.5	2.1	1.1	0.0	0.2
	198.6				-0.7	

The sensitivity of the method resulted in 6.4 µg/mL LOD which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 640 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 21.3 µg/mL. This is equivalent to a formulation concentration of 2130 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 200.1 µg/mL.

4.3.5 Conclusions

The method met all acceptance criteria for specificity, linearity, precision, accuracy, and sensitivity. The method was suitable for the stability study and subsequent formulation analyses.

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 29.08 mg/mL in DMSO for 168 days (24 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A single sample was analyzed on the day of preparation (Day 0), Day 14, Weeks 4, 8 and 12. A second formulation sample was prepared and analyzed on January 24, 2005 (Day 0) and on Week 24. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on January 13, 2005, Day 0 of the storage stability study at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 727 ± 7 mg of lindane into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with

DMSO. The flask was sealed and manually shaken to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

Approximately 6 mL of formulation was transferred into each of four, 8-mL amber glass vials which were then sealed. One vial was used for the Day 0 analysis and the other three were stored at approximately 5°C until use. After the desired storage period, a vial was removed from storage, allowed to warm to room temperature, and triplicate aliquots were prepared and analyzed.

A second formulation (Batch 1-LIN-1) was prepared on January 24, 2005 (Day 0) at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 1.45400 ± 0.058 g into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with DMSO, sealed and mixed well. The contents of the flask was diluted to volume with DMSO and mixed well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0 and 168 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report.

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of IS were pipetted into 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. An appropriate volume of each was transferred to an autoinjector vial and the vials were sealed and analyzed using the chromatographic system in Table 1.

5.4 Results

The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (29.08 mg/mL)

Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
1/13/05	1/13/05	0	29.38	29.48	29.18	29.35 ± 0.15	100 ± 0.5
1/13/05	1/27/05	14	28.56	28.56	28.67	28.60 ± 0.06	97.4 ± 0.2
1/13/05	2/10/05	28	31.36	31.30	31.64	31.43 ± 0.18	107 ± 0.6
1/13/05	3/10/05	56	28.77	28.76	28.65	28.73 ± 0.07	97.9 ± 0.2
1/13/05	4/7/05	84	29.22	29.67	29.47	29.45 ± 0.23	100 ± 0.8
1/24/05	1/24/05	0	30.02	29.88	29.93	29.95 ± 0.07	100 ± 0.2
1/24/05	7/11/05	168	29.64	29.72	29.95	29.77 ± 0.16	99.4 ± 0.5

For the formulation sample prepared on January 13, 2005, the pooled relative standard deviation of the analytical method was 0.5%. This means that there would have to be a difference of more than 1.2% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation sample prepared on January 24, 2005, the pooled RSD of the analytical method was 0.6%. This means that there would have to be a difference of more than 1.3% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

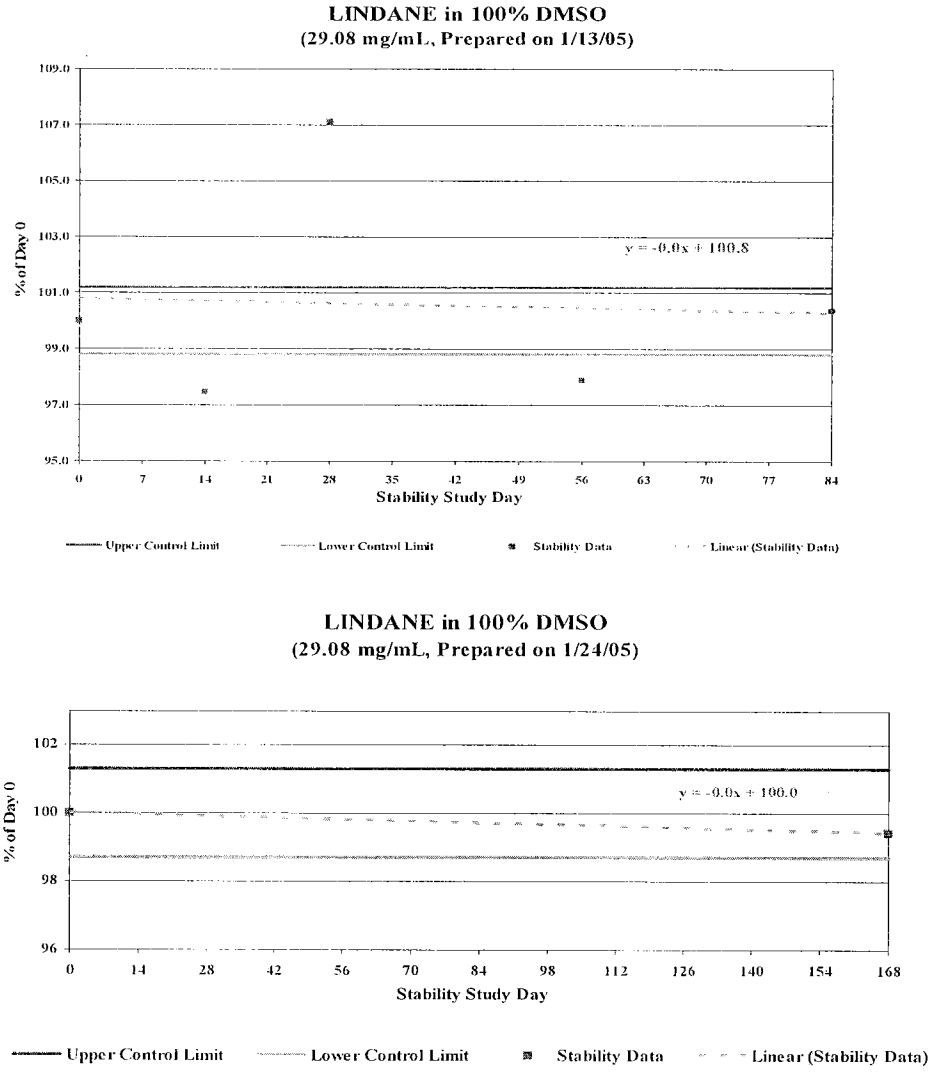

Figure 3 – Control Chart for the Storage Stability Study

5.5 Discussion and Conclusions

The Day 0 determined value for the formulation prepared on January 13, 2005 was approximately 1.0% above nominal (the calculated concentration based on the weight of the chemical). The concentrations of the samples stored at approximately 5°C protected from light in amber glass vials for Days 14 and 56 were below the lower significance level and for Day 28 it was above the upper significance level due to the tight precision of the assay. The average concentrations of the samples were within 2.6% (Day 14), 7.1% (Day 28), 2.1% (Day 56), and 0.4% (Day 84) of the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C for 84 days.

The formulation stability sample prepared on January 24, 2005 (Day 0) and analyzed on Day 0 and Day 168 (July 11, 2005) was approximately 3.0% above nominal for Day 0 (the calculated concentration based on the weight of the chemical) and for Day 168, 0.6% below the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C protected from light for 168 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on January 24, 2005, March 21, 2005 and July 1, 2005, according to SOP COMSPEC.II-029, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Lindane in 100% Dimethylsulfoxide (DMSO)." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Lindane (1.45400 ± 0.058 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

Standards and blanks were prepared as described for the validation (Section 4.3.1 of this report).

6.3 Preparation of Formulation Samples

One (1) mL of the formulation was pipetted into three individual 10-mL volumetric flasks, diluted to volume with methanol, sealed, and mixed well. One (1) mL of the diluted formulation and 1-mL of IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with methanol, sealed, and mixed well.

6.4 Analysis

Autosampler vials were filled with aliquots of each standard, blank and sample. A single injection was made from each vial using the GC conditions from the validation (Table 1). Representative overlaid chromatograms of the high and low vehicle/calibration standards, blank with IS, and a blank are shown in Figure 4.

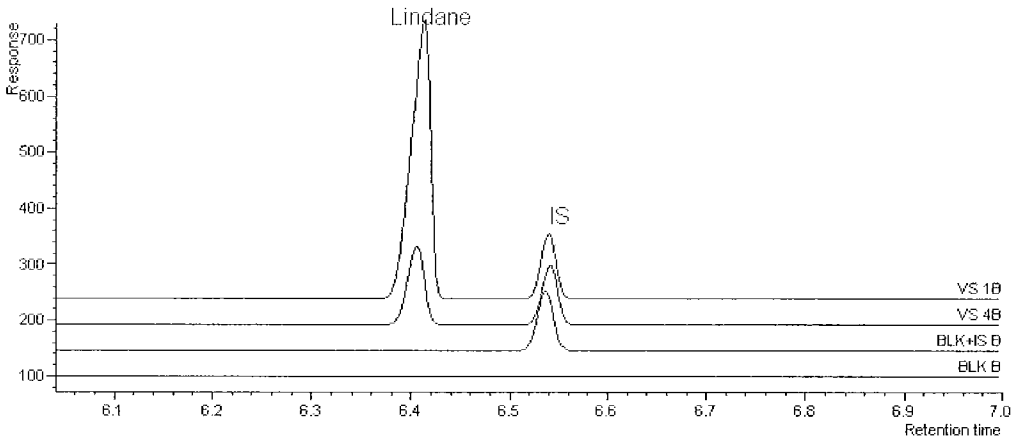

Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

The peaks for lindane and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation was calculated relating the response ratio (lindane/IS) to the concentration of the vehicle/calibration standards. This regression equation and the response ratios were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

The regression analysis results of the vehicle/calibration standard curves indicated linearity and are shown in Table 6.

Table 6 – Formulation Regression Analysis Results

Formulation Date	Slope	y-Intercept	Correlation Coefficient
1/24/05	6.8029	-0.0081	1.000
3/21/05	7.2898	-0.0197	1.000
7/1/05	6.8477	-0.1022	1.000

The results of the formulation analysis are shown in Table 7. Formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 7 – Formulation Analysis Results

Formulation Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1/24/05	30.02	29.88	29.93	29.95	3.0	0.2
3/21/05	29.23	29.67	29.20	29.37	1.0	0.9
7/1/05	29.32	29.26	29.63	29.40	1.1	0.7

6.7 Conclusions

The average concentration of the formulations and its percent RSD were within acceptance criteria. Therefore the formulation was suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Barb Harritos, Darren Brown, John Kelly, Christina Zielinski, Jim Hoskinson, Melinda Pauff, Tudor Fernando, and Sandy Runyon. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

Appendix F

Individual Replicate Spreadsheets (1, 2, and 3)

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0198	28285.11	1428541	
2	0.0198	29507.08	1490257	
3	0.0199	30049.46	1510023	
4	0.0195	30340.47	1555922	
5	0.0195	30528.27	1565552	
			Average DPM/g soln	1510059
			SD	55273
			CV	3.66
			µCi/g soln	0.680

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.2029 g
Mass of dilution B used in substrate prep	4.5998 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.560753 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00770 µg/g soln.
µg/g soln.

a. µCi/g soln	0.680
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.560753 + 0.00770
= 0.568453 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.197 µCi/µg ASDN

760803 dpm/nmol

Assay Date	2/1/2005	Test Chemical ID	RC 1	tested	8		
ID	JG	Replicate #	1	Microsome type	Placental	Microsome ID	MET-0255A #6

Standards:

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	$A_{adj.}$	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

BSA)

Total volume of stock (mL)

Protein stock ID

Assay Date	2/1/2005	Test Chemical ID	RC 1		tested	8		
ID	JG	Replicate #	1	Microsome type	Placental	Microsome ID	MET-0255A #6	
							Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-2
	0.386	0.277	0.194	0.157	0.089	0.051	0.023		
	0.386	0.289	0.224	0.154	0.093	0.053	0.019		
	0.388	0.288	0.214	0.158	0.095	0.056	0.020		

Samples:

0.096
0.093
0.094

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.387	0.366	0.0366	m, b	0.102	-0.001
0.00100	25	0.0250	0.285	0.264	0.0261	se_m, se_b	0.003	0.001
0.00075	25	0.0188	0.211	0.190	0.0186	r , se_y	0.996	0.001
0.00050	25	0.0125	0.156	0.136	0.0130	F, df	1063	4
0.00025	25	0.0063	0.092	0.072	0.0065	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.053	0.033	0.0025			

Regression results are calculated using the function LINEST

Blank 0.021

r^2= 0.996
m= 0.102
b= -0.001

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.096	0.075	0.007	25	80	3980	0.014	0.013	13.244
0.093	0.072	0.006	25	80	3980	0.013		
0.094	0.074	0.007	25	80	3980	0.013		

Assay Date	2/1/2005	Chemical ID	RC 1	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	1

Microsome Dilution Details		
Dilution A	0.08	mL microsome Stock used
	3.98	mL total volume
	49.75	dilution factor
Dilution B	3.9	mL microsome Dilution A used
	42.9	mL total volume
	11	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	547.25	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	13.244
Protein Concentration (dilution added to assay, mg/mL):	0.024201

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	1.00E-06
4	1.00E-07
5	5.00E-08
6	1.00E-08
7	1.00E-09
8	1.00E-10

Assay Date:	2/1/2005	Test Chemical ID: RC 1	# Concentrations tested:	6	Microsome type:	Placental	Microsome ID:	MET-0255A #6	Technician ID:	JG	Replicate #:	1

Sample/ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (dpm/ml)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes uses in assay tube (mL)	assay (mg/mL)	Incubation time (min)	nitrogen containing protein/min
Full activity control	1	2	0.5	1	5008.32	10016.64	10152.96	20305.92	0.1	151000	13.45	20214	0.0266	1	0.012	15	0.0732
			0.5	2	5144.64	10289.28			0.1					1		15	
	2	2	0.5	1	4535.04	9066.08	9005.9	18011.8	0.1	151000	11.93	17920	0.0236	1	0.012	15	0.0649
			0.5	2	4472.96	8945.72			0.1					1		15	
	3	2	0.5	1	4774.70	9549.4	9469.87	18939.74	0.1	151000	12.54	18848	0.0248	1	0.012	15	0.0682
			0.5	2	4695.17	9390.34			0.1					1		15	
	4	2	0.5	1	5243.68	10487.36	10529.44	21058.88	0.1	151000	13.95	20967	0.0276	1	0.012	15	0.0759
			0.5	2	5265.76	10531.52			0.1					1		15	
Background control	1	2	0.5	1	18.28	36.56	39.76	79.52	0.1	151000	0.05	-12	0.0000	1	0.012	15	0.0000
			0.5	2	21.47	42.94			0.1					1		15	
	2	2	0.5	1	31.44	62.88	62.83	125.66	0.1	151000	0.08	34	0.0000	1	0.012	15	0.0001
			0.5	2	31.36	62.78			0.1					1		15	
	3	2	0.5	1	17.00	34	44.05	88.1	0.1	151000	0.06	-4	0.0000	1	0.012	15	0.0000
			0.5	2	27.05	54.1			0.1					1		15	
	4	2	0.5	1	15.21	30.42	36.71	73.42	0.1	151000	0.05	-18	0.0000	1	0.012	15	-0.0001
			0.5	2	21.50	43			0.1					1		15	
Positive control	1	2	0.5	1	2058.86	4118.72	4128.91	8257.82	0.1	151000	5.47	6198	0.0107	1	0.012	15	0.0296
			0.5	2	2069.05	4138.1			0.1					1		15	
	2	2	0.5	1	2018.50	4037	4027.46	8054.92	0.1	151000	5.33	7663	0.0105	1	0.012	15	0.0288
			0.5	2	2010.96	4021.92			0.1					1		15	
	3	2	0.5	1	2346.26	4696.4	4717.66	9435.32	0.1	151000	6.25	9344	0.0123	1	0.012	15	0.0338
			0.5	2	2369.48	4738.92			0.1					1		15	
	4	2	0.5	1	2227.96	4455.92	4453.05	8907.9	0.1	151000	5.90	8816	0.0116	1	0.012	15	0.0319
			0.5	2	2225.98	4451.96			0.1					1		15	
Negative Control	1	2	0.5	1	29.12	58.24	58.94	117.88	0.1	151000	0.08	26	0.0000	1	0.012	15	0.0001
			0.5	2	29.82	59.64			0.1					1		15	
	2	2	0.5	1	27.07	54.14	59.45	118.9	0.1	151000	0.08	27	0.0000	1	0.012	15	0.0001
			0.5	2	32.38	64.76			0.1					1		15	
	3	2	0.5	1	16.15	32.3	44.31	88.62	0.1	151000	0.06	-3	0.0000	1	0.012	15	0.0000
			0.5	2	28.16	56.32			0.1					1		15	
	4	2	0.5	1	12.26	24.52	30.12	60.24	0.1	151000	0.04	-31	0.0000	1	0.012	15	-0.0001
			0.5	2	17.86	35.72			0.1					1		15	
RC 1	1-1	2	0.5	1	301.64	603.28	804.11	1208.22	0.1	151000	0.80	1117	0.0015	1	0.012	15	0.0040
			0.5	2	302.47	604.94			0.1					1		15	
	1-2	2	0.5	1	324.48	648.96	658.63	1317.26	0.1	151000	0.87	1226	0.0016	1	0.012	15	0.0044
			0.5	2	334.15	668.3			0.1					1		15	
	1-3	2	0.5	1	286.21	572.42	569.43	1138.86	0.1	151000	0.75	1047	0.0014	1	0.012	15	0.0038
			0.5	2	283.22	566.44			0.1					1		15	
	2-1	2	0.5	1	1385.08	2770.16	2765.84	5531.68	0.1	151000	3.66	5140	0.0072	1	0.012	15	0.0197
			0.5	2	1380.74	2761.52			0.1					1		15	
	2-2	2	0.5	1	1437.97	2875.94	2646.02	5603.24	0.1	151000	3.77	5602	0.0074	1	0.012	15	0.0203
			0.5	2	1406.65	2813.3			0.1					1		15	
	2-3	2	0.5	1	1373.74	2747.48	2776.51	5553.02	0.1	151000	3.68	5497	0.0072	1	0.012	15	0.0196
			0.5	2	1405.77	2811.54			0.1					1		15	
	3-1	2	0.5	1	4089.31	8178.62	8286.44	16572.88	0.1	151000	10.87	16461	0.0217	1	0.012	15	0.0597
			0.5	2	4197.13	8394.26			0.1					1		15	
	3-2	2	0.5	1	3981.83	7963.66	8121.02	16242.04	0.1	151000	10.76	16150	0.0212	1	0.012	15	0.0585
			0.5	2	4139.19	8278.38			0.1					1		15	
	3-3	2	0.5	1	4253.94	8507.88	8420.46	16840.92	0.1	151000	11.15	16749	0.0220	1	0.012	15	0.0606
			0.5	2	4166.52	8333.04			0.1					1		15	
	4-1	2	0.5	1	5218.24	10436.48	10368.28	20736.56	0.1	151000	13.73	20645	0.0271	1	0.012	15	0.0748
			0.5	2	5150.04	10300.08			0.1					1		15	
	4-2	2	0.5	1	5305.87	10611.74	10492.58	20985.16	0.1	151000	13.90	20893	0.0275	1	0.012	15	0.0757
			0.5	2	5186.71	10373.42			0.1					1		15	
	4-3	2	0.5	1	5253.09	10506.18	10374.68	20749.36	0.1	151000	13.74	20656	0.0272	1	0.012	15	0.0746
			0.5	2	5121.59	10243.18			0.1					1		15	
	5-1	2	0.5	1	5420.68	10847.36	10701.61	21403.22	0.1	151000	14.17	21312	0.0280	1	0.012	15	0.0772
			0.5	2	5277.93	10555.86			0.1					1		15	
	5-2	2	0.5	1	5270.62	10541.24	10565.05	21130.16	0.1	151000	13.99	21038	0.0277	1	0.012	15	0.0762
			0.5	2	5294.46	10588.92			0.1					1		15	
	5-3	2	0.5	1	5371.77	10743.54	10692.33	21384.66	0.1	151000	14.16	21293	0.0280	1	0.012	15	0.0771
			0.5	2	5320.56	10641.12			0.1					1		15	
	6-1	2	0.5	1	5473.21	10946.42	11040.75	22081.5	0.1	151000	14.62	21990	0.0288	1	0.012	15	0.0796
			0.5	2	5567.54	11135.08			0.1					1		15	
	6-2	2	0.5	1	5542.20	11084.4	11107.67	22215.34	0.1	151000	14.71	22124	0.0291	1	0.012	15	0.0801
			0.5	2	5565.47	11130.94			0.1					1		15	
	6-3	2	0.5	1	5308.79	10617.58	10677.78	21355.56	0.1	151000	14.14	21264	0.0279	1	0.012	15	0.0770
			0.5	2	5368.90	10737.98			0.1					1		15	
	7-1	2	0.5	1	5400.89	10801.78	10861.52	21723.04	0.1	151000	14.39	21631	0.0284	1	0.012	15	0.0783
			0.5	2	5460.63	10921.26			0.1					1		15	
	7-2	2	0.5	1	5290.30	10580.6	10731.54	21462.08	0.1	151000	14.21	21371	0.0281	1	0.012	15	0.0774
			0.5	2	5435.24	10870.48			0.1					1		15	
	7-3	2	0.5	1	5386.88	10773.76	10880.66	21761.32	0.1	151000	14.41	21670	0.0285	1	0.012	15	0.0785
			0.5	2	5493.78	10987.56			0.1					1		15	
	8-1	2	0.5	1	5287.09	10574.18	10456.41	20916.82	0.1	151000	13.85	20825	0.0274	1	0.012	15	0.0754
			0.5	2	5171.32	10342.64			0.1					1		15	
	8-2	2	0.5	1	5244.96	10489.92	10512.05	21024.1	0.1	151000	13.92	20932	0.0275	1	0.012	15	0.0758
			0.5	2	5287.09	10534.18			0.1					1		15	
	8-3	2	0.5	1	5303.59	10607.18	10706.33	21416.66	0.1	151000	14.18	21325	0.0280	1	0.012	15	0.0772
			0.5	2	5404.74	10809.48			0.1					1		15	

Assay Date	2/1/2005	ID	RC 1	# Concentrations tested	Microsome type	Placental	Microsome ID	Technician ID	JG	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0690	0.0059
Full activity	End	0.0721	0.0054
Full activity	Overall	0.0706	0.0049
Background	Beginning	0.0000	0.000118131
Background	End	0.0000	3.7585E-05
Background	Overall	0.0000	8.48828E-05
Positive	Beginning	0.0292	0.0005
Positive	End	0.0329	0.0014
Positive	Overall	0.0310	0.0023
Negative	Beginning	0.0001	0.0000
Negative	End	-0.0001	0.0001
Negative	Overall	0.0000	0.0001

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 1	1	1	1.00E-04	-4.00	0.0040
RC 1	1	2	1.00E-04	-4.00	0.0044
RC 1	1	3	1.00E-04	-4.00	0.0038
RC 1	2	1	1.00E-05	-5.00	0.0197
RC 1	2	2	1.00E-05	-5.00	0.0203
RC 1	2	3	1.00E-05	-5.00	0.0198
RC 1	3	1	1.00E-06	-6.00	0.0597
RC 1	3	2	1.00E-06	-6.00	0.0585
RC 1	3	3	1.00E-06	-6.00	0.0606
RC 1	4	1	1.00E-07	-7.00	0.0748
RC 1	4	2	1.00E-07	-7.00	0.0757
RC 1	4	3	1.00E-07	-7.00	0.0748
RC 1	5	1	5.00E-08	-7.30	0.0772
RC 1	5	2	5.00E-08	-7.30	0.0762
RC 1	5	3	5.00E-08	-7.30	0.0771
RC 1	6	1	1.00E-08	-8.00	0.0796
RC 1	6	2	1.00E-08	-8.00	0.0801
RC 1	6	3	1.00E-08	-8.00	0.0770
RC 1	7	1	1.00E-09	-9.00	0.0783
RC 1	7	2	1.00E-09	-9.00	0.0774
RC 1	7	3	1.00E-09	-9.00	0.0785
RC 1	8	1	1.00E-10	-10.00	0.0754
RC 1	8	2	1.00E-10	-10.00	0.0758
RC 1	8	3	1.00E-10	-10.00	0.0772

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-4.00	5.73	6.29	5.37
2	-5.00	27.92	28.74	28.06
3	-6.00	84.57	82.88	85.95
4	-7.00	105.94	107.22	106.01
5	-7.30	109.36	107.96	109.27
6	-8.00	112.84	113.53	109.12
7	-9.00	111.00	109.67	111.20
8	-10.00	106.86	107.42	109.43

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0196	30096.13	1535517	
2	0.0199	31359.14	1575836	
3	0.0196	31608.37	1612672	
4	0.0201	31736.12	1578911	
5	0.0199	31682.78	1592099	
			Average DPM/g soln	1579007
			SD	28312
			CV	1.79
			µCi/g soln	0.711

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.2226 g
Mass of dilution B used in substrate prep	4.6247 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.562438 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00805 µg/g soln.
µg/g soln.

a. µCi/g soln 0.711
b. Specific activity of [H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.562438 + 0.00805
= 0.570489 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.247 µCi/µg ASDN

792701 dpm/nmol

Assay Date	2/3/2005	Test Chemical ID	RC 1	tested	8		
ID	JG	Replicate #	2	Microsome type	Placental	Microsome ID	MET-0255A13

Standards:

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r, se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Total volume of stock (mL)

Protein stock ID

Assay Date	2/3/2005	Test Chemical ID	RC 1	tested	8			
ID	JG	Replicate #	2	Microsome type	Placental	Microsome ID	MET-0255A13	
							Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-3
	0.385	0.315	0.219	0.164	0.100	0.062	0.026		
	0.390	0.321	0.230	0.170	0.104	0.061	0.027		
	0.384	0.314	0.234	0.169	0.107	0.062	0.028		
Samples:									
	0.063								
	0.062								
	0.062								

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.386	0.360	0.0350	m, b	0.101	-0.001
0.00100	25	0.0250	0.317	0.290	0.0279	se_m, se_b	0.007	0.002
0.00075	25	0.0188	0.227	0.201	0.0189	r , se_y	0.980	0.002
0.00050	25	0.0125	0.168	0.141	0.0129	F, df	194	4
0.00025	25	0.0063	0.104	0.077	0.0064	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.062	0.035	0.0021			

Blank 0.027

r^2= 0.980
m= 0.101
b= -0.001

Regression results are calculated using the function LINEST

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Finai vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.063	0.036	0.002	25	90	4390	0.004	0.004	4.352
0.062	0.035	0.002	25	90	4390	0.004		
0.062	0.036	0.002	25	90	4390	0.004		

Assay Date	2/3/2005	Chemical ID	RC 1	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	2

Microsome Dilution Details

Dilution A	0.09	mL microsome Stock used
	4.39	mL total volume
	48.77778	dilution factor
Dilution B	3.9	mL microsome Dilution A used
	43.9	mL total volume
	11.25641	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	549.0627	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	4.352
Protein Concentration (dilution added to assay, mg/mL):	0.007926

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	5.00E-06
4	2.50E-06
5	1.00E-06
6	5.00E-07
7	1.00E-07
8	1.00E-08

Assay Date	2/3/2005	Test Chemical ID	RC 1	# Concentrations tested	6	Microsome type	Placental	Microsome ID	MET-0255A13	Technician ID	JG	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol ^3H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estrogen formed/min protein/min
Full activity control	1	2	0.5	1	1711	3422.10	3366.98	6733.96	0.1	157901	4.30	6642	0.0084	1	0.004	15	0.0705
			0.5	2	1680	3371.82			0.1					1		15	
	2	2	0.5	1	1726	3450.71	3421.67	6843.34	0.1	157901	4.33	6692	0.0084	1	0.004	15	0.0710
			0.5	2	1693	3389.64			0.1					1		15	
	3	2	0.5	1	1576	3151.28	3142.6	6285.2	0.1	157901	3.98	6134	0.0077	1	0.004	15	0.0651
			0.5	2	1567	3133.92			0.1					1		15	
	4	2	0.5	1	1631	3262.76	3206.74	6413.48	0.1	157901	4.06	6262	0.0079	1	0.004	15	0.0664
			0.5	2	1575	3150.71			0.1					1		15	
Background control	1	2	0.5	1	33	66.47	69.19	138.38	0.1	157901	0.09	-13	0.0000	1	0.004	15	-0.0001
			0.5	2	36	71.91			0.1					1		15	
	2	2	0.5	1	43	85.52	81.62	163.24	0.1	157901	0.10	12	0.0000	1	0.004	15	0.0001
			0.5	2	39	77.72			0.1					1		15	
	3	2	0.5	1	35	69.56	72.59	145.18	0.1	157901	0.09	-6	0.0000	1	0.004	15	-0.0001
			0.5	2	38	75.62			0.1					1		15	
	4	2	0.5	1	24	79.66666667	79.66666667	159.33333	0.1	157901	0.10	8	0.0000	1	0.004	15	0.0001
			0	2					0.1					1		15	
Positive control	1	2	0.5	1	1192	2384.64	2394.28	4788.56	0.1	157901	3.03	4637	0.0058	1	0.004	15	0.0492
			0.5	2	1202	2403.74			0.1					1		15	
	2	2	0.5	1	753	1505.06	1506.08	3012.16	0.1	157901	1.91	2861	0.0036	1	0.004	15	0.0304
			0.5	2	753	1506.52			0.1					1		15	
	3	2	0.5	1	735	1469.26	1468.51	2939.02	0.1	157901	1.86	2787	0.0035	1	0.004	15	0.0296
			0.5	2	735	1469.74			0.1					1		15	
	4	2	0.5	1	697	1393.08	1386.73	2773.46	0.1	157901	1.76	2622	0.0033	1	0.004	15	0.0278
			0.5	2	690	1380.41			0.1					1		15	
Negative Control	1	2	0.5	1	1698	3395.94	3351.15	6702.3	0.1	157901	4.24	6551	0.0083	1	0.004	15	0.0695
			0.5	2	1653	3306.36			0.1					1		15	
	2	2	0.5	1	1761	3522.56	3492.85	6985.7	0.1	157901	4.42	6834	0.0086	1	0.004	15	0.0725
			0.5	2	1732	3463.14			0.1					1		15	
	3	2	0.5	1	1492	2984.04	2940.41	5880.82	0.1	157901	3.72	5729	0.0072	1	0.004	15	0.0608
			0.5	2	1448	2896.78			0.1					1		15	
	4	2	0.5	1	1529	3058.72	3022.58	6045.16	0.1	157901	3.83	5894	0.0074	1	0.004	15	0.0625
			0.5	2	1493	2986.44			0.1					1		15	
RC 1	1-1	2	0.5	1	120	239.3	249.26	498.52	0.1	157901	0.32	347	0.0004	1	0.004	15	0.0027
			0.5	2	130	259.22			0.1					1		15	
	1-2	2	0.5	1	106	212.76	211.93	423.86	0.1	157901	0.27	272	0.0003	1	0.004	15	0.0023
			0.5	2	106	211.1			0.1					1		15	
	1-3	2	0.5	1	94	188.28	190.64	380.68	0.1	157901	0.25	248	0.0003	1	0.004	15	0.0020
			0.5	2	100	211.4			0.1					1		15	
	2-1	2	0.5	1	433	866.5	874.97	1749.94	0.1	157901	1.11	1598	0.0020	1	0.004	15	0.0170
			0.5	2	442	883.44			0.1					1		15	
	2-2	2	0.5	1	470	940.8	945.8	1891.6	0.1	157901	1.20	1740	0.0022	1	0.004	15	0.0185
			0.5	2	475	950.8			0.1					1		15	
	2-3	2	0.5	1	455	909.54	903.25	1806.5	0.1	157901	1.14	1655	0.0021	1	0.004	15	0.0176
			0.5	2	448	896.96			0.1					1		15	
	3-1	2	0.5	1	628	1256.16	1278.86	2557.72	0.1	157901	1.62	2400	0.0030	1	0.004	15	0.0255
			0.5	2	651	1301.56			0.1					1		15	
	3-2	2	0.5	1	694	1387.76	1385.26	2770.52	0.1	157901	1.75	2619	0.0033	1	0.004	15	0.0278
			0.5	2	691	1382.74			0.1					1		15	
	3-3	2	0.5	1	735	1469.02	1455.9	2911.8	0.1	157901	1.84	2760	0.0035	1	0.004	15	0.0293
			0.5	2	721	1442.78			0.1					1		15	
	4-1	2	0.5	1	984	1968.2	1942.82	3885.64	0.1	157901	2.46	3734	0.0047	1	0.004	15	0.0396
			0.5	2	959	1917.44			0.1					1		15	
	4-2	2	0.5	1	956	1912.04	1921.58	3843.16	0.1	157901	2.43	3692	0.0047	1	0.004	15	0.0392
			0.5	2	960	1931.12			0.1					1		15	
	4-3	2	0.5	1	942	1883.72	1882	3764	0.1	157901	2.38	3612	0.0046	1	0.004	15	0.0383
			0.5	2	940	1880.28			0.1					1		15	
	5-1	2	0.5	1	1278	2556.46	2555.49	5110.98	0.1	157901	3.24	4959	0.0063	1	0.004	15	0.0526
			0.5	2	1277	2554.52			0.1					1		15	
	5-2	2	0.5	1	2022	4056.1	4044.12	8088.24	0.1	157901	5.12	7937	0.0100	1	0.004	15	0.0842
			0.5	2	2016	4032.14			0.1					1		15	
	5-3	2	0.5	1	1255	2510.46	2538.2	5076.4	0.1	157901	3.21	4925	0.0062	1	0.004	15	0.0523
			0.5	2	1283	2565.94			0.1					1		15	
	6-1	2	0.5	1	1432	2863.96	2888.28	5776.56	0.1	157901	3.66	5625	0.0071	1	0.004	15	0.0597
			0.5	2	1456	2912.6			0.1					1		15	
	6-2	2	0.5	1	1364	2728.12	2723.6	5447.2	0.1	157901	3.45	5296	0.0067	1	0.004	15	0.0562
			0.5	2	1340	2679.08			0.1					1		15	
	6-3	2	0.5	1	1413	2825.08	2798.65	5597.3	0.1	157901	3.54	5446	0.0069	1	0.004	15	0.0578
			0.5	2	1386	2771.22			0.1					1		15	
	7-1	2	0.5	1	1579	3158.64	3192.91	6385.82	0.1	157901	4.04	6234	0.0079	1	0.004	15	0.0661
			0.5	2	1614	3227.18			0.1					1		15	
	7-2	2	0.5	1	1521	3042.28	3063.32	6126.64	0.1	157901	3.88	5975	0.0075	1	0.004	15	0.0634
			0.5	2	1542	3084.36			0.1					1		15	
	7-3	2	0.5	1	1546	3091.68	3135.72	6271.44	0.1	157901	3.97	6120	0.0077	1	0.004	15	0.0649
			0.5	2	1590	3179.76			0.1					1		15	
	8-1	2	0.5	1	1551	3102.72	3133.71	6267.42	0.1	157901	3.97	6116	0.0077	1	0.004	15	0.0649
			0.5	2	1582	3164.7			0.1					1		15	
	8-2	2	0.5	1	1617	3233.14	3224.06	6448.12	0.1	157901	4.06	6297	0.0079	1	0.004	15	0.0668
			0.5	2	1607	3214.98			0.1					1		15	
	8-3	2	0.5	1	1649	3297.62	3309.69	6619.38	0.1	157901	4.19	6468	0.0082	1	0.004	15	0.0686
			0.5	2	1661	3322.36			0.1					1		15	

Assay Date	2/3/2005	ID	RC 1	# Concentrations tested	Microsome 8 type	Placental	Microsome ID	MET-0255A13	Technician ID	JG	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0707	0.0004
Full activity	End	0.0658	0.0010
Full activity	Overall	0.0683	0.0029
Background	Beginning	0.0000	0.000186517
Background	End	0.0000	0.000106188
Background	Overall	0.0000	0.000124231
Positive	Beginning	0.0398	0.0133
Positive	End	0.0287	0.0012
Positive	Overall	0.0342	0.0100
Negative	Beginning	0.0710	0.0021
Negative	End	0.0617	0.0012
Negative	Overall	0.0663	0.0056

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 1	1	1	1.00E-04	-4.00	0.0037
RC 1	1	2	1.00E-04	-4.00	0.0029
RC 1	1	3	1.00E-04	-4.00	0.0026
RC 1	2	1	1.00E-05	-5.00	0.0170
RC 1	2	2	1.00E-05	-5.00	0.0185
RC 1	2	3	1.00E-05	-5.00	0.0176
RC 1	3	1	5.00E-06	-5.30	0.0255
RC 1	3	2	5.00E-06	-5.30	0.0278
RC 1	3	3	5.00E-06	-5.30	0.0293
RC 1	4	1	2.50E-06	-5.60	0.0396
RC 1	4	2	2.50E-06	-5.60	0.0392
RC 1	4	3	2.50E-06	-5.60	0.0383
RC 1	5	1	1.00E-06	-6.00	0.0526
RC 1	5	2	1.00E-06	-6.00	0.0842
RC 1	5	3	1.00E-06	-6.00	0.0523
RC 1	6	1	5.00E-07	-6.30	0.0597
RC 1	6	2	5.00E-07	-6.30	0.0562
RC 1	6	3	5.00E-07	-6.30	0.0578
RC 1	7	1	1.00E-07	-7.00	0.0661
RC 1	7	2	1.00E-07	-7.00	0.0634
RC 1	7	3	1.00E-07	-7.00	0.0649
RC 1	8	1	1.00E-08	-8.00	0.0649
RC 1	8	2	1.00E-08	-8.00	0.0668
RC 1	8	3	1.00E-08	-8.00	0.0686

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-4.00	5.39	4.23	3.86
2	-5.00	24.85	27.05	25.73
3	-5.30	37.41	40.72	42.91
4	-5.60	58.05	57.39	56.16
5	-6.00	77.10	123.39	76.56
6	-6.30	87.45	82.33	84.66
7	-7.00	96.92	92.89	95.14
8	-8.00	95.08	97.89	100.55

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0204	30623.18	1501136	
2	0.0201	31098.76	1547202	
3	0.0200	31597.1	1579855	
4	0.0200	31497.81	1574891	
5	0.0198	31694.85	1600750	
			Average DPM/g soln	1560767
			SD	38413
			CV	2.46
			µCi/g soln	0.703

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.3	10		1030.00
Dilution A			100	10.30
Dilution B			10	1.03

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1808 g
Mass of dilution B used in substrate prep	4.6015 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.57935 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00796 µg/g soln.
µg/g soln.

a. µCi/g soln	0.703
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.579350 + 0.00796
= 0.587308 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.197 µCi/µg ASDN

761105 dpm/nmol

Test

Assay Date 2/4/2005 Chemical ID RC 1 tested 8

ID JG Replicate # 3 Microsome type Placental Microsome ID MET-0255A14

Standards:

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Total volume of stock (mL)

Protein stock ID

BSA)

Assay Date	2/4/2005	Test Chemical ID	RC 1	tested	8		
ID	JG	Replicate #	3	Microsome type	Placental	Microsome ID	MET-0255A14

Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID
	0.380	0.286	0.231	0.164	0.079	0.046	0.027	25	431007-3-4
	0.397	0.296	0.232	0.160	0.092	0.048	0.013		
	0.405	0.306	0.235	0.170	0.095	0.040	0.011		

Samples:

	0.056
	0.066
	0.067

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.394	0.377	0.0357	m, b	0.097	-0.001
0.00100	25	0.0250	0.296	0.279	0.0262	se_m, se_b	0.005	0.001
0.00075	25	0.0188	0.232	0.215	0.0200	r , se_y	0.989	0.002
0.00050	25	0.0125	0.164	0.147	0.0134	F, df	351	4
0.00025	25	0.0063	0.089	0.072	0.0061	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.045	0.028	0.0018			

Regression results are calculated using the function LINEST

	Blank	0.017	r^2=	0.989
			m=	0.097
			b=	-0.001

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.056	0.039	0.003	25	90	4390	0.006	0.007	7.087
0.066	0.049	0.004	25	90	4390	0.008		
0.067	0.050	0.004	25	90	4390	0.008		

Assay Date	2/4/2005	Chemical ID	RC 1	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	3

Microsome Dilution Details

Dilution A	0.09	mL microsome Stock used
	4.39	mL total volume
	48.77778	dilution factor
Dilution B	3.9	mL microsome Dilution A used
	43.9	mL total volume
	11.25641	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	549.0627	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	7.087
Protein Concentration (dilution added to assay, mg/mL):	0.012907

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	5.00E-06
4	2.50E-06
5	1.00E-06
6	5.00E-07
7	1.00E-07
8	1.00E-08

Assay Date	2/4/2005	Test Chemical ID	RC 1	# Concentrations tested		S Microsome type	Placental	Microsome ID	MET-0253A14	Technician ID	JG	Replicate #	3

Sample ID		Calculate DPM in aqueous portion after extraction						Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal Inital volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	Inital DPM in assay tube (dpm)	% conversion to product	Total DPM corrected for blank(ground t)Blank(ground) Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estimated nonenzymatic product/min
Full activity control	1	2	0.5	1	2711	5422.76	5455.86	10911.72	0.1	156077	6.99	10721	0.0141	1	0.009	15	0.0726
			0.5	2	2745	5489.94			0.1					1		15	
	2	2	0.5	1	2721	5441.09	5425.91	10851.82	0.1	156077	6.95	10661	0.0140	1	0.009	15	0.0723
			0.5	2	2705	5410.74			0.1					1		15	
	3	2	0.5	1	2285	4570.72	4585.47	9170.94	0.1	156077	5.86	8980	0.0118	1	0.009	15	0.0609
			0.5	2	2300	4600.22			0.1					1		15	
	4	2	0.5	1	2295	4589.48	4633.89	9267.78	0.1	156077	5.94	9077	0.0119	1	0.009	15	0.0616
			0.5	2	2339	4677.3			0.1					1		15	
Background control	1	2	0.5	1	76	152.54	163.36	326.79	0.1	156077	0.21	136	0.0002	1	0.009	15	0.0009
			0.5	2	87	174.22			0.1					1		15	
	2	2	0.5	1	26	51.44	56.6	113.2	0.1	156077	0.07	-76	-0.0001	1	0.009	15	-0.0005
			0.5	2	31	61.76			0.1					1		15	
	3	2	0.5	1	44	88.2	87.04	174.08	0.1	156077	0.11	-17	0.0000	1	0.009	15	-0.0001
			0.5	2	43	85.88			0.1					1		15	
	4	2	0.5	1	38	76.28	74.74	149.48	0.1	156077	0.10	-41	-0.0001	1	0.009	15	-0.0003
			0.5	2	37	73.2			0.1					1		15	
Positive control	1	2	0.5	1	1265	2530.36	2559.02	5118.04	0.1	156077	3.28	4927	0.0065	1	0.009	15	0.0334
			0.5	2	1294	2587.68			0.1					1		15	
	2	3	0.5	1	2207	4413.14	4461.53	8923.06	0.1	156077	5.72	8732	0.0115	1	0.009	15	0.0593
			0.5	2	2255	4509.92			0.1					1		15	
	3	2	0.5	1	1120	2239.46	2206.82	4413.64	0.1	156077	2.83	4223	0.0055	1	0.009	15	0.0287
			0.5	2	1087	2174.18			0.1					1		15	
	4	2	0.5	1	1057	2113.1	2106.61	4213.22	0.1	156077	2.70	4022	0.0053	1	0.009	15	0.0273
			0.5	2	1050	2100.12			0.1					1		15	
Negative Control	1	2	0.5	1	2624	5246.74	5283.12	10566.24	0.1	156077	6.77	10375	0.0136	1	0.009	15	0.0704
			0.5	2	2650	5317.5			0.1					1		15	
	2	2	0.5	1	3219	6437.12	6451.16	12902.32	0.1	156077	8.27	12711	0.0167	1	0.009	15	0.0863
			0.5	2	3233	6465.2			0.1					1		15	
	3	2	0.5	1	2267	4534.32	4492.8	8985.6	0.1	156077	5.76	8795	0.0116	1	0.009	15	0.0597
			0.5	2	2226	4451.28			0.1					1		15	
	4	2	0.5	1	2187	4374.46	4325.25	8650.5	0.1	156077	5.54	8490	0.0111	1	0.009	15	0.0574
			0.5	2	2138	4276.04			0.1					1		15	
RC	1-1	2	0.5	1	973	1945.58	1941.9	3883.8	0.1	156077	2.49	3693	0.0049	1	0.009	15	0.0251
			0.5	2	969	1938.22			0.1					1		15	
	1-2	2	0.5	1	193	385.64	406.26	812.52	0.1	156077	0.52	622	0.0008	1	0.009	15	0.0042
			0.5	2	213	426.88			0.1					1		15	
	1-3	2	0.5	1	123	246.7	255.11	510.22	0.1	156077	0.33	319	0.0004	1	0.009	15	0.0022
			0.5	2	132	263.52			0.1					1		15	
	2-1	2	0.5	1	586	1172.7	1173.79	2347.58	0.1	156077	1.50	2157	0.0028	1	0.009	15	0.0149
			0.5	2	587	1174.88			0.1					1		15	
	2-2	2	0.5	1	617	1234.52	1219.56	2439.12	0.1	156077	1.56	2248	0.0030	1	0.009	15	0.0153
			0.5	2	602	1204.6			0.1					1		15	
	2-3	2	0.5	1	597	1194.82	1178.78	2357.56	0.1	156077	1.51	2167	0.0028	1	0.009	15	0.0147
			0.5	2	581	1162.74			0.1					1		15	
	3-1	2	0.5	1	954	1907.82	1937.86	3875.72	0.1	156077	2.48	3685	0.0048	1	0.009	15	0.0250
			0.5	2	984	1967.9			0.1					1		15	
	3-2	2	0.5	1	906	1810.68	1827.45	3654.98	0.1	156077	2.34	3464	0.0046	1	0.009	15	0.0235
			0.5	2	919	1838.3			0.1					1		15	
	3-3	2	0.5	1	2033	4066.92	4063.74	8127.48	0.1	156077	5.21	7937	0.0104	1	0.009	15	0.0539
			0.5	2	2030	4060.86			0.1					1		15	
	4-1	2	0.5	1	1558	3115.26	3104.82	6209.64	0.1	156077	3.98	6019	0.0079	1	0.009	15	0.0408
			0.5	2	1547	3094.58			0.1					1		15	
	4-2	2	0.5	1	1295	2590.86	2583.32	5166.64	0.1	156077	3.31	4976	0.0065	1	0.009	15	0.0338
			0.5	2	1288	2575.78			0.1					1		15	
	4-3	2	0.5	1	1364	2728.08	2726.85	5453.7	0.1	156077	3.49	5263	0.0069	1	0.009	15	0.0357
			0.5	2	1363	2725.62			0.1					1		15	
	5-1	2	0.5	1	1778	3555.26	3582.98	7165.96	0.1	156077	4.59	6875	0.0092	1	0.009	15	0.0473
			0.5	2	1805	3610.7			0.1					1		15	
	5-2	2	0.5	1	1931	3861.16	3937.69	7875.38	0.1	156077	5.05	7685	0.0101	1	0.009	15	0.0521
			0.5	2	2007	4014.22			0.1					1		15	
	5-3	2	0.5	1	1729	3457.8	3493.68	6987.36	0.1	156077	4.48	6796	0.0089	1	0.009	15	0.0461
			0.5	2	1765	3529.56			0.1					1		15	
	6-1	2	0.5	1	2538	5076.36	5125.58	10251.16	0.1	156077	6.57	10060	0.0132	1	0.009	15	0.0683
			0.5	2	2587	5174.8			0.1					1		15	
	6-2	2	0.5	1	3122	6244.32	6259.58	12519.16	0.1	156077	8.02	12328	0.0162	1	0.009	15	0.0837
			0.5	2	3137	6274.84			0.1					1		15	
	6-3	2	0.5	1	2825	5649.96	5707.35	11414.7	0.1	156077	7.31	11224	0.0147	1	0.009	15	0.0762
			0.5	2	2882	5764.74			0.1					1		15	
	7-1	2	0.5	1	2291	4582.28	4574.65	9149.7	0.1	156077	5.86	8959	0.0118	1	0.009	15	0.0608
			0.5	2	2314	4627.44			0.1					1		15	
	7-2	2	0.5	1	2144	4287.1	4346.22	8692.44	0.1	156077	5.57	8502	0.0112	1	0.009	15	0.0577
			0.5	2	2203	4405.34			0.1					1		15	
	7-3	2	0.5	1	2219	4438.08	4475.33	8950.66	0.1	156077	5.73	8760	0.0115	1	0.009	15	0.0594
			0.5	2	2256	4512.58			0.1					1		15	
	8-1	2	0.5	1	2259	4515.56	4585.55	9171.1	0.1	156077	5.86	8980	0.0118	1	0.009	15	0.0609
			0.5	2	2328	4655.54			0.1					1		15	
	8-2	2	0.5	1	2236	4472.42	4500.6	9001.2	0.1	156077	5.77	8810	0.0116	1	0.009	15	0.0598
			0.5	2	2264	4528.78			0.1					1		15	
	8-3	2	0.5	1	2267	4533.34	4537.5	9075	0.1	156077	5.81	8884	0.0117	1	0.009	15	0.0603
			0.5	2	2271	4541.66			0.1					1		15	

Assay Date	2/4/2005	ID	RC 1	# Concentrations tested	Microsome 8 type	Placental	Microsome ID MET-0255A14	Technician ID JG	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0725	0.0003
Full activity	End	0.0613	0.0005
Full activity	Overall	0.0669	0.0065
Background	Beginning	0.0002	0.001024775
Background	End	-0.0002	0.000118044
Background	Overall	0.0000	0.000637727
Positive	Beginning	0.0463	0.0183
Positive	End	0.0280	0.0010
Positive	Overall	0.0372	0.0150
Negative	Beginning	0.0783	0.0112
Negative	End	0.0585	0.0016
Negative	Overall	0.0684	0.0132

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 1	1	1	1.00E-04	-4.00	0.0251
RC 1	1	2	1.00E-04	-4.00	0.0042
RC 1	1	3	1.00E-04	-4.00	0.0022
RC 1	2	1	1.00E-05	-5.00	0.0146
RC 1	2	2	1.00E-05	-5.00	0.0153
RC 1	2	3	1.00E-05	-5.00	0.0147
RC 1	3	1	5.00E-06	-5.30	0.0250
RC 1	3	2	5.00E-06	-5.30	0.0235
RC 1	3	3	5.00E-06	-5.30	0.0539
RC 1	4	1	2.50E-06	-5.60	0.0408
RC 1	4	2	2.50E-06	-5.60	0.0338
RC 1	4	3	2.50E-06	-5.60	0.0357
RC 1	5	1	1.00E-06	-6.00	0.0473
RC 1	5	2	1.00E-06	-6.00	0.0521
RC 1	5	3	1.00E-06	-6.00	0.0461
RC 1	6	1	5.00E-07	-6.30	0.0683
RC 1	6	2	5.00E-07	-6.30	0.0837
RC 1	6	3	5.00E-07	-6.30	0.0762
RC 1	7	1	1.00E-07	-7.00	0.0608
RC 1	7	2	1.00E-07	-7.00	0.0577
RC 1	7	3	1.00E-07	-7.00	0.0594
RC 1	8	1	1.00E-08	-8.00	0.0609
RC 1	8	2	1.00E-08	-8.00	0.0598
RC 1	8	3	1.00E-08	-8.00	0.0603

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-4.00	37.45	6.30	3.24
2	-5.00	21.87	22.80	21.98
3	-5.30	37.37	35.13	80.50
4	-5.60	61.05	50.47	53.38
5	-6.00	70.74	77.94	68.93
6	-6.30	102.03	125.04	113.84
7	-7.00	90.86	86.23	88.84
8	-8.00	91.08	89.36	90.11

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	25485.82	1293696	
2	0.0200	26079.86	1303993	
3	0.0198	26119.83	1319183	
4	0.0197	26194.85	1329688	
5	0.0197	25648.65	1301962	
			Average DPM/g soln	1309705
			SD	14474
			CV	1.11
			µCi/g soln	0.590

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.1	10		1010.00
Dilution A			100	10.10
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.179 g
Mass of dilution B used in substrate prep	4.5948 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.567398 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00668 µg/g soln.
µg/g soln.

a. µCi/g soln	0.590
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.567398 + 0.00668
= 0.574076 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.028 µCi/µg ASDN

653396 dpm/nmol

Assay Date	2/9/2005	Test Chemical ID	RC 2	tested	8		
ID	JG	Replicate #	1	Microsome type	Placental	Microsome ID	MET-0255A #15

Standards: BSA) Total volume of stock (mL) Protein stock ID

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date	2/9/2005	Test Chemical ID	RC 2		tested		8		
ID	JG	Replicate #		1	Microsome type		Placental	Microsome ID	MET-0255A #15

Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID
	0.368	0.279	0.229	0.161	0.102	0.063	0.035	25	431007-3-5
	0.376	0.288	0.232	0.165	0.097	0.064	0.027		
	0.382	0.291	0.224	0.178	0.107	0.065	0.029		

Samples:

0.072
0.067
0.073

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.375	0.345	0.0359	m, b	0.109	-0.002
0.00100	25	0.0250	0.286	0.256	0.0261	se_m, se_b	0.005	0.001
0.00075	25	0.0188	0.229	0.198	0.0199	r , se_y	0.991	0.001
0.00050	25	0.0125	0.168	0.137	0.0133	F, df	430	4
0.00025	25	0.0063	0.102	0.072	0.0061	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.064	0.034	0.0020			

Blank 0.030

r^2= 0.991
m= 0.109
b= -0.002

Regression results are calculated using the function LINEST

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.072	0.042	0.003	25	95	5095	0.006	0.006	5.793
0.067	0.036	0.002	25	95	5095	0.005		
0.073	0.043	0.003	25	95	5095	0.006		

Assay Date	2/9/2005	Chemical ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	1

Microsome Dilution Details

Dilution A	0.095	mL microsome Stock used
	5.095	mL total volume
	53.63158	dilution factor
Dilution B	4	mL microsome Dilution A used
	44	mL total volume
	11	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	589.9474	total dilution factor

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	1.00E-06
5	1.00E-07
6	1.00E-08
7	1.00E-09
8	1.00E-10

Protein Concentration (stock microsomes, mg/mL):	5.793
Protein Concentration (dilution added to assay, mg/mL):	0.00982

Assay Date	2/9/2005	Test Chemical ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID	MET-0255A #15	Technician ID	JG	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	incubation time (min)	nmol/min/mg protein
Full activity control	1	2	0.5	1	1738	3475.44	3539.06	7078.12	0.1	130970	5.40	6892	0.0105	1	0.005	15	0.0716
			0.5	2	1801	3602.68			0.1					1		15	
	2	2	0.5	1	1675	3350.48	3343.89	6687.78	0.1	130970	5.11	6502	0.0100	1	0.005	15	0.0676
			0.5	2	1668	3337.3			0.1					1		15	
	3	2	0.5	1	1617	3233.38	3273.29	6546.58	0.1	130970	5.00	6361	0.0097	1	0.005	15	0.0661
			0.5	2	1657	3313.2			0.1					1		15	
	4	2	0.5	1	1630	3259	3262.47	6584.94	0.1	130970	5.01	6379	0.0098	1	0.005	15	0.0663
			0.5	2	1653	3305.94			0.1					1		15	
Background control	1	2	0.5	1	34	68.48	70.42	140.84	0.1	130970	0.11	-45	-0.0001	1	0.005	15	-0.0005
			0.5	2	36	72.36			0.1					1		15	
	2	2	0.5	1	103	206.76	203.66	407.32	0.1	130970	0.31	221	0.0003	1	0.005	15	0.0023
			0.5	2	100	200.56			0.1					1		15	
	3	2	0.5	1	29	58.1	54.92	109.84	0.1	130970	0.08	-79	-0.0001	1	0.005	15	-0.0008
			0.5	2	26	51.74			0.1					1		15	
	4	2	0.5	1	18	36.54	42.87	85.74	0.1	130970	0.07	-100	-0.0002	1	0.005	15	-0.0010
			0.5	2	25	49.2			0.1					1		15	
Positive control	1	2	0.5	1	907	1814.92	1826.84	3653.68	0.1	130970	2.79	3469	0.0053	1	0.005	15	0.0366
			0.5	2	919	1838.76			0.1					1		15	
	2	2	0.5	1	774	1549.48	1514.29	3028.58	0.1	130970	2.31	2843	0.0044	1	0.005	15	0.0299
			0.5	2	740	1480.12			0.1					1		15	
	3	2	0.5	1	740	1480.34	1511.35	3022.7	0.1	130970	2.31	2837	0.0043	1	0.005	15	0.0295
			0.5	2	771	1542.36			0.1					1		15	
	4	2	0.5	1	750	1500.16	1462.44	2864.88	0.1	130970	2.28	2799	0.0043	1	0.005	15	0.0291
			0.5	2	712	1424.7			0.1					1		15	
Negative Control	1	2	0.5	1	1693	3385.58	3380.84	6761.68	0.1	130970	5.16	6570	0.0101	1	0.005	15	0.0683
			0.5	2	1688	3376.1			0.1					1		15	
	2	2	0.5	1	1665	3329.08	3333.15	6666.3	0.1	130970	5.09	6480	0.0099	1	0.005	15	0.0673
			0.5	2	1669	3337.24			0.1					1		15	
	3	2	0.5	1	1543	3083.36	3082.33	6164.66	0.1	130970	4.71	5979	0.0092	1	0.005	15	0.0621
			0.5	2	1540	3079.3			0.1					1		15	
	4	2	0.5	1	1520	3040.99	3015.63	6031.26	0.1	130970	4.61	5845	0.0089	1	0.005	15	0.0607
			0.5	2	1495	2990.3			0.1					1		15	
RC 2	1-1	2	0.5	1	46	92.7	81.35	162.7	0.1	130970	0.12	-23	0.0000	1	0.005	15	-0.0002
			0.5	2	35	70			0.1					1		15	
	1-2	2	0.5	1	41	81.34	71.51	143.02	0.1	130970	0.11	-43	-0.0001	1	0.005	15	-0.0004
			0.5	2	31	61.68			0.1					1		15	
	1-3	2	0.5	1	33	65.14	74.38	148.76	0.1	130970	0.11	-37	-0.0001	1	0.005	15	-0.0004
			0.5	2	42	83.62			0.1					1		15	
	2-1	2	0.5	1	116	235.26	226.72	453.44	0.1	130970	0.35	268	0.0004	1	0.005	15	0.0026
			0.5	2	109	218.18			0.1					1		15	
	2-2	2	0.5	1	99	197.80	186.72	373.44	0.1	130970	0.29	188	0.0003	1	0.005	15	0.0018
			0.5	2	88	175.58			0.1					1		15	
	2-3	2	0.5	1	102	203.08	188.6	377.2	0.1	130970	0.29	191	0.0003	1	0.005	15	0.0020
			0.5	2	87	174.12			0.1					1		15	
	3-1	2	0.5	1	548	1096	1079.76	2159.58	0.1	130970	1.65	1974	0.0030	1	0.005	15	0.0205
			0.5	2	531	1063.58			0.1					1		15	
	3-2	2	0.5	1	530	1059.62	1066.57	2133.14	0.1	130970	1.63	1947	0.0030	1	0.005	15	0.0202
			0.5	2	537	1073.52			0.1					1		15	
	3-3	2	0.5	1	506	1011.1	1012.91	2025.82	0.1	130970	1.55	1840	0.0028	1	0.005	15	0.0191
			0.5	2	507	1014.72			0.1					1		15	
	4-1	2	0.5	1	1345	2689.04	2727.86	5455.72	0.1	130970	4.17	5270	0.0081	1	0.005	15	0.0546
			0.5	2	1383	2766.68			0.1					1		15	
	4-2	2	0.5	1	1297	2594.44	2588.74	5177.48	0.1	130970	3.95	4992	0.0076	1	0.005	15	0.0518
			0.5	2	1292	2583.04			0.1					1		15	
	4-3	2	0.5	1	1317	2633.92	2671.27	5342.54	0.1	130970	4.08	5157	0.0079	1	0.005	15	0.0536
			0.5	2	1354	2708.62			0.1					1		15	
	5-1	2	0.5	1	1626	3256.2	3272.63	6545.26	0.1	130970	5.00	6350	0.0097	1	0.005	15	0.0661
			0.5	2	1645	3289.06			0.1					1		15	
	5-2	2	0.5	1	1653	3306.74	3288.73	6577.46	0.1	130970	5.02	6392	0.0098	1	0.005	15	0.0664
			0.5	2	1635	3270.72			0.1					1		15	
	5-3	2	0.5	1	1636	3271.88	3240.24	6480.48	0.1	130970	4.95	6295	0.0096	1	0.005	15	0.0654
			0.5	2	1604	3208.6			0.1					1		15	
	6-1	2	0.5	1	1667	3334.92	3331.72	6663.44	0.1	130970	5.09	6478	0.0099	1	0.005	15	0.0673
			0.5	2	1664	3328.52			0.1					1		15	
	6-2	2	0.5	1	1643	3285.08	3269.39	6538.78	0.1	130970	4.99	6353	0.0097	1	0.005	15	0.0660
			0.5	2	1027	3253.7			0.1					1		15	
	6-3	2	0.5	1	1683	3365.2	3354.68	6709.76	0.1	130970	5.12	6524	0.0100	1	0.005	15	0.0678
			0.5	2	1672	3344.56			0.1					1		15	
	7-1	2	0.5	1	1619	3229.48	3294.15	6588.3	0.1	130970	5.03	6402	0.0098	1	0.005	15	0.0665
			0.5	2	1675	3358.82			0.1					1		15	
	7-2	2	0.5	1	1672	3344.16	3332.77	6665.54	0.1	130970	5.09	6480	0.0099	1	0.005	15	0.0673
			0.5	2	1661	3321.38			0.1					1		15	
	7-3	2	0.5	1	1576	3152.6	3219.09	6438.18	0.1	130970	4.92	6252	0.0096	1	0.005	15	0.0650
			0.5	2	1643	3285.58			0.1					1		15	
	8-1	2	0.5	1	1629	3243.36	3226.7	6453.4	0.1	130970	4.93	6267	0.0096	1	0.005	15	0.0651
			0.5	2	1605	3210.02			0.1					1		15	
	8-2	2	0.5	1	1658	3316.9	3302.33	6604.66	0.1	130970	5.04	6419	0.0098	1	0.005	15	0.0667
			0.5	2	1644	3287.76			0.1					1		15	
	8-3	2	0.5	1	1689	3376.52	3347.6	6695.2	0.1	130970	5.11	6509	0.0100	1	0.005	15	0.0678
			0.5	2	1658	3318.68			0.1					1		15	

Assay Date	2/9/2005	ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0696	0.0029
Full activity	End	0.0662	0.0001
Full activity	Overall	0.0679	0.0026
Background	Beginning	0.0009	0.001957904
Background	End	-0.0009	0.00017707
Background	Overall	0.0000	0.001551354
Positive	Beginning	0.0328	0.0046
Positive	End	0.0293	0.0003
Positive	Overall	0.0310	0.0033
Negative	Beginning	0.0678	0.0007
Negative	End	0.0614	0.0010
Negative	Overall	0.0646	0.0038

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 2	1	1	1.00E-03	-3.00	-0.0002
RC 2	1	2	1.00E-03	-3.00	-0.0004
RC 2	1	3	1.00E-03	-3.00	-0.0004
RC 2	2	1	1.00E-04	-4.00	0.0028
RC 2	2	2	1.00E-04	-4.00	0.0019
RC 2	2	3	1.00E-04	-4.00	0.0020
RC 2	3	1	1.00E-05	-5.00	0.0205
RC 2	3	2	1.00E-05	-5.00	0.0202
RC 2	3	3	1.00E-05	-5.00	0.0191
RC 2	4	1	1.00E-06	-6.00	0.0548
RC 2	4	2	1.00E-06	-6.00	0.0519
RC 2	4	3	1.00E-06	-6.00	0.0536
RC 2	5	1	1.00E-07	-7.00	0.0661
RC 2	5	2	1.00E-07	-7.00	0.0664
RC 2	5	3	1.00E-07	-7.00	0.0654
RC 2	6	1	1.00E-08	-8.00	0.0673
RC 2	6	2	1.00E-08	-8.00	0.0660
RC 2	6	3	1.00E-08	-8.00	0.0678
RC 2	7	1	1.00E-09	-9.00	0.0665
RC 2	7	2	1.00E-09	-9.00	0.0673
RC 2	7	3	1.00E-09	-9.00	0.0650
RC 2	8	1	1.00E-10	-10.00	0.0651
RC 2	8	2	1.00E-10	-10.00	0.0667
RC 2	8	3	1.00E-10	-10.00	0.0676

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-3.00	-0.36	-0.66	-0.57
2	-4.00	4.09	2.87	2.93
3	-5.00	30.21	29.80	28.16
4	-6.00	80.66	76.40	78.93
5	-7.00	97.34	97.83	96.34
6	-8.00	99.14	97.24	99.85
7	-9.00	97.99	99.18	95.70
8	-10.00	95.93	98.24	99.63

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	27982	1434974	
2	0.0195	29631	1519538	
3	0.0195	29923	1534513	
4	0.0200	29890	1494500	
5	0.0199	30362	1525729	
			Average DPM/g soln	1501851
			SD	40238
			CV	2.68
			µCi/g soln	0.677

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1645 g
Mass of dilution B used in substrate prep	4.5845 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.561516 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00766 µg/g soln.
µg/g soln.

a. µCi/g soln	0.677
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.561516 + 0.00766
= 0.569175 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.189 µCi/µg ASDN

755709 dpm/nmol

Test

Assay Date 2/14/2005 Chemical ID RC 2 tested 8

ID JG Replicate # 2 Microsome type Placental Microsome ID MET-0255A #16 & 17

Standards:

BSA)	Total volume of stock (mL)	Protein stock ID

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date 2/14/2005 Test Chemical ID RC 2 tested 8

ID JG Replicate # 2 Microsome type Placental Microsome ID MET-0255A #16 & 17

Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	Protein stock (mg/10 mL)	Protein stock ID
	0.401	0.296	0.230	0.171	0.084	0.062	0.043	25	431007-3-6
	0.427	0.308	0.233	0.170	0.116	0.061	0.034		
	0.405	0.308	0.238	0.176	0.107	0.058	0.031		

Samples:
0.158
0.161
0.146

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output
0.00150	25	0.0375	0.411	0.375	0.0364
0.00100	25	0.0250	0.304	0.268	0.0260
0.00075	25	0.0188	0.234	0.198	0.0191
0.00050	25	0.0125	0.172	0.137	0.0132
0.00025	25	0.0063	0.102	0.066	0.0063
0.00013	25	0.0033	0.060	0.025	0.0022
	Blank 0.036				

Variables	Regression results	
m, b	0.097	0.000
se_m, se_b	0.003	0.001
r , se_y	0.995	0.001
F, df	860	4
ss_{reg}, ss_{resid}	0.001	0.000

Regression results are calculated using the function LINEST

r^2= 0.995
m= 0.097
b= 0.000

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.158	0.122	0.012	25	190	5190	0.013	0.013	12.509
0.161	0.125	0.012	25	190	5190	0.013		
0.146	0.110	0.011	25	190	5190	0.012		

Assay Date	2/14/2005	Chemical ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	2

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	2.5	mL microsome Dilution A used
	44	mL total volume
	17.6	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	480.7579	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.509
Protein Concentration (dilution added to assay, mg/mL):	0.026019

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	7.50E-06
5	2.50E-06
6	1.00E-06
7	1.00E-07
8	1.00E-09

Assay Date	2/14/2005	Test Chemical ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID	MET-0255A #16 & 17	Technician ID	JG	Replicate #	2

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	Initial DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	5715.05	11430.1	11393.47	22786.94	0.1	150185	15.17	22557	0.0298	1	0.013	15	0.0765
			0.5	2	5678.42	11356.84			0.1					1		15	
	2	2	0.5	1	5555.81	11111.62	11073.31	22146.62	0.1	150185	14.75	21917	0.0290	1	0.013	15	0.0743
			0.5	2	5517.50	11035			0.1					1		15	
	3	2	0.5	1	5169.33	10338.66	10395.99	20791.98	0.1	150185	13.84	20562	0.0272	1	0.013	15	0.0697
			0.5	2	5226.66	10453.32			0.1					1		15	
	4	2	0.5	1	5042.91	10085.82	10139.94	20279.88	0.1	150185	13.50	20050	0.0265	1	0.013	15	0.0680
			0.5	2	5097.03	10194.06			0.1					1		15	
Background control	1	2	0.5	1	30.55	61.1	65.56	131.16	0.1	150185	0.09	-99	-0.0001	1	0.013	15	-0.0003
			0.5	2	35.03	70.06			0.1					1		15	
	2	2	0.5	1	117.28	234.56	239.56	479.12	0.1	150185	0.32	249	0.0003	1	0.013	15	0.0008
			0.5	2	122.28	244.56			0.1					1		15	
	3	2	0.5	1	30.98	61.96	67.92	135.84	0.1	150185	0.09	-94	-0.0001	1	0.013	15	-0.0003
			0.5	2	36.94	73.88			0.1					1		15	
	4	2	0.5	1	40.21	80.42	86.9	173.8	0.1	150185	0.12	-56	-0.0001	1	0.013	15	-0.0002
			0.5	2	46.69	93.38			0.1					1		15	
Positive control	1	2	0.5	1	3698.97	7397.94	7414.4	14828.8	0.1	150185	9.87	14568	0.0193	1	0.013	15	0.0496
			0.5	2	3715.43	7430.86			0.1					1		15	
	2	2	0.5	1	2804.82	5609.64	5558.42	11116.84	0.1	150185	7.40	10887	0.0144	1	0.013	15	0.0369
			0.5	2	2753.60	5507.2			0.1					1		15	
	3	2	0.5	1	2398.66	4797.32	4776.09	9552.18	0.1	150185	6.36	9322	0.0123	1	0.013	15	0.0316
			0.5	2	2377.43	4754.86			0.1					1		15	
	4	2	0.5	1	2262.45	4524.9	4680.19	9360.38	0.1	150185	6.23	9130	0.0121	1	0.013	15	0.0310
			0.5	2	2417.74	4835.48			0.1					1		15	
Negative Control	1	2	0.5	1	5325.06	10650.12	10736.94	21473.88	0.1	150185	14.30	21244	0.0281	1	0.013	15	0.0720
			0.5	2	5411.88	10823.76			0.1					1		15	
	2	2	0.5	1	5547.26	11094.52	11101.93	22203.86	0.1	150185	14.78	21974	0.0291	1	0.013	15	0.0745
			0.5	2	5554.67	11109.34			0.1					1		15	
	3	2	0.5	1	4827.78	9655.56	9651.2	19302.4	0.1	150185	12.85	19072	0.0252	1	0.013	15	0.0647
			0.5	2	4823.42	9646.84			0.1					1		15	
	4	2	0.5	1	4912.44	9824.88	9911.83	19823.26	0.1	150185	13.20	19593	0.0259	1	0.013	15	0.0664
			0.5	2	4999.19	9998.38			0.1					1		15	
RC 2	1-1	2	0.5	1	364.89	729.78	692.64	1385.28	0.1	150185	0.92	1155	0.0015	1	0.013	15	0.0039
			0.5	2	327.75	655.5			0.1					1		15	
	1-2	2	0.5	1	52.71	105.42	110.04	220.08	0.1	150185	0.15	-10	0.0000	1	0.013	15	0.0000
			0.5	2	57.33	114.66			0.1					1		15	
	1-3	2	0.5	1	218.28	436.56	444.21	888.42	0.1	150185	0.59	658	0.0009	1	0.013	15	0.0022
			0.5	2	225.93	451.86			0.1					1		15	
	2-1	2	0.5	1	285.03	570.06	564.42	1128.84	0.1	150185	0.75	899	0.0012	1	0.013	15	0.0030
			0.5	2	279.39	558.78			0.1					1		15	
	2-2	2	0.5	1	272.23	544.46	552.81	1105.62	0.1	150185	0.74	876	0.0012	1	0.013	15	0.0030
			0.5	2	280.58	561.16			0.1					1		15	
	2-3	2	0.5	1	267.94	535.88	538.23	1076.46	0.1	150185	0.72	846	0.0011	1	0.013	15	0.0029
			0.5	2	270.29	540.58			0.1					1		15	
	3-1	2	0.5	1	1645.53	3291.06	3261.39	6522.78	0.1	150185	4.34	6293	0.0083	1	0.013	15	0.0213
			0.5	2	1615.86	3231.72			0.1					1		15	
	3-2	2	0.5	1	1558.60	3117.2	3153.12	6306.24	0.1	150185	4.20	6076	0.0080	1	0.013	15	0.0206
			0.5	2	1594.52	3189.04			0.1					1		15	
	3-3	2	0.5	1	1644.25	3288.5	3293.34	6586.68	0.1	150185	4.39	6357	0.0084	1	0.013	15	0.0216
			0.5	2	1649.09	3298.18			0.1					1		15	
	4-1	2	0.5	1	2112.42	4224.84	4256.47	8512.94	0.1	150185	5.67	8283	0.0110	1	0.013	15	0.0281
			0.5	2	2144.05	4288.1			0.1					1		15	
	4-2	2	0.5	1	1907.51	3815.02	3782.67	7565.34	0.1	150185	5.04	7335	0.0097	1	0.013	15	0.0249
			0.5	2	1875.16	3750.32			0.1					1		15	
	4-3	2	0.5	1	1943.89	3887.78	3908.71	7817.42	0.1	150185	5.21	7587	0.0100	1	0.013	15	0.0257
			0.5	2	1964.82	3929.64			0.1					1		15	
	5-1	2	0.5	1	3442.13	6884.26	6870.04	13740.08	0.1	150185	9.15	13510	0.0179	1	0.013	15	0.0458
			0.5	2	3427.91	6855.82			0.1					1		15	
	5-2	2	0.5	1	3244.57	6489.14	6519.91	13039.82	0.1	150185	8.68	12810	0.0170	1	0.013	15	0.0434
			0.5	2	3275.24	6550.48			0.1					1		15	
	5-3	2	0.5	1	3200.08	6478.16	6541.68	13083.36	0.1	150185	8.71	12853	0.0170	1	0.013	15	0.0436
			0.5	2	3302.60	6605.2			0.1					1		15	
	6-1	2	0.5	1	4077.88	8155.76	8203.34	16406.68	0.1	150185	10.92	16177	0.0214	1	0.013	15	0.0548
			0.5	2	4125.46	8250.92			0.1					1		15	
	6-2	2	0.5	1	4142.64	8285.28	8380.23	16760.46	0.1	150185	11.16	16530	0.0219	1	0.013	15	0.0560
			0.5	2	4237.59	8475.18			0.1					1		15	
	6-3	2	0.5	1	4118.57	8237.14	8238.18	16476.36	0.1	150185	10.97	16246	0.0215	1	0.013	15	0.0551
			0.5	2	4119.61	8239.22			0.1					1		15	
	7-1	2	0.5	1	4761.51	9523.02	9584.29	19168.58	0.1	150185	12.76	18938	0.0251	1	0.013	15	0.0642
			0.5	2	4822.78	9645.56			0.1					1		15	
	7-2	2	0.5	1	4880.26	9760.4	9865.21	19730.42	0.1	150185	13.14	19500	0.0258	1	0.013	15	0.0661
			0.5	2	4985.01	9970.02			0.1					1		15	
	7-3	2	0.5	1	5105.96	10211.92	10321.21	20642.42	0.1	150185	13.74	20412	0.0270	1	0.013	15	0.0692
			0.5	2	5215.25	10430.5			0.1					1		15	
	8-1	2	0.5	1	4945.51	9891.02	9990.81	19981.62	0.1	150185	13.30	19752	0.0261	1	0.013	15	0.0670
			0.5	2	5045.30	10090.6			0.1					1		15	
	8-2	2	0.5	1	5002.94	10005.88	9968.06	19936.12	0.1	150185	13.27	19706	0.0261	1	0.013	15	0.0666
			0.5	2	4965.12	9930.24			0.1					1		15	
	8-3	2	0.5	1	4822.07	9644.14	9728.68	19457.36	0.1	150185	12.96	19227	0.0254	1	0.013	15	0.0652
			0.5	2	4906.61	9813.22			0.1					1		15	

Assay Date	2/14/2005	ID	RC 2	# Concentrations tested	Microsome 8 type	Placental	Microsome ID	Technician ID	JG	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0754	0.0015
Full activity	End	0.0688	0.0012
Full activity	Overall	0.0721	0.0039
Background	Beginning	0.0003	0.000834204
Background	End	-0.0003	9.10059E-05
Background	Overall	0.0000	0.000566841
Positive	Beginning	0.0432	0.0089
Positive	End	0.0313	0.0005
Positive	Overall	0.0372	0.0086
Negative	Beginning	0.0733	0.0018
Negative	End	0.0655	0.0012
Negative	Overall	0.0694	0.0046

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 2	1	1	1.00E-03	-3.00	0.0039
RC 2	1	2	1.00E-03	-3.00	0.0000
RC 2	1	3	1.00E-03	-3.00	0.0022
RC 2	2	1	1.00E-04	-4.00	0.0030
RC 2	2	2	1.00E-04	-4.00	0.0030
RC 2	2	3	1.00E-04	-4.00	0.0029
RC 2	3	1	1.00E-05	-5.00	0.0213
RC 2	3	2	1.00E-05	-5.00	0.0206
RC 2	3	3	1.00E-05	-5.00	0.0216
RC 2	4	1	7.50E-06	-5.12	0.0281
RC 2	4	2	7.50E-06	-5.12	0.0249
RC 2	4	3	7.50E-06	-5.12	0.0257
RC 2	5	1	2.50E-06	-5.60	0.0458
RC 2	5	2	2.50E-06	-5.60	0.0434
RC 2	5	3	2.50E-06	-5.60	0.0436
RC 2	6	1	1.00E-06	-6.00	0.0548
RC 2	6	2	1.00E-06	-6.00	0.0560
RC 2	6	3	1.00E-06	-6.00	0.0551
RC 2	7	1	1.00E-07	-7.00	0.0642
RC 2	7	2	1.00E-07	-7.00	0.0661
RC 2	7	3	1.00E-07	-7.00	0.0692
RC 2	8	1	1.00E-09	-9.00	0.0670
RC 2	8	2	1.00E-09	-9.00	0.0668
RC 2	8	3	1.00E-09	-9.00	0.0652

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-3.00	5.43	-0.05	3.10
2	-4.00	4.23	4.12	3.98
3	-5.00	29.58	28.57	29.88
4	-5.12	38.94	34.48	35.67
5	-5.60	63.51	60.22	60.43
6	-6.00	76.05	77.71	76.38
7	-7.00	89.03	91.67	95.96
8	-9.00	92.86	92.64	90.39

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0199	29839.58	1499476	
2	0.0199	31448.85	1580344	
3	0.0200	30904.31	1545216	
4	0.0200	31497.06	1574853	
5	0.0201	31746.57	1579431	
			Average DPM/g soln	1555864
			SD	34671
			CV	2.23
			µCi/g soln	0.701

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.1	10		1010.00
Dilution A			100	10.10
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.161 g
Mass of dilution B used in substrate prep	4.5921 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.568315 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00793 µg/g soln.
µg/g soln.

a. µCi/g soln 0.701
b. Specific activity of [H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.568315 + 0.00793
= 0.576249 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.216 µCi/µg ASDN

773276 dpm/nmol

Test

Assay Date 2/15/2005 Chemical ID RC 2 tested 8

ID JG Replicate # 3 Microsome type Placental Microsome ID MET-0255A 18&19

Standards: BSA) Total volume of stock (mL) Protein stock ID

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank r^2= Regression results are calculated using the function LINEST
m=
b=

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date	2/15/2005	Test Chemical ID	RC 2	tested	8			
ID	JG	Replicate #	3	Microsome type	Placental	Microsome ID	MET-0255A 18&19	
							Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-7
	0.383	0.280	0.229	0.161	0.060	0.057	0.035		
	0.402	0.298	0.236	0.175	0.079	0.057	0.029		
	0.394	0.290	0.236	0.167	0.084	0.052	0.029		

Samples: microsomes
0.121
0.117
0.119

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.393	0.362	0.0358	m, b	0.098	0.000
0.00100	25	0.0250	0.289	0.258	0.0257	se_m, se_b	0.005	0.001
0.00075	25	0.0188	0.234	0.203	0.0203	r , se_y	0.988	0.002
0.00050	25	0.0125	0.168	0.137	0.0138	F, df	327	4
0.00025	25	0.0063	0.075	0.044	0.0047	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.055	0.024	0.0028			

Regression results are calculated using the function LINEST

Blank 0.031

r^2= 0.988
m= 0.098
b= 0.000

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.121	0.090	0.009	25	190	5190	0.010	0.010	9.934
0.117	0.086	0.009	25	190	5190	0.010		
0.119	0.088	0.009	25	190	5190	0.010		

Assay Date	2/15/2005	Chemical ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	3

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	3	mL microsome Dilution A used
	43	mL total volume
	14.33333	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	391.5263	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	9.934
Protein Concentration (dilution added to assay, mg/mL):	0.025372

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	7.50E-06
5	2.50E-06
6	1.00E-06
7	1.00E-07
8	1.00E-09

Assay Date	2/15/2005	Test Chemical ID	RC 2	# Concentrations tested	8	Microsome type	Placental	Microsome ID	MET-0255A 18A19	Technician ID	JC	Replicate #	3

Sample ID					Calculate DPM in aqueous portion after extraction				Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (nmol)	% conversion to product	Total DPM corrected for background (Background Tube=)	nmol ^3H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	incubation time (min)	ug/pgen formed/min protein/min
Full activity curves	1	2	0.5	1	2634.22	5268.44	5387.48	10774.96	0.1	155586	6.93	10054	0.0138	1	0.013	15	0.0062
			0.5	2	2753.26	5506.52			0.1					1		15	
	2	2	0.5	1	2930.72	5861.44	5849.56	11699.12	0.1	155586	7.52	11578	0.0150	1	0.013	15	0.0361
			0.5	2	2918.84	5837.68			0.1					1		15	
	3	2	0.5	1	2745.85	5491.7	5488.27	10976.54	0.1	155586	7.06	10857	0.0140	1	0.013	15	0.0338
			0.5	2	2743.42	5486.84			0.1					1		15	
	4	2	0.5	1	2757.91	5515.82	5516.25	11032.5	0.1	155586	7.09	10911	0.0141	1	0.013	15	0.037
			0.5	2	2758.34	5516.68			0.1					1		15	
Background control	1	2	0.5	1	35.82	71.64	58.25	116.5	0.1	155586	0.06	3	0.0000	1	0.013	15	0.0000
			0.5	2	23.43	46.86			0.1					1		15	
	2	2	0.5	1	30.81	61.62	58.88	117.76	0.1	155586	0.06	-3	0.0000	1	0.013	15	0.0000
			0.5	2	28.07	56.14			0.1					1		15	
	3	2	0.5	1	34.57	69.14	63.11	126.22	0.1	155586	0.08	5	0.0000	1	0.013	15	0.0000
			0.5	2	28.54	57.08			0.1					1		15	
	4	2	0.5	1	32.28	64.56	61.03	122.06	0.1	155586	0.06	1	0.0000	1	0.013	15	0.0000
			0.5	2	29.75	57.5			0.1					1		15	
Positive control	1	2	0.5	1	1349.42	2698.84	2680.93	5361.86	0.1	155586	3.45	5241	0.0068	1	0.013	15	0.0178
			0.5	2	1331.51	2663.02			0.1					1		15	
	2	2	0.5	1	1324.08	2648.16	2680.01	5360.02	0.1	155586	3.45	5239	0.0068	1	0.013	15	0.0176
			0.5	2	1355.93	2711.86			0.1					1		15	
	3	2	0.5	1	1237.45	2474.9	2466.39	4932.78	0.1	155586	3.17	4812	0.0062	1	0.013	15	0.0163
			0.5	2	1228.94	2457.88			0.1					1		15	
	4	2	0.5	1	1234.48	2468.96	2470.74	4941.48	0.1	155586	3.18	4820	0.0062	1	0.013	15	0.0164
			0.5	2	1246.26	2492.52			0.1					1		15	
Negative Control	1	2	0.5	1	2903.74	5807.48	5777.21	11554.42	0.1	155586	7.23	11433	0.0146	1	0.013	15	0.0386
			0.5	2	2875.47	5748.94			0.1					1		15	
	2	2	0.5	1	2883.80	5767.6	5747.19	11494.32	0.1	155586	7.39	11373	0.0147	1	0.013	15	0.0385
			0.5	2	2863.36	5726.72			0.1					1		15	
	3	2	0.5	1	2646.05	5292.1	5261.45	10522.9	0.1	155586	6.75	10402	0.0135	1	0.013	15	0.0353
			0.5	2	2612.40	5224.8			0.1					1		15	
	4	2	0.5	1	2634.54	5269.08	5213.37	10426.74	0.1	155586	6.70	10306	0.0133	1	0.013	15	0.0350
			0.5	2	2578.83	5157.66			0.1					1		15	
RC 2	1-1	2	0.5	1	49.91	99.82	100.36	200.72	0.1	155586	0.13	80	0.0001	1	0.013	15	0.0003
			0.5	2	50.45	100.91			0.1					1		15	
	1-2	2	0.5	1	41.70	83.4	79.62	159.24	0.1	155586	0.10	38	0.0000	1	0.013	15	0.0001
			0.5	2	37.92	75.84			0.1					1		15	
	1-3	2	0.5	1	34.50	69	71.58	143.16	0.1	155586	0.09	22	0.0000	1	0.013	15	0.0001
			0.5	2	37.08	74.16			0.1					1		15	
	2-1	2	0.5	1	172.14	344.28	346.1	692.2	0.1	155586	0.44	571	0.0007	1	0.013	15	0.0019
			0.5	2	173.96	347.92			0.1					1		15	
	2-2	2	0.5	1	171.84	343.68	343.45	686.9	0.1	155586	0.44	586	0.0007	1	0.013	15	0.0019
			0.5	2	171.61	343.22			0.1					1		15	
	2-3	2	0.5	1	174.98	349.96	329.27	658.54	0.1	155586	0.42	537	0.0007	1	0.013	15	0.0018
			0.5	2	154.29	308.58			0.1					1		15	
	3-1	2	0.5	1	948.74	1893.48	1872.65	3745.3	0.1	155586	2.41	3624	0.0047	1	0.013	15	0.0123
			0.5	2	925.91	1851.82			0.1					1		15	
	3-2	2	0.5	1	937.91	1875.82	1871.28	3742.56	0.1	155586	2.41	3621	0.0047	1	0.013	15	0.0123
			0.5	2	933.37	1866.74			0.1					1		15	
	3-3	2	0.5	1	939.51	1879.02	1898.93	3797.88	0.1	155586	2.44	3677	0.0048	1	0.013	15	0.0125
			0.5	2	959.42	1918.84			0.1					1		15	
	4-1	2	0.5	1	1118.92	2237.84	2227.78	4455.56	0.1	155586	2.86	4334	0.0056	1	0.013	15	0.0147
			0.5	2	1108.86	2217.72			0.1					1		15	
	4-2	2	0.5	1	1134.43	2268.86	2255.24	4510.48	0.1	155586	2.90	4389	0.0057	1	0.013	15	0.0149
			0.5	2	1120.81	2241.62			0.1					1		15	
	4-3	2	0.5	1	1112.24	2224.48	2242.84	4485.68	0.1	155586	2.88	4366	0.0056	1	0.013	15	0.0146
			0.5	2	1130.60	2261.2			0.1					1		15	
	5-1	2	0.5	1	1824.64	3649.28	3635.68	7271.36	0.1	155586	4.67	7150	0.0092	1	0.013	15	0.0243
			0.5	2	1811.04	3622.08			0.1					1		15	
	5-2	2	0.5	1	1883.40	3766.8	3756.63	7517.26	0.1	155586	4.83	7396	0.0095	1	0.013	15	0.0251
			0.5	2	1875.23	3750.46			0.1					1		15	
	5-3	2	0.5	1	1824.09	3648.18	3679.63	7358.99	0.1	155586	4.73	7239	0.0094	1	0.013	15	0.0246
			0.5	2	1855.74	3711.48			0.1					1		15	
	6-1	2	0.5	1	2336.21	4672.42	4666.34	9332.68	0.1	155586	6.00	9212	0.0119	1	0.013	15	0.0313
			0.5	2	2330.13	4660.26			0.1					1		15	
	6-2	2	0.5	1	2306.75	4613.5	4627.79	9255.58	0.1	155586	5.95	9134	0.0118	1	0.013	15	0.0310
			0.5	2	2321.04	4642.08			0.1					1		15	
	6-3	2	0.5	1	2231.91	4463.82	4478.41	8956.82	0.1	155586	5.76	8836	0.0114	1	0.013	15	0.0300
			0.5	2	2246.50	4493			0.1					1		15	
	7-1	2	0.5	1	2749.31	5498.62	5429.91	10859.82	0.1	155586	6.98	10739	0.0139	1	0.013	15	0.0365
			0.5	2	2681.60	5363.2			0.1					1		15	
	7-2	2	0.5	1	2679.47	5358.94	5363.92	10727.84	0.1	155586	6.90	10607	0.0137	1	0.013	15	0.0360
			0.5	2	2684.45	5368.9			0.1					1		15	
	7-3	2	0.5	1	2665.85	5331.7	5363.47	10726.94	0.1	155586	6.89	10606	0.0137	1	0.013	15	0.0360
			0.5	2	2697.62	5395.24			0.1					1		15	
	8-1	2	0.5	1	2732.22	5464.44	5528.4	11056.8	0.1	155586	7.11	10936	0.0141	1	0.013	15	0.0372
			0.5	2	2796.18	5592.36			0.1					1		15	
	8-2	2	0.5	1	2751.15	5502.3	5419.8	10839.6	0.1	155586	6.97	10718	0.0139	1	0.013	15	0.0364
			0.5	2	2668.65	5337.3			0.1					1		15	
	8-3	2	0.5	1	2669.79	5339.58	5390.8	10781.6	0.1	155586	6.93	10690	0.0138	1	0.013	15	0.0362
			0.5	2	2721.01	5442.02			0.1					1		15	

Assay Date	2/15/2005	ID	RC 2	# Concentrations tested	Microsome type	Placental	Microsome ID	Technician ID	JG	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0378	0.0022
Full activity	End	0.0370	0.0001
Full activity	Overall	0.0374	0.0014
Background	Beginning	0.0000	1.77798E-06
Background	End	0.0000	9.99515E-06
Background	Overall	0.0000	1.31668E-05
Positive	Beginning	0.0178	0.0000
Positive	End	0.0164	0.0000
Positive	Overall	0.0171	0.0008
Negative	Beginning	0.0387	0.0001
Negative	End	0.0352	0.0002
Negative	Overall	0.0370	0.0021

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 2	1	1	1.00E-03	-3.00	0.0003
RC 2	1	2	1.00E-03	-3.00	0.0001
RC 2	1	3	1.00E-03	-3.00	0.0001
RC 2	2	1	1.00E-04	-4.00	0.0019
RC 2	2	2	1.00E-04	-4.00	0.0019
RC 2	2	3	1.00E-04	-4.00	0.0018
RC 2	3	1	1.00E-05	-5.00	0.0123
RC 2	3	2	1.00E-05	-5.00	0.0123
RC 2	3	3	1.00E-05	-5.00	0.0125
RC 2	4	1	7.50E-06	-5.12	0.0147
RC 2	4	2	7.50E-06	-5.12	0.0149
RC 2	4	3	7.50E-06	-5.12	0.0148
RC 2	5	1	2.50E-06	-5.60	0.0243
RC 2	5	2	2.50E-06	-5.60	0.0251
RC 2	5	3	2.50E-06	-5.60	0.0246
RC 2	6	1	1.00E-06	-6.00	0.0313
RC 2	6	2	1.00E-06	-6.00	0.0310
RC 2	6	3	1.00E-06	-6.00	0.0300
RC 2	7	1	1.00E-07	-7.00	0.0365
RC 2	7	2	1.00E-07	-7.00	0.0360
RC 2	7	3	1.00E-07	-7.00	0.0360
RC 2	8	1	1.00E-09	-9.00	0.0372
RC 2	8	2	1.00E-09	-9.00	0.0364
RC 2	8	3	1.00E-09	-9.00	0.0362

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-3.00	0.72	0.35	0.20
2	-4.00	5.19	5.14	4.89
3	-5.00	32.95	32.92	33.42
4	-5.12	39.40	39.90	39.68
5	-5.60	65.00	67.24	65.80
6	-6.00	83.74	83.04	80.32
7	-7.00	97.62	96.42	96.42
8	-9.00	99.41	97.44	96.91

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	31342.02	1590965	
2	0.0199	32131.41	1614644	
3	0.0198	33454.07	1689599	
4	0.0197	33646.03	1707920	
5	0.0198	33493.96	1691614	
			Average DPM/g soln	1658949
			SD	52415
			CV	3.16
			µCi/g soln	0.747

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1009 g
Mass of dilution B used in substrate prep	4.5747 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.564715 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00846 µg/g soln.
µg/g soln.

a. µCi/g soln 0.747
b. Specific activity of [H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.564715 + 0.00846
= 0.573174 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.304 µCi/µg ASDN

828933 dpm/nmol

Assay Date 2/17/2005 Test Chemical ID RC 3 tested 8

ID JRH Replicate # 1 Microsome type Placental Microsome ID MET-0255A #20 & 21

Standards: BSA) Total volume of stock (mL) Protein stock ID

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank r^2= m= b=

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date 2/17/2005 Test Chemical ID RC 3 tested 8

ID JRH Replicate # 1 Microsome type Placental Microsome ID MET-0255A #20 & 21

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-8
	0.401	0.296	0.239	0.172	0.103	0.059	0.037		
	0.395	0.290	0.234	0.177	0.106	0.075	0.035		
	0.427	0.292	0.232	0.169	0.105	0.063	0.041		

Samples:	microsomes
	0.143
	0.162
	0.165

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.407	0.370	0.0368	m, b	0.101	0.000
0.00100	25	0.0250	0.293	0.255	0.0252	se_m, se_b	0.003	0.001
0.00075	25	0.0188	0.235	0.197	0.0194	r , se_y	0.997	0.001
0.00050	25	0.0125	0.173	0.135	0.0131	F, df	1473	4
0.00025	25	0.0063	0.105	0.067	0.0063	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.066	0.028	0.0024			

Regression results are calculated using the function LINEST

Blank 0.038

r^2= 0.997
m= 0.101
b= 0.000

A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.143	0.105	0.010	25	190	5190	0.011	0.013	12.585
0.162	0.124	0.012	25	190	5190	0.013		
0.165	0.127	0.012	25	190	5190	0.014		

Assay Date	2/17/2005	Chemical ID	RC 3	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JRH	Replicate #	1

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	2.5	mL microsome Dilution A used
	44	mL total volume
	17.6	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	480.7579	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.585
Protein Concentration (dilution added to assay, mg/mL):	0.026177

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-04
2	1.00E-05
3	1.00E-06
4	1.00E-07
5	5.00E-08
6	1.00E-08
7	1.00E-09
8	1.00E-10

Assay Date	2/17/2005	Test Chemical ID	RC 3	# Concentrations tested	8	Microsome type	Placental	Microsome ID	MET-0255A #20 & 21	Technician ID	JRH	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	nmol/pmol formed/mg protein/min
Full activity control	1	2	0.5	1	1158.89	2317.38	2307.61	4615.62	0.1	185695	2.78	4476	0.0054	1	0.013	15	0.0138
			0.5	2	1149.22	2298.44			0.1					1		15	
	2	2	0.5	1	1202.22	2404.44	2439.91	4879.82	0.1	185695	2.94	4740	0.0057	1	0.013	15	0.0146
			0.5	2	1237.69	2475.38			0.1					1		15	
	3	2	0.5	1	1091.56	2183.12	2219.39	4438.78	0.1	185695	2.68	4299	0.0052	1	0.013	15	0.0132
			0.5	2	1127.63	2255.66			0.1					1		15	
	4	2	0.5	1	1108.12	2216.24	2230.58	4461.16	0.1	185695	2.69	4322	0.0052	1	0.013	15	0.0133
			0.5	2	1122.46	2244.92			0.1					1		15	
Background control	1	2	0.5	1	44.58	89.76	84.4	168.8	0.1	185695	0.10	29	0.0000	1	0.013	15	0.0001
			0.5	2	39.92	79.04			0.1					1		15	
	2	2	0.5	1	40.94	81.88	83.98	167.96	0.1	185695	0.10	28	0.0000	1	0.013	15	0.0001
			0.5	2	43.04	86.08			0.1					1		15	
	3	2	0.5	1	28.73	57.46	56.57	113.14	0.1	185695	0.07	-20	0.0000	1	0.013	15	-0.0001
			0.5	2	30.84	61.68			0.1					1		15	
	4	2	0.5	1	25.43	50.86	51.3	102.6	0.1	185695	0.06	-37	0.0000	1	0.013	15	-0.0001
			0.5	2	25.87	51.74			0.1					1		15	
Positive control	1	2	0.5	1	654.05	1308.1	1289.31	2578.62	0.1	185695	1.55	2439	0.0029	1	0.013	15	0.0075
			0.5	2	635.26	1270.52			0.1					1		15	
	2	2	0.5	1	583.65	1167.3	1175.63	2351.26	0.1	185695	1.42	2212	0.0027	1	0.013	15	0.0068
			0.5	2	591.98	1183.96			0.1					1		15	
	3	2	0.5	1	542.71	1085.42	1110.89	2221.78	0.1	185695	1.34	2082	0.0025	1	0.013	15	0.0064
			0.5	2	568.18	1136.36			0.1					1		15	
	4	2	0.5	1	526.60	1053.2	1122.52	2245.04	0.1	185695	1.35	2105	0.0025	1	0.013	15	0.0065
			0.5	2	595.92	1191.84			0.1					1		15	
Negative Control	1	2	0.5	1	1107.84	2215.68	2287.07	4574.14	0.1	185695	2.76	4435	0.0053	1	0.013	15	0.0136
			0.5	2	1179.23	2358.46			0.1					1		15	
	2	2	0.5	1	1111.34	2222.68	2306.4	4612.8	0.1	185695	2.78	4473	0.0054	1	0.013	15	0.0137
			0.5	2	1195.08	2390.12			0.1					1		15	
	3	2	0.5	1	1085.83	2171.66	2122.19	4244.38	0.1	185695	2.56	4105	0.0050	1	0.013	15	0.0126
			0.5	2	1036.36	2072.72			0.1					1		15	
	4	2	0.5	1	1010.72	2021.44	2040.95	4081.9	0.1	185695	2.46	3942	0.0048	1	0.013	15	0.0121
			0.5	2	1030.23	2060.46			0.1					1		15	
RC 3	1-1	2	0.5	1	999.85	1999.7	1931.13	3862.26	0.1	185695	2.33	3723	0.0045	1	0.013	15	0.0114
			0.5	2	931.28	1862.56			0.1					1		15	
	1-2	2	0.5	1	122.92	245.84	256.85	513.7	0.1	185695	0.31	374	0.0005	1	0.013	15	0.0011
			0.5	2	133.93	267.86			0.1					1		15	
	1-3	2	0.5	1	113.74	227.48	231.04	462.08	0.1	185695	0.28	322	0.0004	1	0.013	15	0.0010
			0.5	2	117.30	234.6			0.1					1		15	
	2-1	2	0.5	1	570.75	1141.5	1128.9	2257.8	0.1	185695	1.36	2118	0.0026	1	0.013	15	0.0065
			0.5	2	558.15	1116.3			0.1					1		15	
	2-2	2	0.5	1	554.55	1109.1	1108.42	2216.84	0.1	185695	1.34	2077	0.0025	1	0.013	15	0.0064
			0.5	2	553.87	1107.74			0.1					1		15	
	2-3	2	0.5	1	566.19	1132.38	1154	2308	0.1	185695	1.39	2165	0.0026	1	0.013	15	0.0067
			0.5	2	587.81	1175.62			0.1					1		15	
	3-1	2	0.5	1	995.26	1990.52	2074.41	4148.82	0.1	185695	2.50	4009	0.0048	1	0.013	15	0.0125
			0.5	2	1079.13	2158.26			0.1					1		15	
	3-2	2	0.5	1	1021.17	2042.34	2052.65	4105.3	0.1	185695	2.47	3966	0.0048	1	0.013	15	0.0122
			0.5	2	1031.48	2062.96			0.1					1		15	
	3-3	2	0.5	1	1011.07	2022.14	2009.77	4019.54	0.1	185695	2.42	3880	0.0047	1	0.013	15	0.0119
			0.5	2	998.70	1997.4			0.1					1		15	
	4-1	2	0.5	1	1123.53	2247.06	2247.38	4494.76	0.1	185695	2.71	4355	0.0053	1	0.013	15	0.0134
			0.5	2	1123.86	2247.72			0.1					1		15	
	4-2	2	0.5	1	1113.68	2227.36	2246.68	4493.76	0.1	185695	2.71	4354	0.0053	1	0.013	15	0.0134
			0.5	2	1133.20	2266.4			0.1					1		15	
	4-3	2	0.5	1	1118.13	2236.26	2243.59	4487.18	0.1	185695	2.70	4348	0.0052	1	0.013	15	0.0134
			0.5	2	1125.46	2250.92			0.1					1		15	
	5-1	2	0.5	1	1123.27	2246.54	2245.32	4490.64	0.1	185695	2.71	4351	0.0052	1	0.013	15	0.0134
			0.5	2	1122.05	2244.1			0.1					1		15	
	5-2	2	0.5	1	1079.03	2159.26	2120.58	4241.16	0.1	185695	2.56	4102	0.0049	1	0.013	15	0.0126
			0.5	2	1040.99	2081.92			0.1					1		15	
	5-3	2	0.5	1	1171.97	2343.94	2306.71	4613.42	0.1	185695	2.78	4474	0.0054	1	0.013	15	0.0137
			0.5	2	1134.74	2269.48			0.1					1		15	
	6-1	2	0.5	1	1193.91	2387.82	2354.96	4709.92	0.1	185695	2.84	4570	0.0055	1	0.013	15	0.0140
			0.5	2	1161.05	2322.1			0.1					1		15	
	6-2	2	0.5	1	1210.85	2421.7	2406.44	4812.88	0.1	185695	2.90	4673	0.0056	1	0.013	15	0.0144
			0.5	2	1195.59	2391.18			0.1					1		15	
	6-3	2	0.5	1	1133.73	2267.46	2299.35	4598.7	0.1	185695	2.74	4399	0.0053	1	0.013	15	0.0135
			0.5	2	1135.62	2271.24			0.1					1		15	
	7-1	2	0.5	1	1118.01	2236.02	2234.53	4469.06	0.1	185695	2.69	4329	0.0052	1	0.013	15	0.0133
			0.5	2	1115.52	2231.04			0.1					1		15	
	7-2	2	0.5	1	1070.18	2140.36	2172.86	4345.72	0.1	185695	2.62	4206	0.0051	1	0.013	15	0.0129
			0.5	2	1102.68	2205.36			0.1					1		15	
	7-3	2	0.5	1	1125.48	2250.96	2268.4	4536.8	0.1	185695	2.73	4397	0.0053	1	0.013	15	0.0135
			0.5	2	1142.92	2285.84			0.1					1		15	
	8-1	2	0.5	1	1071.60	2143.2	2128.18	4256.36	0.1	185695	2.57	4119	0.0050	1	0.013	15	0.0127
			0.5	2	1067.58	2135.16			0.1					1		15	
	8-2	2	0.5	1	1074.39	2148.78	2224.52	4449.04	0.1	185695	2.68	4309	0.0052	1	0.013	15	0.0132
			0.5	2	1150.13	2300.26			0.1					1		15	
	8-3	2	0.5	1	1086.07	2172.14	2218.42	4436.84	0.1	185695	2.67	4297	0.0052	1	0.013	15	0.0132
			0.5	2	1130.35	2260.7			0.1					1		15	

Assay Date	2/17/2005	ID	RC 3	# Concentrations tested	Microsome type	Placental	Microsome ID	Technician ID	JRH	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0142	0.0006
Full activity	End	0.0132	0.0000
Full activity	Overall	0.0137	0.0006
Background	Beginning	0.0001	1.82485E-06
Background	End	-0.0001	3.59322E-05
Background	Overall	0.0000	0.000104104
Positive	Beginning	0.0071	0.0005
Positive	End	0.0064	0.0001
Positive	Overall	0.0068	0.0005
Negative	Beginning	0.0137	0.0001
Negative	End	0.0124	0.0004
Negative	Overall	0.0130	0.0008

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 3	1	1	1.00E-04	-4.00	0.0114
RC 3	1	2	1.00E-04	-4.00	0.0011
RC 3	1	3	1.00E-04	-4.00	0.0010
RC 3	2	1	1.00E-05	-5.00	0.0065
RC 3	2	2	1.00E-05	-5.00	0.0064
RC 3	2	3	1.00E-05	-5.00	0.0067
RC 3	3	1	1.00E-06	-6.00	0.0123
RC 3	3	2	1.00E-06	-6.00	0.0122
RC 3	3	3	1.00E-06	-6.00	0.0119
RC 3	4	1	1.00E-07	-7.00	0.0134
RC 3	4	2	1.00E-07	-7.00	0.0134
RC 3	4	3	1.00E-07	-7.00	0.0134
RC 3	5	1	5.00E-08	-7.30	0.0134
RC 3	5	2	5.00E-08	-7.30	0.0126
RC 3	5	3	5.00E-08	-7.30	0.0137
RC 3	6	1	1.00E-08	-8.00	0.0140
RC 3	6	2	1.00E-08	-8.00	0.0144
RC 3	6	3	1.00E-08	-8.00	0.0135
RC 3	7	1	1.00E-09	-9.00	0.0133
RC 3	7	2	1.00E-09	-9.00	0.0129
RC 3	7	3	1.00E-09	-9.00	0.0135
RC 3	8	1	1.00E-10	-10.00	0.0127
RC 3	8	2	1.00E-10	-10.00	0.0132
RC 3	8	3	1.00E-10	-10.00	0.0132

	Percent of control values			
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-4.00	83.48	8.39	7.23
2	-5.00	47.50	46.58	48.63
3	-6.00	89.91	88.93	87.01
4	-7.00	97.67	97.64	97.49
5	-7.30	97.57	91.98	100.33
6	-8.00	102.49	104.80	98.65
7	-9.00	97.09	94.32	98.61
8	-10.00	92.36	96.64	96.37

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0201	27155.54	1351022	
2	0.0201	27424.04	1364380	
3	0.0200	28490.39	1424520	
4	0.0201	28899.26	1437774	
5	0.0202	28924.55	1431908	
			Average DPM/g soln	1401921
			SD	40913
			CV	2.92
			µCi/g soln	0.631

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1147 g
Mass of dilution B used in substrate prep	4.5915 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.565825 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00715 µg/g soln.
µg/g soln.

a. µCi/g soln 0.631
b. Specific activity of [H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.565825 + 0.00715
= 0.572974 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.102 µCi/µg ASDN

700748 dpm/nmol

Assay Date 2/18/2005 Test Chemical ID RC 3 tested 8

ID JRH Replicate # 2 Microsome type Placental Microsome ID MET-0255A #22 & 23

Standards:

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)		mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date	2/18/2005	Test Chemical ID	RC 3		tested		8		
ID	JRH	Replicate #		2	Microsome type	Placental	Microsome ID	MET-0255A #22 & 23	
								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-9
	0.431	0.315	0.239	0.183	0.089	0.067	0.038		
	0.438	0.297	0.251	0.170	0.092	0.069	0.042		
		0.309	0.257	0.177	0.093	0.071	0.043		
Samples:	microsomes								
	0.160								
	0.157								
	0.168								

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.434	0.393	0.0368	m, b	0.092	0.001
0.00100	25	0.0250	0.307	0.266	0.0251	se_m, se_b	0.003	0.001
0.00075	25	0.0188	0.249	0.208	0.0198	r^2, se_y	0.996	0.001
0.00050	25	0.0125	0.177	0.136	0.0131	F, df	1118	4
0.00025	25	0.0063	0.091	0.050	0.0053	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.069	0.028	0.0032			
Blank	0.041	r^2= 0.996				Regression results are calculated using the function LINEST		
		m= 0.092						
		b= 0.001						

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.160	0.119	0.012	25	190	5190	0.013	0.013	12.834
0.157	0.116	0.011	25	190	5190	0.012		
0.168	0.127	0.012	25	190	5190	0.014		

Assay Date	2/18/2005	Chemical ID	RC 3	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JRH	Replicate #	2

Microsome Dilution Details		
Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	2.4	mL microsome Dilution A used
	44	mL total volume
	18.33333	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	500.7895	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.834
Protein Concentration (dilution added to assay, mg/mL):	0.025628

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-04
2	4.00E-05
3	1.60E-05
4	6.40E-06
5	2.50E-06
6	1.00E-06
7	5.00E-08
8	1.00E-09

Assay Date	2/18/2005	Test Chemical ID	RC 3	# Concentrations tested		B Microsome type	Placental	Microsome ID	MET-0255A #22 & 23	Technician ID	JRH	Replicate #	2

Sample ID			Calculate DPM in aqueous portion after extraction						Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal conc (mM)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (n/aliq)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	nanogram formed/mg protein/min
Full activity control	1	1	0.5	1	749.93	1499.86	1500.93	3001.86	0.1	140192	2.14	2689	0.0041	1	0.013	15	0.0107
			0.5	2	751.00	1502			0.1					1		15	
	2	2	0.5	1	709.26	1418.52	1429.67	2859.34	0.1	140192	2.04	2749	0.0039	1	0.013	15	0.0102
			0.5	2	720.41	1440.82			0.1					1		15	
	3	2	0.5	1	743.42	1486.84	1484.07	2969.34	0.1	140192	2.12	2856	0.0041	1	0.013	15	0.0106
			0.5	2	741.25	1482.5			0.1					1		15	
	4	2	0.5	1	680.00	1360	1353.96	2707.92	0.1	140192	1.93	2595	0.0037	1	0.013	15	0.0096
			0.5	2	673.96	1347.92			0.1					1		15	
Background control	1	2	0.5	1	29.53	59.06	58.52	117.04	0.1	140192	0.08	4	0.0000	1	0.013	15	0.0000
			0.5	2	28.99	57.98			0.1					1		15	
	2	2	0.5	1	19.82	39.64	55.07	110.14	0.1	140192	0.08	-3	0.0000	1	0.013	15	0.0000
			0.5	2	35.25	70.5			0.1					1		15	
	3	2	0.5	1	33.53	67.06	59.53	119.06	0.1	140192	0.08	5	0.0000	1	0.013	15	0.0000
			0.5	2	26.00	52			0.1					1		15	
	4	2	0.5	1	24.78	49.56	53.14	106.28	0.1	140192	0.08	-7	0.0000	1	0.013	15	0.0000
			0.5	2	28.36	56.72			0.1					1		15	
Positive control	1	2	0.5	1	401.67	803.34	838.8	1677.6	0.1	140192	1.20	1564	0.0022	1	0.013	15	0.0056
			0.5	2	437.13	874.26			0.1					1		15	
	2	2	0.5	1	420.83	841.66	848.99	1697.98	0.1	140192	1.21	1585	0.0023	1	0.013	15	0.0059
			0.5	2	428.10	856.32			0.1					1		15	
	3	2	0.5	1	400.72	801.44	801.36	1602.72	0.1	140192	1.14	1490	0.0021	1	0.013	15	0.0055
			0.5	2	400.64	801.28			0.1					1		15	
	4	2	0.5	1	381.17	762.34	768.7	1537.4	0.1	140192	1.10	1424	0.0020	1	0.013	15	0.0053
			0.5	2	387.53	775.06			0.1					1		15	
Negative Control	1	2	0.5	1	692.44	1384.88	1391.9	2783.8	0.1	140192	1.99	2671	0.0038	1	0.013	15	0.0099
			0.5	2	699.46	1398.92			0.1					1		15	
	2	2	0.5	1	735.44	1470.88	1470.43	2940.86	0.1	140192	2.10	2828	0.0040	1	0.013	15	0.0105
			0.5	2	734.99	1469.98			0.1					1		15	
	3	2	0.5	1	667.73	1335.46	1377.03	2754.06	0.1	140192	1.96	2641	0.0038	1	0.013	15	0.0098
			0.5	2	709.30	1418.6			0.1					1		15	
	4	2	0.5	1	658.04	1316.08	1337.85	2675.7	0.1	140192	1.91	2563	0.0037	1	0.013	15	0.0095
			0.5	2	679.81	1359.62			0.1					1		15	
RC 3	1-1	2	0.5	1	91.07	182.14	179.99	359.98	0.1	140192	0.26	247	0.0004	1	0.013	15	0.0009
			0.5	2	88.92	177.84			0.1					1		15	
	1-2	2	0.5	1	88.77	177.54	182.89	365.78	0.1	140192	0.26	253	0.0004	1	0.013	15	0.0009
			0.5	2	94.12	188.24			0.1					1		15	
	1-3	2	0.5	1	93.46	186.92	171	342	0.1	140192	0.24	229	0.0003	1	0.013	15	0.0008
			0.5	2	77.54	155.08			0.1					1		15	
	2-1	2	0.5	1	187.79	375.58	386.33	772.66	0.1	140192	0.55	660	0.0009	1	0.013	15	0.0024
			0.5	2	198.54	397.08			0.1					1		15	
	2-2	2	0.5	1	168.60	337.36	366.82	733.64	0.1	140192	0.52	621	0.0009	1	0.013	15	0.0023
			0.5	2	198.13	396.26			0.1					1		15	
	2-3	2	0.5	1	180.95	361.9	372.5	745	0.1	140192	0.53	632	0.0009	1	0.013	15	0.0023
			0.5	2	191.55	383.1			0.1					1		15	
	3-1	2	0.5	1	315.89	631.78	630.84	1261.68	0.1	140192	0.90	1149	0.0016	1	0.013	15	0.0043
			0.5	2	314.95	629.9			0.1					1		15	
	3-2	2	0.5	1	300.41	600.82	607.13	1214.26	0.1	140192	0.87	1101	0.0016	1	0.013	15	0.0041
			0.5	2	306.72	613.44			0.1					1		15	
	3-3	2	0.5	1	300.20	600.4	632.37	1264.74	0.1	140192	0.90	1152	0.0016	1	0.013	15	0.0043
			0.5	2	332.17	664.34			0.1					1		15	
	4-1	2	0.5	1	477.01	954.02	931.88	1863.76	0.1	140192	1.33	1751	0.0025	1	0.013	15	0.0065
			0.5	2	454.87	909.74			0.1					1		15	
	4-2	2	0.5	1	452.40	904.8	914.02	1828.04	0.1	140192	1.30	1715	0.0024	1	0.013	15	0.0064
			0.5	2	461.62	923.24			0.1					1		15	
	4-3	2	0.5	1	465.79	931.58	937.02	1874.04	0.1	140192	1.34	1761	0.0025	1	0.013	15	0.0065
			0.5	2	471.23	942.46			0.1					1		15	
	5-1	2	0.5	1	562.34	1124.68	1160.8	2321.6	0.1	140192	1.66	2208	0.0032	1	0.013	15	0.0082
			0.5	2	598.46	1196.92			0.1					1		15	
	5-2	2	0.5	1	584.88	1169.76	1171.68	2343.32	0.1	140192	1.67	2230	0.0032	1	0.013	15	0.0083
			0.5	2	606.08	1212.16			0.1					1		15	
	5-3	2	0.5	1	561.24	1122.48	1177.4	2354.8	0.1	140192	1.68	2242	0.0032	1	0.013	15	0.0083
			0.5	2	616.16	1232.32			0.1					1		15	
	6-1	2	0.5	1	638.08	1276.16	1305.69	2611.38	0.1	140192	1.86	2498	0.0036	1	0.013	15	0.0093
			0.5	2	667.61	1335.22			0.1					1		15	
	6-2	2	0.5	1	617.94	1235.88	1238.27	2476.54	0.1	140192	1.77	2383	0.0034	1	0.013	15	0.0088
			0.5	2	620.33	1240.66			0.1					1		15	
	6-3	2	0.5	1	630.15	1260.3	1285.85	2571.7	0.1	140192	1.83	2459	0.0035	1	0.013	15	0.0091
			0.5	2	655.70	1311.4			0.1					1		15	
	7-1	2	0.5	1	691.58	1383.16	1404.39	2808.78	0.1	140192	2.00	2696	0.0038	1	0.013	15	0.0100
			0.5	2	712.81	1425.62			0.1					1		15	
	7-2	2	0.5	1	787.79	1575.58	1629.79	3259.58	0.1	140192	2.33	3146	0.0045	1	0.013	15	0.0117
			0.5	2	842.03	1684.06			0.1					1		15	
	7-3	2	0.5	1	750.35	1500.7	1520.47	3040.94	0.1	140192	2.17	2928	0.0042	1	0.013	15	0.0109
			0.5	2	770.12	1540.24			0.1					1		15	
	8-1	2	0.5	1	654.52	1309.04	1344.44	2688.88	0.1	140192	1.92	2570	0.0037	1	0.013	15	0.0096
			0.5	2	690.12	1380.24			0.1					1		15	
	8-2	2	0.5	1	687.73	1375.46	1413.47	2826.94	0.1	140192	2.02	2714	0.0039	1	0.013	15	0.0101
			0.5	2	725.74	1451.48			0.1					1		15	
	8-3	2	0.5	1	697.39	1394.78	1369.15	2736.3	0.1	140192	1.95	2625	0.0037	1	0.013	15	0.0097
			0.5	2	671.76	1343.52			0.1					1		15	

Assay Date	2/18/2005	ID	RC 3	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JRH	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0105	0.0004
Full activity	End	0.0101	0.0007
Full activity	Overall	0.0103	0.0005
Background	Beginning	0.0000	1.81123E-05
Background	End	0.0000	3.35472E-05
Background	Overall	0.0000	2.20993E-05
Positive	Beginning	0.0058	0.0001
Positive	End	0.0054	0.0002
Positive	Overall	0.0056	0.0003
Negative	Beginning	0.0102	0.0004
Negative	End	0.0097	0.0002
Negative	Overall	0.0099	0.0004

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 3	1	1	1.00E-04	-4.00	0.0009
RC 3	1	2	1.00E-04	-4.00	0.0009
RC 3	1	3	1.00E-04	-4.00	0.0008
RC 3	2	1	4.00E-05	-4.40	0.0024
RC 3	2	2	4.00E-05	-4.40	0.0023
RC 3	2	3	4.00E-05	-4.40	0.0023
RC 3	3	1	1.60E-05	-4.80	0.0043
RC 3	3	2	1.60E-05	-4.80	0.0041
RC 3	3	3	1.60E-05	-4.80	0.0043
RC 3	4	1	6.40E-06	-5.19	0.0065
RC 3	4	2	6.40E-06	-5.19	0.0064
RC 3	4	3	6.40E-06	-5.19	0.0065
RC 3	5	1	2.50E-06	-5.60	0.0082
RC 3	5	2	2.50E-06	-5.60	0.0083
RC 3	5	3	2.50E-06	-5.60	0.0083
RC 3	6	1	1.00E-06	-6.00	0.0093
RC 3	6	2	1.00E-06	-6.00	0.0088
RC 3	6	3	1.00E-06	-6.00	0.0091
RC 3	7	1	5.00E-08	-7.30	0.0100
RC 3	7	2	5.00E-08	-7.30	0.0117
RC 3	7	3	5.00E-08	-7.30	0.0109
RC 3	8	1	1.00E-09	-9.00	0.0096
RC 3	8	2	1.00E-09	-9.00	0.0101
RC 3	8	3	1.00E-09	-9.00	0.0097

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-4.00	8.91	9.12	8.26
2	-4.40	23.80	22.39	22.80
3	-4.80	41.44	39.73	41.55
4	-5.19	63.17	61.88	63.54
5	-5.60	79.69	80.47	80.88
6	-6.00	90.14	85.28	88.71
7	-7.30	97.26	113.53	105.64
8	-9.00	92.94	97.92	94.72

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	29753.74	1510342	
2	0.0200	30919.54	1545977	
3	0.0200	30926.91	1546346	
4	0.0201	31072.4	1545891	
5	0.0198	31126.6	1572051	
			Average DPM/g soln	1544121
			SD	21981
			CV	1.42
			µCi/g soln	0.696

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.1	10		1010.00
Dilution A			100	10.10
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.2025 g
Mass of dilution B used in substrate prep	4.6095 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.567582 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00787 µg/g soln.
µg/g soln.

a. µCi/g soln 0.696
b. Specific activity of [H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.567582 + 0.00787
= 0.575456 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.209 µCi/µg ASDN

768497 dpm/nmol

Assay Date 3/8/2005 | Test Chemical ID RC 3 | tested 8

ID JRH | Replicate # 3 | Microsome type Placental | Microsome ID MET-0255A #30 & 31

Standards:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Total volume of BSA)	stock (mL)	Protein stock ID
0			#DIV/0!	25	#DIV/0!						
0			#DIV/0!	25	#DIV/0!						
0			#DIV/0!	25	#DIV/0!						
0			#DIV/0!	25	#DIV/0!						
0			#DIV/0!	25	#DIV/0!						
0			#DIV/0!	25	#DIV/0!						

Blank

r^2=
m=
b=

Variables	Regression results
m, b	
se_m, se_b	
r^2, se_y	
F, df	
ss_{reg}, ss_{resid}	

Regression results are calculated using the function LINEST

Samples:

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date 3/8/2005 Test Chemical ID RC 3 tested 8

ID JRH Replicate # 3 Microsome type Placental Microsome ID MET-0255A #30 & 31

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk		
	0.382	0.294	0.235	0.171	0.114	0.077	0.038	25	431007-3-13
	0.397	0.278	0.234	0.170	0.122	0.077	0.036		
	0.397	0.290	0.226	0.176	0.116	0.073	0.046		

Samples:	microsomes	0.125	0.5	1
	0.148	0.069	0.181	0.310
	0.132	0.067	0.182	0.322
	0.137			

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.392	0.352	0.0368	m, b	0.109	-0.002
0.00100	25	0.0250	0.287	0.247	0.0254	se_m, se_b	0.003	0.001
0.00075	25	0.0188	0.232	0.192	0.0193	r , se_y	0.997	0.001
0.00050	25	0.0125	0.172	0.132	0.0128	F, df	1403	4
0.00025	25	0.0063	0.117	0.077	0.0068	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.075	0.036	0.0022			

Blank 0.040

r^2= 0.997
m= 0.109
b= -0.002

Regression results are calculated using the function LINEST

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
	0.148	0.108	0.010	25	190	5190	0.011	0.010	10.026
	0.132	0.093	0.008	25	190	5190	0.009		
	0.137	0.098	0.009	25	190	5190	0.010		
0.125	0.069	0.029	0.001	25	1	1	0.000	0.000	0.056
0.125	0.067	0.028	0.001	25	1	1	0.000		
0.125									
0.5	0.181	0.141	0.014	25	1	1	0.001	0.001	0.552
0.5	0.182	0.143	0.014	25	1	1	0.001		
0.5									
1	0.310	0.270	0.028	25	1	1	0.001	0.001	1.140
1	0.322	0.282	0.029	25	1	1	0.001		
1									

Assay Date	3/8/2005	Chemical ID	RC 3	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JRH	Replicate #	3

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	3	mL microsome Dilution A used
	44	mL total volume
	14.66667	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	400.6316	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	10.026
Protein Concentration (dilution added to assay, mg/mL):	0.025025

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-04
2	4.00E-05
3	1.60E-05
4	6.40E-06
5	2.50E-06
6	1.00E-06
7	5.00E-08
8	1.00E-09

Assay Date	3/8/2005	Test Chemical ID	RC 3	# Concentrations tested	6	Microsome type	Placental	Microsome ID	MET-0255A #30 & 31	Technician ID	JRH	Replicate #	3

Sample ID			Calculate DPM in aqueous portion after extraction						Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/tube	DPM/mL	Ave. DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2003	4005.36	4148.46	8296.92	0.1	154412	5.37	7801	0.0102	1	0.013	15	0.0270
			0.5	2	2146	4291.54			0.1					1		15	
	2	2	0.5	1	2444	4887.4	4852.56	9705.12	0.1	154412	6.29	9210	0.0120	1	0.013	15	0.0319
			0.5	2	2409	4817.72			0.1					1		15	
	3	2	0.5	1	2542	5084.02	5092.69	10185.38	0.1	154412	6.60	9690	0.0126	1	0.013	15	0.0336
			0.5	2	2550	5100.76			0.1					1		15	
	4	2	0.5	1	2261	4521.5	4487.45	8974.9	0.1	154412	5.81	8479	0.0110	1	0.013	15	0.0294
			0.5	2	2227	4453.4			0.1					1		15	
Background control	1	2	0.5	1	114	227.2	223.63	447.06	0.1	154412	0.29	46	0.0001	1	0.013	15	0.0002
			0.5	2	110	220.46			0.1					1		15	
	2	2	0.5	1	103	205.22	215.84	431.68	0.1	154412	0.28	64	0.0001	1	0.013	15	0.0002
			0.5	2	113	226.46			0.1					1		15	
	3	2	0.5	1	133	265.8	275.24	550.48	0.1	154412	0.36	55	0.0001	1	0.013	15	0.0002
			0.5	2	142	284.98			0.1					1		15	
	4	2	0.5	1	136	271.56	279.25	562.5	0.1	154412	0.36	57	0.0001	1	0.013	15	0.0002
			0.5	2	140	280.94			0.1					1		15	
Positive control	1	2	0.5	1	1006	2015.22	2015.22	4030.44	0.1	154412	2.61	3535	0.0046	1	0.013	15	0.0123
			0.5	2	1006	2015.22			0.1					1		15	
	2	2	0.5	1	1018	2035.52	2060.51	4121.02	0.1	154412	2.67	3625	0.0047	1	0.013	15	0.0126
			0.5	2	1043	2085.5			0.1					1		15	
	3	2	0.5	1	929	1857.96	1900.96	3801.92	0.1	154412	2.46	3306	0.0043	1	0.013	15	0.0115
			0.5	2	972	1944.26			0.1					1		15	
	4	2	0.5	1	877	1753.46	1777.36	3554.72	0.1	154412	2.30	3059	0.0040	1	0.013	15	0.0109
			0.5	2	901	1801.26			0.1					1		15	
Negative Control	1	2	0.5	1	2198	4396.58	4420.2	8840.4	0.1	154412	5.73	8345	0.0109	1	0.013	15	0.0289
			0.5	2	2251	4501.82			0.1					1		15	
	2	2	0.5	1	2298	4595.51	4958.21	9916.42	0.1	154412	6.03	8821	0.0115	1	0.013	15	0.0306
			0.5	2	2660	4720.92			0.1					1		15	
	3	2	0.5	1	2163	4325.8	4361	8722	0.1	154412	5.65	8226	0.0107	1	0.013	15	0.0285
			0.5	2	2198	4396.2			0.1					1		15	
	4	2	0.5	1	2055	4110.04	4303.19	8606.38	0.1	154412	5.57	8111	0.0106	1	0.013	15	0.0281
			0.5	2	2246	4496.34			0.1					1		15	
RC 3	1-1	2	0.5	1	198	395.08	388.39	776.78	0.1	154412	0.50	281	0.0004	1	0.013	15	0.0010
			0.5	2	191	381.7			0.1					1		15	
	1-2	2	0.5	1	228	451.54	471.55	943.1	0.1	154412	0.61	448	0.0006	1	0.013	15	0.0016
			0.5	2	248	491.56			0.1					1		15	
	1-3	2	0.5	1	510	1019.7	1017.5	2035	0.1	154412	1.32	1539	0.0020	1	0.013	15	0.0053
			0.5	2	508	1015.3			0.1					1		15	
	2-1	2	0.5	1	407	814.42	851.39	1702.78	0.1	154412	1.10	1207	0.0016	1	0.013	15	0.0042
			0.5	2	444	888.36			0.1					1		15	
	2-2	2	0.5	1	403	805.78	820.91	1641.82	0.1	154412	1.06	1146	0.0015	1	0.013	15	0.0040
			0.5	2	418	836.04			0.1					1		15	
	2-3	2	0.5	1	407	813.56	839.7	1679.4	0.1	154412	1.09	1184	0.0015	1	0.013	15	0.0041
			0.5	2	433	865.84			0.1					1		15	
	3-1	2	0.5	1	786	1571.38	1605.78	3211.56	0.1	154412	2.08	2716	0.0035	1	0.013	15	0.0094
			0.5	2	820	1640.18			0.1					1		15	
	3-2	2	0.5	1	773	1546.94	1596.33	3192.66	0.1	154412	2.07	2697	0.0035	1	0.013	15	0.0093
			0.5	2	823	1645.72			0.1					1		15	
	3-3	2	0.5	1	783	1565	1587.24	3174.48	0.1	154412	2.06	2679	0.0035	1	0.013	15	0.0093
			0.5	2	805	1609.48			0.1					1		15	
	4-1	2	0.5	1	1240	2481.46	2516.82	5033.64	0.1	154412	3.26	4538	0.0059	1	0.013	15	0.0157
			0.5	2	1271	2542.18			0.1					1		15	
	4-2	2	0.5	1	1218	2435.8	2441.45	4882.9	0.1	154412	3.16	4367	0.0057	1	0.013	15	0.0152
			0.5	2	1224	2447.1			0.1					1		15	
	4-3	2	0.5	1	1234	2467.42	2433.25	4866.5	0.1	154412	3.15	4371	0.0057	1	0.013	15	0.0152
			0.5	2	1200	2399.08			0.1					1		15	
	5-1	2	0.5	1	1648	3295.48	3373.79	6747.58	0.1	154412	4.37	6252	0.0081	1	0.013	15	0.0217
			0.5	2	1726	3452.1			0.1					1		15	
	5-2	2	0.5	1	1648	3296.56	3422.07	6844.14	0.1	154412	4.43	6349	0.0083	1	0.013	15	0.0220
			0.5	2	1774	3548.46			0.1					1		15	
	5-3	2	0.5	1	1636	3276.92	3375.95	6751.9	0.1	154412	4.37	6256	0.0081	1	0.013	15	0.0217
			0.5	2	1737	3473.88			0.1					1		15	
	6-1	2	0.5	1	1961	3962.52	3970.12	7940.24	0.1	154412	5.14	7445	0.0097	1	0.013	15	0.0258
			0.5	2	1980	3977.72			0.1					1		15	
	6-2	2	0.5	1	1922	3843.06	3918.17	7832.34	0.1	154412	5.07	7337	0.0095	1	0.013	15	0.0254
			0.5	2	1996	3989.28			0.1					1		15	
	6-3	2	0.5	1	1951	3902	3890.01	7780.02	0.1	154412	5.04	7284	0.0095	1	0.013	15	0.0253
			0.5	2	1939	3878.02			0.1					1		15	
	7-1	2	0.5	1	2230	4466.48	4535.95	9071.9	0.1	154412	5.88	8576	0.0112	1	0.013	15	0.0297
			0.5	2	2303	4605.42			0.1					1		15	
	7-2	2	0.5	1	2212	4424.9	4484.38	8968.76	0.1	154412	5.81	8473	0.0110	1	0.013	15	0.0294
			0.5	2	2272	4543.86			0.1					1		15	
	7-3	2	0.5	1	2238	4476.34	4535.64	9073.28	0.1	154412	5.88	8578	0.0112	1	0.013	15	0.0297
			0.5	2	2296	4595.94			0.1					1		15	
	8-1	2	0.5	1	2335	4669.46	4629.67	9259.34	0.1	154412	5.99	8758	0.0114	1	0.013	15	0.0304
			0.5	2	2292	4583.88			0.1					1		15	
	8-2	2	0.5	1	2288	4575.1	4682.92	9365.84	0.1	154412	6.07	8870	0.0115	1	0.013	15	0.0307
			0.5	2	2395	4788.74			0.1					1		15	
	8-3	2	0.5	1	2270	4539.06	4596.95	9157.9	0.1	154412	5.90	8702	0.0113	1	0.013	15	0.0302
			0.5	2	2329	4656.84			0.1					1		15	

Assay Date	3/8/2005	ID	RC 3	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JRH	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0295	0.0035
Full activity	End	0.0315	0.0030
Full activity	Overall	0.0305	0.0029
Background	Beginning	-0.0002	3.91693E-05
Background	End	0.0002	4.95131E-06
Background	Overall	0.0000	0.000224949
Positive	Beginning	0.0124	0.0002
Positive	End	0.0110	0.0006
Positive	Overall	0.0117	0.0009
Negative	Beginning	0.0298	0.0012
Negative	End	0.0283	0.0003
Negative	Overall	0.0290	0.0011

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 3	1	1	1.00E-04	-4.00	0.0010
RC 3	1	2	1.00E-04	-4.00	0.0016
RC 3	1	3	1.00E-04	-4.00	0.0053
RC 3	2	1	4.00E-05	-4.40	0.0042
RC 3	2	2	4.00E-05	-4.40	0.0040
RC 3	2	3	4.00E-05	-4.40	0.0041
RC 3	3	1	1.60E-05	-4.80	0.0094
RC 3	3	2	1.60E-05	-4.80	0.0093
RC 3	3	3	1.60E-05	-4.80	0.0093
RC 3	4	1	6.40E-06	-5.19	0.0157
RC 3	4	2	6.40E-06	-5.19	0.0152
RC 3	4	3	6.40E-06	-5.19	0.0152
RC 3	5	1	2.50E-06	-5.60	0.0217
RC 3	5	2	2.50E-06	-5.60	0.0220
RC 3	5	3	2.50E-06	-5.60	0.0217
RC 3	6	1	1.00E-06	-6.00	0.0258
RC 3	6	2	1.00E-06	-6.00	0.0254
RC 3	6	3	1.00E-06	-6.00	0.0253
RC 3	7	1	5.00E-08	-7.30	0.0297
RC 3	7	2	5.00E-08	-7.30	0.0294
RC 3	7	3	5.00E-08	-7.30	0.0297
RC 3	8	1	1.00E-09	-9.00	0.0304
RC 3	8	2	1.00E-09	-9.00	0.0307
RC 3	8	3	1.00E-09	-9.00	0.0302

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-4.00	3.20	5.09	17.50
2	-4.40	13.73	13.03	13.46
3	-4.80	30.88	30.67	30.46
4	-5.19	51.60	49.88	49.70
5	-5.60	71.09	72.18	71.13
6	-6.00	84.65	83.42	82.82
7	-7.30	97.51	96.34	97.53
8	-9.00	99.58	100.86	98.95

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0197	29782.21	1511787	
2	0.0200	29764.08	1488204	
3	0.0196	31374.85	1600758	
4	0.0199	30852.37	1550370	
5	0.0199	31132.88	1564466	
			Average DPM/g soln	1543117
			SD	44238
			CV	2.87
			µCi/g soln	0.695

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.2	10		1020.00
Dilution A			100	10.20
Dilution B			10	1.02

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1719 g
Mass of dilution B used in substrate prep	4.6007 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.57425 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00787 µg/g soln.
µg/g soln.

a. µCi/g soln	0.695
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.574250 + 0.00787
= 0.582119 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.194 µCi/µg ASDN

759207 dpm/nmol

Assay Date	2/23/2005	Test Chemical ID	RC 4	tested	8		
ID	JG	Replicate #	1	Microsome type	Placental	Microsome ID	MET-0255A 24&25

Standards:

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	Total volume of stock (mL)	Protein stock ID
0			#DIV/0!	25	#DIV/0!				m, b		BSA)	
0			#DIV/0!	25	#DIV/0!				se_m, se_b			
0			#DIV/0!	25	#DIV/0!				r , se_y			
0			#DIV/0!	25	#DIV/0!				F, df			
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}			
0			#DIV/0!	25	#DIV/0!							

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date 2/23/2005 Test Chemical ID RC 4 tested 8

ID JG Replicate # 1 Microsome type Placental Microsome ID MET-0255A 24&25

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-10
	0.407	0.284	0.225	0.175	0.101	0.059	0.053		
	0.408	0.303	0.240	0.175	0.095	0.047	0.032		
	0.403	0.311	0.238	0.178	0.096	0.048	0.035		
Samples:	microsomes								
	0.142	0.080	0.183	0.311					
	0.138	0.066	0.187	0.322					
	0.141								

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.406	0.366	0.0361	m, b	0.097	0.001
0.00100	25	0.0250	0.299	0.259	0.0258	se_m, se_b	0.005	0.001
0.00075	25	0.0188	0.234	0.194	0.0195	r , se_y	0.991	0.001
0.00050	25	0.0125	0.176	0.136	0.0138	F, df	457	4
0.00025	25	0.0063	0.098	0.057	0.0063	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.051	0.011	0.0018			

Blank 0.040 r^2= 0.991 m= 0.097 b= 0.001

Regression results are calculated using the function LINEST

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.142	0.102	0.011	25	190	5190	0.012	0.011	11.367
0.138	0.098	0.010	25	190	5190	0.011		
0.141	0.101	0.010	25	190	5190	0.011		
0.080	0.040	0.005	25	1	1	0.000	0.000	0.155
0.066	0.026	0.003	25	1	1	0.000		
				1	1			
0.183	0.143	0.015	25	1	1	0.001	0.001	0.588
0.187	0.146	0.015	25	1	1	0.001		
				1	1			
0.311	0.271	0.027	25	1	1	0.001	0.001	1.097
0.322	0.282	0.028	25	1	1	0.001		
				1	1			
				1	1			
				1	1			
				1	1			

Assay Date	2/23/2005	Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	1

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	2.8	mL microsome Dilution A used
	44	mL total volume
	15.71429	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	429.2481	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	11.367
Protein Concentration (dilution added to assay, mg/mL):	0.026481

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-03
2	1.00E-04
3	1.00E-05
4	1.00E-06
5	1.00E-07
6	1.00E-08
7	1.00E-09
8	1.00E-10

Assay Date	2/23/2005	Test Chemical ID	RC 4	# Concentrations tested	6	Microsome type	Placental	Microsome ID	MET-0255A 2+A25	Technician ID	JG	Replicate #	1

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2162.67	4325.34	4362.9	8725.8	0.1	154312	5.65	8614	0.0113	1	0.013	15	0.0286
			0.5	2	2200.23	4400.46			0.1					1		15	
	2	2	0.5	1	1877.74	3755.48	3763.07	7526.14	0.1	154312	4.86	7415	0.0098	1	0.013	15	0.0246
			0.5	2	1885.33	3770.66			0.1					1		15	
	3	2	0.5	1	2218.17	4436.34	4483.56	8967.12	0.1	154312	5.81	8850	0.0117	1	0.013	15	0.0294
			0.5	2	2265.39	4530.78			0.1					1		15	
	4	2	0.5	1	2282.91	4565.82	4539.72	9079.44	0.1	154312	5.88	8968	0.0118	1	0.013	15	0.0297
			0.5	2	2256.61	4513.02			0.1					1		15	
Background control	1	2	0.5	1	23.23	46.45	44.86	89.72	0.1	154312	0.06	-22	0.0000	1	0.013	15	-0.0001
			0.5	2	21.63	43.26			0.1					1		15	
	2	2	0.5	1	40.53	81.06	62.32	124.64	0.1	154312	0.08	13	0.0000	1	0.013	15	0.0000
			0.5	2	21.79	43.58			0.1					1		15	
	3	2	0.5	1	31.76	63.52	63.3	126.6	0.1	154312	0.08	15	0.0000	1	0.013	15	0.0000
			0.5	2	31.54	63.08			0.1					1		15	
	4	2	0.5	1	23.6	47.2	52.63	105.26	0.1	154312	0.07	-6	0.0000	1	0.013	15	0.0000
			0.5	2	29.03	58.06			0.1					1		15	
Positive control	1	2	0.5	1	1112.01	2224.02	2194.58	4389.16	0.1	154312	2.84	4278	0.0059	1	0.013	15	0.0142
			0.5	2	1082.57	2165.14			0.1					1		15	
	2	2	0.5	1	1134.08	2268.16	2301.77	4603.54	0.1	154312	2.98	4492	0.0059	1	0.013	15	0.0149
			0.5	2	1167.69	2335.38			0.1					1		15	
	3	2	0.5	1	1017.09	2034.18	2064.96	4129.92	0.1	154312	2.68	4018	0.0053	1	0.013	15	0.0133
			0.5	2	1047.87	2095.74			0.1					1		15	
	4	2	0.5	1	1051.65	2103.3	2114.77	4228.54	0.1	154312	2.74	4118	0.0054	1	0.013	15	0.0137
			0.5	2	1063.12	2126.24			0.1					1		15	
Negative Control	1	2	0.5	1	2299.76	4599.52	4562.28	9124.56	0.1	154312	5.91	9015	0.0119	1	0.013	15	0.0299
			0.5	2	2262.52	4525.04			0.1					1		15	
	2	2	0.5	1	2343.96	4687.92	4613.41	9226.82	0.1	154312	5.98	9115	0.0120	1	0.013	15	0.0302
			0.5	2	2269.45	4538.9			0.1					1		15	
	3	2	0.5	1	2014	4028	4117.68	8235.36	0.1	154312	5.34	8124	0.0107	1	0.013	15	0.0269
			0.5	2	2103.68	4207.36			0.1					1		15	
	4	2	0.5	1	2086.35	4130.7	4218.53	8437.06	0.1	154312	5.47	8329	0.0110	1	0.013	15	0.0276
			0.5	2	2150.18	4300.36			0.1					1		15	
RC 4	1-1	2	0.5	1	52.02	104.04	130.99	261.98	0.1	154312	0.17	150	0.0002	1	0.013	15	0.0005
			0.5	2	78.97	157.94			0.1					1		15	
	1-2	2	0.5	1	24.38	48.76	47.51	95.02	0.1	154312	0.06	-17	0.0000	1	0.013	15	-0.0001
			0.5	2	23.13	46.26			0.1					1		15	
	1-3	2	0.5	1	28.18	56.36	61.99	123.98	0.1	154312	0.08	12	0.0000	1	0.013	15	0.0000
			0.5	2	33.81	67.62			0.1					1		15	
	2-1	2	0.5	1	91.64	183.28	130.88	261.76	0.1	154312	0.17	150	0.0002	1	0.013	15	0.0005
			0.5	2	39.24	78.48			0.1					1		15	
	2-2	2	0.5	1	27.16	54.32	52.16	104.32	0.1	154312	0.07	-7	0.0000	1	0.013	15	0.0000
			0.5	2	25	50			0.1					1		15	
	2-3	2	0.5	1	31.76	63.52	53.54	107.08	0.1	154312	0.07	-4	0.0000	1	0.013	15	0.0000
			0.5	2	21.78	43.56			0.1					1		15	
	3-1	2	0.5	1	24.52	49.04	52.04	104.08	0.1	154312	0.07	-7	0.0000	1	0.013	15	0.0000
			0.5	2	27.22	54.441			0.1					1		15	
	3-2	2	0.5	1	27.14	54.28	56.74	113.48	0.1	154312	0.07	2	0.0000	1	0.013	15	0.0000
			0.5	2	29.6	59.2			0.1					1		15	
	3-3	2	0.5	1	30.27	60.54	54.82	109.64	0.1	154312	0.07	-2	0.0000	1	0.013	15	0.0000
			0.5	2	24.55	49.1			0.1					1		15	
	4-1	2	0.5	1	36.91	73.82	65.44	130.88	0.1	154312	0.08	19	0.0000	1	0.013	15	0.0001
			0.5	2	28.53	57.06			0.1					1		15	
	4-2	2	0.5	1	32.59	65.18	62.4	124.8	0.1	154312	0.08	13	0.0000	1	0.013	15	0.0000
			0.5	2	29.81	59.62			0.1					1		15	
	4-3	2	0.5	1	31.17	62.34	60.55	121.1	0.1	154312	0.08	10	0.0000	1	0.013	15	0.0000
			0.5	2	29.36	58.76			0.1					1		15	
	5-1	2	0.5	1	76.55	157.1	151.26	302.52	0.1	154312	0.20	191	0.0003	1	0.013	15	0.0006
			0.5	2	72.71	145.42			0.1					1		15	
	5-2	2	0.5	1	76.19	156.38	166.04	332.08	0.1	154312	0.22	221	0.0003	1	0.013	15	0.0007
			0.5	2	87.85	175.7			0.1					1		15	
	5-3	2	0.5	1	70.27	140.54	146.93	293.86	0.1	154312	0.19	182	0.0002	1	0.013	15	0.0006
			0.5	2	76.66	153.32			0.1					1		15	
	6-1	2	0.5	1	390.4	780.8	777.44	1554.88	0.1	154312	1.01	1443	0.0019	1	0.013	15	0.0048
			0.5	2	387.04	774.08			0.1					1		15	
	6-2	2	0.5	1	357	714	719.56	1439.12	0.1	154312	0.93	1328	0.0017	1	0.013	15	0.0044
			0.5	2	362.56	725.12			0.1					1		15	
	6-3	2	0.5	1	381.51	763.02	772.97	1545.94	0.1	154312	1.00	1434	0.0019	1	0.013	15	0.0048
			0.5	2	391.46	782.92			0.1					1		15	
	7-1	2	0.5	1	1440.49	2880.98	2905.78	5811.56	0.1	154312	3.77	5702	0.0075	1	0.013	15	0.0189
			0.5	2	1466.29	2932.58			0.1					1		15	
	7-2	2	0.5	1	1478.03	2956.06	2994.12	5988.24	0.1	154312	3.88	5877	0.0077	1	0.013	15	0.0196
			0.5	2	1510.09	3020.18			0.1					1		15	
	7-3	2	0.5	1	1349.03	2698.06	2703.2	5406.4	0.1	154312	3.50	5295	0.0070	1	0.013	15	0.0176
			0.5	2	1353.57	2707.14			0.1					1		15	
	8-1	2	0.5	1	2021.76	4043.52	4035.7	8071.4	0.1	154312	5.23	7920	0.0105	1	0.013	15	0.0264
			0.5	2	2013.94	4027.88			0.1					1		15	
	8-2	2	0.5	1	2008.03	4016.06	3994.89	7989.78	0.1	154312	5.18	7878	0.0104	1	0.013	15	0.0261
			0.5	2	1986.86	3973.72			0.1					1		15	
	8-3	2	0.5	1	1976.2	3952.4	3941.38	7882.76	0.1	154312	5.11	7771	0.0102	1	0.013	15	0.0258
			0.5	2	1905.18	3930.36			0.1					1		15	

Assay Date	2/23/2005	ID	RC 4	# Concentrations tested	Microsome 8 type	Placental	Microsome ID	Technician ID JG	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0266	0.0028
Full activity	End	0.0296	0.0003
Full activity	Overall	0.0281	0.0024
Background	Beginning	0.0000	8.18785E-05
Background	End	0.0000	5.00369E-05
Background	Overall	0.0000	5.78782E-05
Positive	Beginning	0.0145	0.0005
Positive	End	0.0135	0.0002
Positive	Overall	0.0140	0.0007
Negative	Beginning	0.0301	0.0002
Negative	End	0.0273	0.0005
Negative	Overall	0.0287	0.0016

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 4	1	1	1.00E-03	-3.00	0.0005
RC 4	1	2	1.00E-03	-3.00	-0.0001
RC 4	1	3	1.00E-03	-3.00	0.0000
RC 4	2	1	1.00E-04	-4.00	0.0005
RC 4	2	2	1.00E-04	-4.00	0.0000
RC 4	2	3	1.00E-04	-4.00	0.0000
RC 4	3	1	1.00E-05	-5.00	0.0000
RC 4	3	2	1.00E-05	-5.00	0.0000
RC 4	3	3	1.00E-05	-5.00	0.0000
RC 4	4	1	1.00E-06	-6.00	0.0001
RC 4	4	2	1.00E-06	-6.00	0.0000
RC 4	4	3	1.00E-06	-6.00	0.0000
RC 4	5	1	1.00E-07	-7.00	0.0006
RC 4	5	2	1.00E-07	-7.00	0.0007
RC 4	5	3	1.00E-07	-7.00	0.0006
RC 4	6	1	1.00E-08	-8.00	0.0048
RC 4	6	2	1.00E-08	-8.00	0.0044
RC 4	6	3	1.00E-08	-8.00	0.0048
RC 4	7	1	1.00E-09	-9.00	0.0189
RC 4	7	2	1.00E-09	-9.00	0.0195
RC 4	7	3	1.00E-09	-9.00	0.0176
RC 4	8	1	1.00E-10	-10.00	0.0264
RC 4	8	2	1.00E-10	-10.00	0.0261
RC 4	8	3	1.00E-10	-10.00	0.0258

	Percent of control values			
		Replicate		
.evel	Log[test ostance]	1	2	3
1	-3.00	1.78	-0.20	0.15
2	-4.00	1.77	-0.09	-0.05
3	-5.00	-0.09	0.02	-0.02
4	-6.00	0.23	0.16	0.11
5	-7.00	2.26	2.61	2.15
6	-8.00	17.05	15.69	16.95
7	-9.00	67.38	69.44	62.56
8	-10.00	94.05	93.09	91.82

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0199	29103.08	1462466	
2	0.0199	29792.54	1497113	
3	0.0198	30411.37	1535928	
4	0.0198	30836.8	1557414	
5	0.0197	30098.66	1527851	
			Average DPM/g soln	1516154
			SD	36997
			CV	2.44
			µCi/g soln	0.683

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1572 g
Mass of dilution B used in substrate prep	4.5877 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.562411 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00773 µg/g soln.
µg/g soln.

a. µCi/g soln	0.683
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.562411 + 0.00773
= 0.570142 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.198 µCi/µg ASDN

761611 dpm/nmol

Test

Assay Date 2/28/2005 Chemical ID RC 4 tested 8

ID JG Replicate # 2 Microsome type Placental Microsome ID MET-0255A 26&27

Standards: BSA) Total volume of stock (mL) Protein stock ID

Samples:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r^2, se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{regr}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

	A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date 2/28/2005 Test Chemical ID RC 4 tested 8

ID JG Replicate # 2 Microsome type Placental Microsome ID MET-0255A 26&27

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-11
	0.402	0.303	0.239	0.174	0.099	0.062	0.032		
	0.439	0.318	0.252	0.180	0.103	0.061	0.032		
	0.427	0.308	0.256	0.187	0.102	0.059	0.038		

Samples:	microsomes		0.5 mg/mL BSA	
	0.169	0.063	0.195	0.336
	0.150	0.071	0.197	0.328
	0.158			

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.422	0.388	0.0362	m, b	0.094	0.000
0.00100	25	0.0250	0.310	0.276	0.0256	se_m, se_b	0.004	0.001
0.00075	25	0.0188	0.249	0.215	0.0199	r , se_y	0.993	0.001
0.00050	25	0.0125	0.180	0.146	0.0134	F, df	584	4
0.00025	25	0.0063	0.101	0.067	0.0060	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.060	0.026	0.0021			

Blank 0.034

r^2= 0.993
m= 0.094
b= 0.000

Regression results are calculated using the function LINEST

A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
0.169	0.135	0.012	25	190	5190	0.013	0.012	12.471
0.150	0.116	0.011	25	190	5190	0.012		
0.158	0.124	0.011	25	190	5190	0.012		
0.063	0.029	0.002	25	1	1	0.000	0.000	0.109
0.071	0.036	0.003	25	1	1	0.000		
0.195	0.160	0.015	25	1	1	0.001	0.001	0.594
0.197	0.162	0.015	25	1	1	0.001		
0.336	0.302	0.028	25	1	1	0.001	0.001	1.107
0.328	0.293	0.027	25	1	1	0.001		

Assay Date	2/28/2005	Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	2

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.3157895	dilution factor
Dilution B	2.5	mL microsome Dilution A used
	44	mL total volume
	17.6	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	480.757895	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	12.471
Protein Concentration (dilution added to assay, mg/mL):	0.02594

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	1.00E-08
4	6.50E-09
5	3.00E-09
6	1.00E-09
7	5.00E-10
8	1.00E-10

Assay Date:	2/26/2005	Test Chemical ID: RC 1	# Concentrations tested:	6	Microsome type:	Placental	Microsome ID:	MET-0255A 26K27	Technician ID:	JG	Replicate #:	2

Sample ID					Calculate DPM in aqueous portion after extraction				Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (nMol)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	nmol/mg protein/min
Full activity control	1	2	0.5	1	340.15	680.3	692.16	1384.32	0.1	151615	0.91	1215	0.0016	1	0.013	15	0.0041
			0.5	2	352.01	704.02			0.1					1		15	
	2	2	0.5	1	2115.99	4231.98	4261.25	8522.5	0.1	151615	5.62	8353	0.0110	1	0.013	15	0.0282
			0.5	2	2149.27	4298.54			0.1					1		15	
	3	2	0.5	1	3773.92	7547.84	7501.72	15003.44	0.1	151615	9.90	14834	0.0195	1	0.013	15	0.0501
			0.5	2	3727.8	7455.6			0.1					1		15	
	4	2	0.5	1	4336.98	8677.96	8700.88	17401.76	0.1	151615	11.48	17233	0.0226	1	0.013	15	0.0582
			0.5	2	4361.9	8723.8			0.1					1		15	
Background control	1	2	0.5	1	43.31	86.62	78.32	156.64	0.1	151615	0.10	-12	0.0000	1	0.013	15	0.0000
			0.5	2	35.01	70.02			0.1					1		15	
	2	2	0.5	1	41.39	82.78	82.42	164.84	0.1	151615	0.11	4	0.0000	1	0.013	15	0.0000
			0.5	2	41.03	82.06			0.1					1		15	
	3	2	0.5	1	45.84	91.68	87.81	175.62	0.1	151615	0.12	7	0.0000	1	0.013	15	0.0000
			0.5	2	41.97	83.94			0.1					1		15	
	4	2	0.5	1	43.87	87.74	89.68	179.36	0.1	151615	0.12	10	0.0000	1	0.013	15	0.0000
			0.5	2	45.81	91.62			0.1					1		15	
Positive control	1	2	0.5	1	2319.92	4639.84	4597.3	9394.6	0.1	151615	6.20	9225	0.0121	1	0.013	15	0.0311
			0.5	2	2377.38	4754.76			0.1					1		15	
	2	2	0.5	1	1821.15	3642.31	3685.35	7330.7	0.1	151615	4.84	7162	0.0094	1	0.013	15	0.0242
			0.5	2	1844.2	3688.4			0.1					1		15	
	3	2	0.5	1	2118.55	4237.1	4223.38	8446.76	0.1	151615	5.57	8278	0.0109	1	0.013	15	0.0279
			0.5	2	2104.83	4209.66			0.1					1		15	
	4	2	0.5	1	2168.76	4337.52	4321.83	8643.66	0.1	151615	5.70	8475	0.0111	1	0.013	15	0.0286
			0.5	2	2153.07	4306.14			0.1					1		15	
Negative Control	1	2	0.5	1	5417.36	10834.72	10890.59	21781.18	0.1	151615	14.37	21612	0.0284	1	0.013	15	0.0729
			0.5	2	5473.23	10946.46			0.1					1		15	
	2	2	0.5	1	5811.75	11623.5	11610.03	23220.06	0.1	151615	15.32	23051	0.0303	1	0.013	15	0.0778
			0.5	2	5798.26	11596.56			0.1					1		15	
	3	2	0.5	1	5267.78	10575.56	10625.5	21251	0.1	151615	14.02	21082	0.0277	1	0.013	15	0.0711
			0.5	2	5337.72	10675.44			0.1					1		15	
	4	2	0.5	1	5010.9	10021.8	9986.65	19973.3	0.1	151615	13.17	19804	0.0260	1	0.013	15	0.0668
			0.5	2	4975.75	9951.5			0.1					1		15	
RC 1	1-1	2	0.5	1	45.79	91.58	85.89	171.78	0.1	151615	0.11	3	0.0000	1	0.013	15	0.0000
			0.5	2	40.1	80.2			0.1					1		15	
	1-2	2	0.5	1	40.01	80.02	87.26	174.52	0.1	151615	0.12	5	0.0000	1	0.013	15	0.0000
			0.5	2	47.25	94.5			0.1					1		15	
	1-3	2	0.5	1	46.08	92.16	95.33	190.66	0.1	151615	0.13	22	0.0000	1	0.013	15	0.0001
			0.5	2	49.25	98.5			0.1					1		15	
	2-1	2	0.5	1	125.19	250.38	238.66	477.32	0.1	151615	0.31	308	0.0004	1	0.013	15	0.0010
			0.5	2	113.47	226.94			0.1					1		15	
	2-2	2	0.5	1	108.62	217.24	216.4	432.8	0.1	151615	0.29	264	0.0003	1	0.013	15	0.0009
			0.5	2	107.58	215.16			0.1					1		15	
	2-3	2	0.5	1	100.32	212.64	213.18	426.36	0.1	151615	0.26	257	0.0003	1	0.013	15	0.0009
			0.5	2	106.86	213.72			0.1					1		15	
	3-1	2	0.5	1	635.25	1270.5	1246.21	2492.42	0.1	151615	1.64	2320	0.0031	1	0.013	15	0.0076
			0.5	2	610.96	1221.92			0.1					1		15	
	3-2	2	0.5	1	649.43	1298.86	1269.73	2539.46	0.1	151615	1.67	2370	0.0031	1	0.013	15	0.0080
			0.5	2	620.3	1240.6			0.1					1		15	
	3-3	2	0.5	1	658.35	1316.7	1326.71	2653.42	0.1	151615	1.75	2484	0.0033	1	0.013	15	0.0084
			0.5	2	668.36	1336.72			0.1					1		15	
	4-1	2	0.5	1	931.41	1862.82	1869.97	3739.64	0.1	151615	2.47	3571	0.0047	1	0.013	15	0.0120
			0.5	2	938.56	1877.12			0.1					1		15	
	4-2	2	0.5	1	1040.06	2080.12	2044.3	4088.6	0.1	151615	2.70	3919	0.0051	1	0.013	15	0.0132
			0.5	2	1004.24	2008.48			0.1					1		15	
	4-3	2	0.5	1	951.1	1902.2	1866.49	3732.98	0.1	151615	2.46	3584	0.0047	1	0.013	15	0.0120
			0.5	2	915.39	1830.78			0.1					1		15	
	5-1	2	0.5	1	1732.34	3464.68	3474.83	6949.66	0.1	151615	4.58	6781	0.0089	1	0.013	15	0.0229
			0.5	2	1742.49	3484.98			0.1					1		15	
	5-2	2	0.5	1	1799.92	3599.84	3567.05	7134.1	0.1	151615	4.71	6985	0.0091	1	0.013	15	0.0235
			0.5	2	1767.13	3534.26			0.1					1		15	
	5-3	2	0.5	1	1034.79	3269.58	3319.4	6639.8	0.1	151615	4.38	6476	0.0085	1	0.013	15	0.0216
			0.5	2	1684.01	3369.22			0.1					1		15	
	6-1	2	0.5	1	3248.54	6497.08	6525.94	13051.88	0.1	151615	8.61	12883	0.0169	1	0.013	15	0.0435
			0.5	2	3277.4	6554.8			0.1					1		15	
	6-2	2	0.5	1	3215.02	6430.04	6439.42	12878.84	0.1	151615	6.49	12710	0.0167	1	0.013	15	0.0429
			0.5	2	3224.4	6448.8			0.1					1		15	
	6-3	2	0.5	1	3209.77	6419.54	6462.57	12925.14	0.1	151615	6.52	12756	0.0167	1	0.013	15	0.0430
			0.5	2	3252.8	6505.6			0.1					1		15	
	7-1	2	0.5	1	4309.53	8739.06	8708.41	17416.82	0.1	151615	11.49	17248	0.0226	1	0.013	15	0.0582
			0.5	2	4338.86	8677.76			0.1					1		15	
	7-2	2	0.5	1	4087.79	8175.58	8230.49	16460.98	0.1	151615	10.86	16292	0.0214	1	0.013	15	0.0550
			0.5	2	4142.7	8285.4			0.1					1		15	
	7-3	2	0.5	1	4168.12	8336.24	8316.89	16637.78	0.1	151615	10.97	16459	0.0216	1	0.013	15	0.0556
			0.5	2	4150.77	8301.54			0.1					1		15	
	8-1	2	0.5	1	5330.88	10661.76	10782.73	21565.46	0.1	151615	14.22	21396	0.0281	1	0.013	15	0.0722
			0.5	2	5451.85	10903.7			0.1					1		15	
	8-2	2	0.5	1	5251.55	10503.1	10451.3	20902.6	0.1	151615	13.79	20733	0.0272	1	0.013	15	0.0700
			0.5	2	5199.75	10399.5			0.1					1		15	
	8-3	2	0.5	1	5126.34	10252.68	10385.71	20777.42	0.1	151615	13.70	20608	0.0271	1	0.013	15	0.0695
			0.5	2	5262.37	10524.74			0.1					1		15	

Assay Date	2/28/2005	ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0161	0.0170
Full activity	End	0.0541	0.0057
Full activity	Overall	0.0351	0.0242
Background	Beginning	0.0000	1.95659E-05
Background	End	0.0000	8.92395E-06
Background	Overall	0.0000	3.4915E-05
Positive	Beginning	0.0276	0.0049
Positive	End	0.0283	0.0005
Positive	Overall	0.0280	0.0029
Negative	Beginning	0.0754	0.0034
Negative	End	0.0690	0.0030
Negative	Overall	0.0722	0.0045

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 4	1	1	1.00E-06	-6.00	0.0000
RC 4	1	2	1.00E-06	-6.00	0.0000
RC 4	1	3	1.00E-06	-6.00	0.0001
RC 4	2	1	1.00E-07	-7.00	0.0010
RC 4	2	2	1.00E-07	-7.00	0.0009
RC 4	2	3	1.00E-07	-7.00	0.0009
RC 4	3	1	1.00E-08	-8.00	0.0078
RC 4	3	2	1.00E-08	-8.00	0.0080
RC 4	3	3	1.00E-08	-8.00	0.0084
RC 4	4	1	6.50E-09	-8.19	0.0120
RC 4	4	2	6.50E-09	-8.19	0.0132
RC 4	4	3	6.50E-09	-8.19	0.0120
RC 4	5	1	3.00E-09	-8.52	0.0229
RC 4	5	2	3.00E-09	-8.52	0.0235
RC 4	5	3	3.00E-09	-8.52	0.0218
RC 4	6	1	1.00E-09	-9.00	0.0435
RC 4	6	2	1.00E-09	-9.00	0.0429
RC 4	6	3	1.00E-09	-9.00	0.0430
RC 4	7	1	5.00E-10	-9.30	0.0582
RC 4	7	2	5.00E-10	-9.30	0.0550
RC 4	7	3	5.00E-10	-9.30	0.0556
RC 4	8	1	1.00E-10	-10.00	0.0722
RC 4	8	2	1.00E-10	-10.00	0.0700
RC 4	8	3	1.00E-10	-10.00	0.0695

Percent of control values				
		Replicate		
Level	Log[test substance]	1	2	3
1	-6.00	0.03	0.05	0.21
2	-7.00	2.96	2.53	2.47
3	-8.00	22.32	22.77	23.87
4	-8.19	34.31	37.66	34.24
5	-8.52	65.14	66.91	62.16
6	-9.00	123.77	122.10	122.55
7	-9.30	165.70	156.52	158.22
8	-10.00	205.56	199.19	197.99

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0198	39232.87	1981458	
2	0.0196	40032.34	2042466	
3	0.0198	40758.95	2058533	
4	0.0200	41333.79	2066690	
5	0.0198	40502.45	2045578	
			Average DPM/g soln	2038945
			SD	33595
			CV	1.65
			µCi/g soln	0.918

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10	10		1000.00
Dilution A			100	10.00
Dilution B			10	1.00

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.124 g
Mass of dilution B used in substrate prep	4.5605 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.561361 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.01040 µg/g soln.
µg/g soln.

a. µCi/g soln	0.918
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.561361 + 0.01040
= 0.571758 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.606 µCi/µg ASDN

1021330 dpm/nmol

Assay Date	3/1/2005	Test Chemical ID	RC 4	tested	8				
ID	JG	Replicate #	3	Microsome type	Placental	Microsome ID	MET-0255A 28&29		

Standards:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Total volume of stock (mL)	Protein stock ID
0			#DIV/0!	25	#DIV/0!					
0			#DIV/0!	25	#DIV/0!					
0			#DIV/0!	25	#DIV/0!					
0			#DIV/0!	25	#DIV/0!					
0			#DIV/0!	25	#DIV/0!					
0			#DIV/0!	25	#DIV/0!					

Samples:

			Blank		r^2=					
					m=					
					b=					

			A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	Final vol. Diluted usomes prep. (µL)	mg protein/µL Prep.	average mg/µL	mg/mL

Variables: m, b; se_m, se_b; r, se_y; F, df; ss_{reg}, ss_{resid}

Regression results

Regression results are calculated using the function LINEST

Assay Date 3/1/2005 Test Chemical ID RC 4 tested 8

ID JG Replicate # 3 Microsome type Placental Microsome ID MET-0255A 28&29

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk		
	0.406	0.294	0.231	0.169	0.107	0.067	0.033	25	431007-3-12
	0.430	0.311	0.240	0.171	0.102	0.065	0.030		
	0.422	0.312	0.245	0.180	0.106	0.062	0.035		

Samples:	microsomes	0.125	0.5	1
	0.144	0.069	0.187	0.329
	0.144	0.069	0.191	0.335
	0.145			

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.419	0.387	0.0367	m, b	0.096	-0.001
0.00100	25	0.0250	0.306	0.273	0.0257	se_m, se_b	0.003	0.001
0.00075	25	0.0188	0.238	0.206	0.0192	r , se_y	0.997	0.001
0.00050	25	0.0125	0.173	0.141	0.0129	F, df	1426	4
0.00025	25	0.0063	0.105	0.073	0.0063	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.064	0.032	0.0024			

Blank 0.032

r^2= 0.997
m= 0.096
b= -0.001

Regression results are calculated using the function LINEST

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
	0.144	0.112	0.010	25	190	5190	0.011	0.011	11.045
	0.144	0.111	0.010	25	190	5190	0.011		
	0.145	0.113	0.010	25	190	5190	0.011		
0.125	0.069	0.037	0.003	25	1	1	0.000	0.000	0.115
0.125	0.069	0.037	0.003	25	1	1	0.000		
0.125									
0.5	0.187	0.155	0.014	25	1	1	0.001	0.001	0.578
0.5	0.191	0.159	0.015	25	1	1	0.001		
0.5									
1	0.329	0.297	0.028	25	1	1	0.001	0.001	1.130
1	0.335	0.303	0.029	25	1	1	0.001		
1									

Assay Date	3/1/2005	Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	3

Microsome Dilution Details

Dilution A	0.19	mL microsome Stock used
	5.19	mL total volume
	27.31579	dilution factor
Dilution B	2.8	mL microsome Dilution A used
	44	mL total volume
	15.71429	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	429.2481	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	11.045
Protein Concentration (dilution added to assay, mg/mL):	0.025731

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	1.00E-08
4	6.50E-09
5	3.00E-09
6	1.00E-09
7	5.00E-10
8	1.00E-10

Assay Date	3/1/2005	Test Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID	MET-0255A 28&29	Technician ID	JG	Replicate #	3

Sample ID		Calculate DPM in aqueous portion after extraction							Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estrogen formed/mg protein/min
Full activity control	1	2	0.5	1	2987.11	5834.22	5872.64	11745.28	0.1	203895	5.76	11605	0.0114	1	0.013	15	0.0204
			0.5	2	2905.73	5811.46			0.1					1		15	
	2	2	0.5	1	879.23	1752.46	1776.76	3553.56	0.1	203895	1.74	3414	0.0033	1	0.013	15	0.0087
			0.5	2	900.55	1801.1			0.1					1		15	
	3	2	0.5	1	5180.58	10361.16	10383.78	20767.56	0.1	203895	10.19	20628	0.0202	1	0.013	15	0.0523
			0.5	2	5203.2	10406.4			0.1					1		15	
	4	2	0.5	1	5005.16	10010.32	10038.65	20077.3	0.1	203895	9.85	19938	0.0195	1	0.013	15	0.0506
			0.5	2	5033.49	10066.98			0.1					1		15	
Background control	1	2	0.5	1	33.18	66.36	73.04	146.08	0.1	203895	0.07	7	0.0000	1	0.013	15	0.0000
			0.5	2	39.86	79.72			0.1					1		15	
	2	2	0.5	1	42.43	84.86	71.84	143.68	0.1	203895	0.07	4	0.0000	1	0.013	15	0.0000
			0.5	2	29.41	58.82			0.1					1		15	
	3	2	0.5	1	31.39	62.78	64.04	128.08	0.1	203895	0.06	-11	0.0000	1	0.013	15	0.0000
			0.5	2	32.65	65.3			0.1					1		15	
	4	2	0.5	1	37.32	74.64	69.74	139.48	0.1	203895	0.07	0	0.0000	1	0.013	15	0.0000
			0.5	2	32.42	64.84			0.1					1		15	
Positive control	1	2	0.5	1	2465.23	4930.46	4915.79	9831.58	0.1	203895	4.82	9092	0.0095	1	0.013	15	0.0246
			0.5	2	2450.56	4901.12			0.1					1		15	
	2	2	0.5	1	2553.55	5107.1	5137.99	10275.98	0.1	203895	5.04	10137	0.0099	1	0.013	15	0.0257
			0.5	2	2584.44	5168.88			0.1					1		15	
	3	2	0.5	1	2041.19	4082.38	4104.36	8208.72	0.1	203895	4.03	8089	0.0079	1	0.013	15	0.0205
			0.5	2	2063.17	4126.34			0.1					1		15	
	4	2	0.5	1	2193.27	4386.54	4408.61	8817.22	0.1	203895	4.32	8674	0.0085	1	0.013	15	0.0220
			0.5	2	2243.34	4486.68			0.1					1		15	
Negative Control	1	2	0.5	1	6057.16	12114.32	12205.27	24410.54	0.1	203895	11.97	24271	0.0238	1	0.013	15	0.0616
			0.5	2	6148.11	12296.22			0.1					1		15	
	2	2	0.5	1	5358.32	10716.64	10876.97	21753.94	0.1	203895	10.67	21614	0.0212	1	0.013	15	0.0548
			0.5	2	5518.35	11036.7			0.1					1		15	
	3	2	0.5	1	5835.81	11671.62	11694.37	23388.74	0.1	203895	11.47	23249	0.0228	1	0.013	15	0.0590
			0.5	2	5858.56	11717.12			0.1					1		15	
	4	2	0.5	1	5672.9	11345.8	11386.56	22773.12	0.1	203895	11.17	22634	0.0222	1	0.013	15	0.0574
			0.5	2	5713.66	11427.32			0.1					1		15	
RC 4	1-1	2	0.5	1	64.65	129.3	126.5	253	0.1	203895	0.12	114	0.0001	1	0.013	15	0.0003
			0.5	2	61.85	123.7			0.1					1		15	
	1-2	2	0.5	1	61.16	122.32	110.66	221.36	0.1	203895	0.11	92	0.0001	1	0.013	15	0.0002
			0.5	2	49.52	99.04			0.1					1		15	
	1-3	2	0.5	1	53.41	106.82	112.8	225.6	0.1	203895	0.11	86	0.0001	1	0.013	15	0.0002
			0.5	2	59.39	118.78			0.1					1		15	
	2-1	2	0.5	1	138.11	276.22	274.45	548.95	0.1	203895	0.27	410	0.0004	1	0.013	15	0.0010
			0.5	2	136.37	272.74			0.1					1		15	
	2-2	2	0.5	1	137.99	275.98	295.02	590.04	0.1	203895	0.29	451	0.0004	1	0.013	15	0.0011
			0.5	2	157.03	314.06			0.1					1		15	
	2-3	2	0.5	1	143.95	287.9	295.56	591.12	0.1	203895	0.29	452	0.0004	1	0.013	15	0.0011
			0.5	2	151.61	303.22			0.1					1		15	
	3-1	2	0.5	1	938.33	1876.66	1875.76	3751.52	0.1	203895	1.84	3612	0.0035	1	0.013	15	0.0092
			0.5	2	937.43	1874.86			0.1					1		15	
	3-2	2	0.5	1	954.04	1908.08	1973.7	3947.4	0.1	203895	1.94	3808	0.0037	1	0.013	15	0.0097
			0.5	2	1010.66	2039.32			0.1					1		15	
	3-3	2	0.5	1	960.21	1920.42	1958.45	3918.9	0.1	203895	1.92	3780	0.0037	1	0.013	15	0.0096
			0.5	2	996.24	1998.48			0.1					1		15	
	4-1	2	0.5	1	1372.12	2744.24	2753.65	5507.3	0.1	203895	2.70	5368	0.0053	1	0.013	15	0.0136
			0.5	2	1381.53	2763.06			0.1					1		15	
	4-2	2	0.5	1	1435.42	2870.84	2879.92	5759.84	0.1	203895	2.82	5621	0.0055	1	0.013	15	0.0143
			0.5	2	1444.5	2889			0.1					1		15	
	4-3	2	0.5	1	1383.67	2767.34	2740.82	5481.64	0.1	203895	2.69	5342	0.0052	1	0.013	15	0.0136
			0.5	2	1357.15	2714.3			0.1					1		15	
	5-1	2	0.5	1	2616.8	5233.6	5247.92	10495.84	0.1	203895	5.15	10357	0.0101	1	0.013	15	0.0263
			0.5	2	2628.12	5256.24			0.1					1		15	
	5-2	2	0.5	1	2765.9	5531.8	5487.52	10975.04	0.1	203895	5.36	10839	0.0106	1	0.013	15	0.0275
			0.5	2	2701.82	5403.24			0.1					1		15	
	5-3	2	0.5	1	2510.88	5021.76	5079.43	10158.86	0.1	203895	4.98	10020	0.0098	1	0.013	15	0.0254
			0.5	2	2568.55	5137.1			0.1					1		15	
	6-1	2	0.5	1	4285.67	8571.34	8523.01	17046.02	0.1	203895	8.36	16907	0.0166	1	0.013	15	0.0429
			0.5	2	4237.34	8474.68			0.1					1		15	
	6-2	2	0.5	1	4495.01	8990.02	8918.88	17833.76	0.1	203895	8.75	17694	0.0173	1	0.013	15	0.0449
			0.5	2	4421.87	8843.74			0.1					1		15	
	6-3	2	0.5	1	4056.3	8112.6	8277.4	16554.8	0.1	203895	8.12	16416	0.0161	1	0.013	15	0.0416
			0.5	2	4221.1	8442.2			0.1					1		15	
	7-1	2	0.5	1	5049.09	10098.18	10072.2	20144.4	0.1	203895	9.85	20005	0.0196	1	0.013	15	0.0507
			0.5	2	5023.11	10046.22			0.1					1		15	
	7-2	2	0.5	1	5457.03	10914.06	10624.08	21248.16	0.1	203895	10.42	21109	0.0207	1	0.013	15	0.0535
			0.5	2	5167.05	10334.1			0.1					1		15	
	7-3	2	0.5	1	5059.69	10119.38	10114.51	20229.02	0.1	203895	9.92	20090	0.0197	1	0.013	15	0.0510
			0.5	2	5054.82	10109.64			0.1					1		15	
	8-1	2	0.5	1	5837.16	11674.32	11563.22	23126.44	0.1	203895	11.36	23027	0.0225	1	0.013	15	0.0584
			0.5	2	5746.06	11492.12			0.1					1		15	
	8-2	2	0.5	1	6245.17	12490.34	12340.5	24681	0.1	203895	12.10	24542	0.0240	1	0.013	15	0.0623
			0.5	2	6095.33	12190.66			0.1					1		15	
	8-3	2	0.5	1	5664.23	11328.46	11499.24	22998.48	0.1	203895	11.28	22859	0.0224	1	0.013	15	0.0580
			0.5	2	5835.01	11670.02			0.1					1		15	

Assay Date	3/1/2005	ID	RC 4	# Concentrations tested	Microsome 8 type	Placental	Microsome ID	Technician ID	JG	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0191	0.0147
Full activity	End	0.0515	0.0012
Full activity	Overall	0.0353	0.0206
Background	Beginning	0.0000	4.30508E-06
Background	End	0.0000	2.04491E-05
Background	Overall	0.0000	2.02452E-05
Positive	Beginning	0.0252	0.0008
Positive	End	0.0212	0.0011
Positive	Overall	0.0232	0.0024
Negative	Beginning	0.0582	0.0048
Negative	End	0.0582	0.0011
Negative	Overall	0.0582	0.0028

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 4	1	1	1.00E-06	-6.00	0.0003
RC 4	1	2	1.00E-06	-6.00	0.0002
RC 4	1	3	1.00E-06	-6.00	0.0002
RC 4	2	1	1.00E-07	-7.00	0.0010
RC 4	2	2	1.00E-07	-7.00	0.0011
RC 4	2	3	1.00E-07	-7.00	0.0011
RC 4	3	1	1.00E-08	-8.00	0.0092
RC 4	3	2	1.00E-08	-8.00	0.0097
RC 4	3	3	1.00E-08	-8.00	0.0096
RC 4	4	1	6.50E-09	-8.19	0.0136
RC 4	4	2	6.50E-09	-8.19	0.0143
RC 4	4	3	6.50E-09	-8.19	0.0136
RC 4	5	1	3.00E-09	-8.52	0.0263
RC 4	5	2	3.00E-09	-8.52	0.0275
RC 4	5	3	3.00E-09	-8.52	0.0254
RC 4	6	1	1.00E-09	-9.00	0.0429
RC 4	6	2	1.00E-09	-9.00	0.0449
RC 4	6	3	1.00E-09	-9.00	0.0416
RC 4	7	1	5.00E-10	-9.30	0.0507
RC 4	7	2	5.00E-10	-9.30	0.0535
RC 4	7	3	5.00E-10	-9.30	0.0510
RC 4	8	1	1.00E-10	-10.00	0.0584
RC 4	8	2	1.00E-10	-10.00	0.0623
RC 4	8	3	1.00E-10	-10.00	0.0580

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-6.00	0.82	0.59	0.62
2	-7.00	2.95	3.24	3.25
3	-8.00	25.99	27.40	27.20
4	-8.19	38.63	40.44	38.44
5	-8.52	74.52	77.97	72.10
6	-9.00	121.66	127.33	118.12
7	-9.30	143.96	151.90	144.56
8	-10.00	165.70	176.60	164.49

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0195	28385.97	1455691	
2	0.0197	29349.43	1489819	
3	0.0198	29662.54	1498108	
4	0.0201	29979.97	1491541	
5	0.0201	30077.75	1496405	
			Average DPM/g soln	1486313
			SD	17452
			CV	1.17
			µCi/g soln	0.670

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.1	10		1010.00
Dilution A			100	10.10
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1741 g
Mass of dilution B used in substrate prep	4.5927 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.567479 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00758 µg/g soln.
µg/g soln.

a. µCi/g soln	0.670
b. Specific activity of [H]ASDN (µCi/mmol)	25300000
c. Molecular wt of ASDN (mg/mmol)	286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.567479 + 0.00758
= 0.575058 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.164 µCi/µg ASDN

740239 dpm/nmol

Assay Date	3/18/2005	Test Chemical ID	RC 4	tested	8		
ID	JG	Replicate #	4	Microsome type	Placental	Microsome ID	MET-0255A #32

Standards:

concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results
0			#DIV/0!	25	#DIV/0!				m, b	
0			#DIV/0!	25	#DIV/0!				se_m, se_b	
0			#DIV/0!	25	#DIV/0!				r , se_y	
0			#DIV/0!	25	#DIV/0!				F, df	
0			#DIV/0!	25	#DIV/0!				ss_{reg}, ss_{resid}	
0			#DIV/0!	25	#DIV/0!					

Blank

r^2=
m=
b=

Regression results are calculated using the function LINEST

Samples:

			A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)		mg protein/µL Prep.	average mg/µL	mg/mL

Assay Date	3/18/2005	Test Chemical ID	RC 4		tested		8		
ID	JG	Replicate #		4	Microsome type		Placental	Microsome ID	MET-0255A #32

								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-14
	0.383	0.279	0.216	0.166	0.109	0.057	0.034		
	0.408	0.303	0.232	0.164	0.109	0.061	0.033		
	0.398	0.301	0.229	0.172	0.110	0.062	0.034		

Samples:	microsomes	0.125	0.5	1
	0.079	0.069	0.182	0.314
	0.076	0.071	0.186	0.326
	0.080			

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.397	0.363	0.0365	m, b	0.103	-0.001
0.00100	25	0.0250	0.294	0.260	0.0260	se_m, se_b	0.004	0.001
0.00075	25	0.0188	0.226	0.192	0.0190	r , se_y	0.995	0.001
0.00050	25	0.0125	0.167	0.133	0.0129	F, df	733	4
0.00025	25	0.0063	0.109	0.075	0.0070	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.060	0.026	0.0019			

Regression results are calculated using the function LINEST

Blank	0.034	r^2=	0.995	
		m=	0.103	
		b=	-0.001	

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
	0.079	0.045	0.004	25	95	5095	0.008	0.008	8.174
	0.076	0.043	0.004	25	95	5095	0.008		
	0.080	0.046	0.004	25	95	5095	0.008		
0.125	0.069	0.036	0.003	25	1	1	0.000	0.000	0.118
0.125	0.071	0.037	0.003	25	1	1	0.000		
0.125									
0.5	0.182	0.148	0.014	25	1	1	0.001	0.001	0.586
0.5	0.186	0.152	0.015	25	1	1	0.001		
0.5									
1	0.314	0.280	0.028	25	1	1	0.001	0.001	1.144
1	0.326	0.292	0.029	25	1	1	0.001		
1									

Assay Date	3/18/2005	Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	4

Microsome Dilution Details

Dilution A	0.095	mL microsome Stock used
	5.095	mL total volume
	53.63158	dilution factor
Dilution B	5	mL microsome Dilution A used
	41	mL total volume
	8.2	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	439.7789	total dilution factor

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	1.00E-08
4	6.50E-09
5	3.00E-09
6	1.00E-09
7	5.00E-10
8	1.00E-10

Protein Concentration (stock microsomes, mg/mL):	8.174
Protein Concentration (dilution added to assay, mg/mL):	0.018587

Assay Date	3/16/2005	Test Chemical ID	RC 4	# Concentrations tested		# Microsome type	Placental	Microsome ID	MET-0255A #32	Technician ID	JG	Replicate #	4

Sample ID					Calculate DPM in aqueous portion after extraction				Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal Initial volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	Incubation time (min)	estrogen formed(mg protein/min
Full activity control	1	2	0.5	1	26.11	52.22	49.86	99.78	0.1	148631	0.07	25	0.0000	1	0.009	15	0.0001
			0.5	2	23.77	47.54			0.1					1		15	
	2	2	0.5	1	55.60	111.2	95.8	191.6	0.1	148631	0.13	116	0.0002	1	0.009	15	0.0006
			0.5	2	40.20	80.4			0.1					1		15	
	3	2	0.5	1	248.91	497.82	503.46	1006.92	0.1	148631	0.68	932	0.0013	1	0.009	15	0.0045
			0.5	2	254.55	509.1			0.1					1		15	
	4	2	0.5	1	442.70	885.4	875.09	1750.18	0.1	148631	1.18	1875	0.0023	1	0.009	15	0.0061
			0.5	2	432.39	864.78			0.1					1		15	
Background control	1	2	0.5	1	18.10	36.2	44.41	88.82	0.1	148631	0.06	14	0.0000	1	0.009	15	0.0001
			0.5	2	26.31	52.62			0.1					1		15	
	2	2	0.5	1	3.34	6.68	24.64	49.28	0.1	148631	0.03	-26	0.0000	1	0.009	15	-0.0001
			0.5	2	21.30	42.6			0.1					1		15	
	3	2	0.5	1	19.82	39.64	34.99	69.98	0.1	148631	0.05	-5	0.0000	1	0.009	15	0.0000
			0.5	2	15.17	30.34			0.1					1		15	
	4	2	0.5	1	28.48	56.96	46.39	92.78	0.1	148631	0.06	18	0.0000	1	0.009	15	0.0001
			0.5	2	17.91	35.82			0.1					1		15	
Positive control	1	2	0.5	1	183.99	367.98	354.15	708.3	0.1	148631	0.48	633	0.0009	1	0.009	15	0.0031
			0.5	2	170.16	340.32			0.1					1		15	
	2	2	0.5	1	169.11	338.22	338.02	676.04	0.1	148631	0.45	601	0.0008	1	0.009	15	0.0029
			0.5	2	168.91	337.82			0.1					1		15	
	3	2	0.5	1	239.21	478.42	483.58	967.16	0.1	148631	0.65	892	0.0012	1	0.009	15	0.0043
			0.5	2	244.37	488.74			0.1					1		15	
	4	2	0.5	1	140.95	281.9	279.14	558.28	0.1	148631	0.38	483	0.0007	1	0.009	15	0.0023
			0.5	2	136.19	272.38			0.1					1		15	
Negative Control	1	2	0.5	1	304.22	608.44	617.39	1234.78	0.1	148631	0.83	1180	0.0016	1	0.009	15	0.0056
			0.5	2	313.17	626.34			0.1					1		15	
	2	2	0.5	1	360.91	721.82	715.19	1430.38	0.1	148631	0.99	1355	0.0018	1	0.009	15	0.0066
			0.5	2	354.28	708.56			0.1					1		15	
	3	2	0.5	1	438.80	877.6	878.45	1756.9	0.1	148631	1.18	1682	0.0023	1	0.009	15	0.0081
			0.5	2	439.65	879.3			0.1					1		15	
	4	2	0.5	1	132.39	264.78	243.82	487.64	0.1	148631	0.33	412	0.0006	1	0.009	15	0.0020
			0.5	2	111.43	222.86			0.1					1		15	
RC 4	1-1	2	0.5	1	21.62	43.24	39.43	78.86	0.1	148631	0.05	4	0.0000	1	0.009	15	0.0000
			0.5	2	17.81	35.62			0.1					1		15	
	1-2	2	0.5	1	19.93	39.86	41.9	83.8	0.1	148631	0.06	9	0.0000	1	0.009	15	0.0000
			0.5	2	21.97	43.94			0.1					1		15	
	1-3	2	0.5	1	24.39	48.78	43	86	0.1	148631	0.06	11	0.0000	1	0.009	15	0.0001
			0.5	2	18.61	37.22			0.1					1		15	
	2-1	2	0.5	1	46.39	92.78	91.3	182.6	0.1	148631	0.12	107	0.0001	1	0.009	15	0.0005
			0.5	2	44.91	89.82			0.1					1		15	
	2-2	2	0.5	1	52.33	104.66	101.67	203.34	0.1	148631	0.14	128	0.0002	1	0.009	15	0.0006
			0.5	2	49.34	98.68			0.1					1		15	
	2-3	2	0.5	1	40.13	80.26	73.88	147.76	0.1	148631	0.10	73	0.0001	1	0.009	15	0.0004
			0.5	2	33.75	67.5			0.1					1		15	
	3-1	2	0.5	1	202.79	405.4	399.59	799.18	0.1	148631	0.54	724	0.0010	1	0.009	15	0.0035
			0.5	2	196.86	393.78			0.1					1		15	
	3-2	2	0.5	1	188.83	377.66	389.84	779.68	0.1	148631	0.52	704	0.0010	1	0.009	15	0.0034
			0.5	2	201.01	402.02			0.1					1		15	
	3-3	2	0.5	1	197.23	394.48	393.13	786.26	0.1	148631	0.53	731	0.0010	1	0.009	15	0.0034
			0.5	2	195.90	391.8			0.1					1		15	
	4-1	2	0.5	1	282.39	564.78	575.69	1151.38	0.1	148631	0.77	1076	0.0015	1	0.009	15	0.0052
			0.5	2	293.30	586.6			0.1					1		15	
	4-2	2	0.5	1	296.90	593.8	590.18	1180.36	0.1	148631	0.79	1105	0.0015	1	0.009	15	0.0054
			0.5	2	293.28	586.56			0.1					1		15	
	4-3	2	0.5	1	282.42	564.84	572.11	1144.22	0.1	148631	0.77	1069	0.0014	1	0.009	15	0.0052
			0.5	2	289.68	579.36			0.1					1		15	
	5-1	2	0.5	1	427.72	855.44	855.52	1711.04	0.1	148631	1.15	1630	0.0022	1	0.009	15	0.0079
			0.5	2	427.80	855.6			0.1					1		15	
	5-2	2	0.5	1	441.01	882.02	905.19	1810.38	0.1	148631	1.22	1735	0.0023	1	0.009	15	0.0084
			0.5	2	464.18	928.35			0.1					1		15	
	5-3	2	0.5	1	370.24	740.48	770.99	1541.98	0.1	148631	1.04	1467	0.0020	1	0.009	15	0.0071
			0.5	2	400.75	801.5			0.1					1		15	
	6-1	2	0.5	1	653.68	1307.36	1304.62	2609.24	0.1	148631	1.76	2534	0.0034	1	0.009	15	0.0123
			0.5	2	650.94	1301.88			0.1					1		15	
	6-2	2	0.5	1	586.45	1172.9	1182.69	2365.78	0.1	148631	1.59	2291	0.0031	1	0.009	15	0.0111
			0.5	2	596.44	1192.88			0.1					1		15	
	6-3	2	0.5	1	654.50	1309	1305.07	2610.14	0.1	148631	1.76	2541	0.0034	1	0.009	15	0.0123
			0.5	2	653.57	1307.14			0.1					1		15	
	7-1	2	0.5	1	552.03	1104.06	1137.85	2275.7	0.1	148631	1.53	2200	0.0030	1	0.009	15	0.0107
			0.5	2	583.82	1167.64			0.1					1		15	
	7-2	2	0.5	1	912.74	1825.48	1818.73	3637.46	0.1	148631	2.45	3502	0.0046	1	0.009	15	0.0171
			0.5	2	905.99	1811.98			0.1					1		15	
	7-3	2	0.5	1	664.30	1328.6	1360.29	2720.58	0.1	148631	1.83	2645	0.0036	1	0.009	15	0.0128
			0.5	2	695.99	1391.98			0.1					1		15	
	8-1	3	0.5	1	866.30	1732.6	1715.5	3431	0.1	148631	2.31	3356	0.0045	1	0.009	15	0.0163
			0.5	2	849.20	1698.4			0.1					1		15	
	8-2	2	0.5	1	808.82	1617.64	1605.45	3210.9	0.1	148631	2.16	3136	0.0042	1	0.009	15	0.0152
			0.5	2	796.63	1593.26			0.1					1		15	
	8-3	2	0.5	1	821.33	1642.66	1676.52	3353.04	0.1	148631	2.26	3278	0.0044	1	0.009	15	0.0159
			0.5	2	855.19	1710.38			0.1					1		15	

Assay Date	3/18/2005	ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	4

Control Type	Portion	Average	SD
Full activity	Beginning	0.0003	0.0003
Full activity	End	0.0063	0.0025
Full activity	Overall	0.0033	0.0038
Background	Beginning	0.0000	0.000135475
Background	End	0.0000	7.8119E-05
Background	Overall	0.0000	9.6653E-05
Positive	Beginning	0.0030	0.0001
Positive	End	0.0033	0.0014
Positive	Overall	0.0032	0.0008
Negative	Beginning	0.0061	0.0007
Negative	End	0.0051	0.0043
Negative	Overall	0.0056	0.0026

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 4	1	1	1.00E-06	-6.00	0.0000
RC 4	1	2	1.00E-06	-6.00	0.0000
RC 4	1	3	1.00E-06	-6.00	0.0001
RC 4	2	1	1.00E-07	-7.00	0.0005
RC 4	2	2	1.00E-07	-7.00	0.0006
RC 4	2	3	1.00E-07	-7.00	0.0004
RC 4	3	1	1.00E-08	-8.00	0.0035
RC 4	3	2	1.00E-08	-8.00	0.0034
RC 4	3	3	1.00E-08	-8.00	0.0034
RC 4	4	1	6.50E-09	-8.19	0.0052
RC 4	4	2	6.50E-09	-8.19	0.0054
RC 4	4	3	6.50E-09	-8.19	0.0052
RC 4	5	1	3.00E-09	-8.52	0.0079
RC 4	5	2	3.00E-09	-8.52	0.0084
RC 4	5	3	3.00E-09	-8.52	0.0071
RC 4	6	1	1.00E-09	-9.00	0.0123
RC 4	6	2	1.00E-09	-9.00	0.0111
RC 4	6	3	1.00E-09	-9.00	0.0123
RC 4	7	1	5.00E-10	-9.30	0.0107
RC 4	7	2	5.00E-10	-9.30	0.0173
RC 4	7	3	5.00E-10	-9.30	0.0128
RC 4	8	1	1.00E-10	-10.00	0.0163
RC 4	8	2	1.00E-10	-10.00	0.0152
RC 4	8	3	1.00E-10	-10.00	0.0159

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-6.00	0.53	1.25	1.57
2	-7.00	15.63	18.65	10.56
3	-8.00	105.40	102.56	103.52
4	-8.19	156.67	160.89	155.63
5	-8.52	238.15	252.61	213.53
6	-9.00	368.91	333.46	369.91
7	-9.30	320.35	518.60	385.12
8	-10.00	488.54	456.50	477.19

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.	
1	0.0199	34322.82	1724765	
2	0.0195	35313.26	1810936	
3	0.0195	35779.2	1834831	
4	0.0197	36715.46	1863729	
5	0.0199	36259.46	1822083	
			Average DPM/g soln	1811269
			SD	52219
			CV	2.88
			µCi/g soln	0.816

Calculation of actual concentration of nonradiolabeled ASDN in solution used to prepare substrate solution:

ASDN solution	mg ASDN added	(mL)	dilution factor	[ASDN] in solution (µg/mL)
Stock	10.1	10		1010.00
Dilution A			100	10.10
Dilution B			10	1.01

Calculation of concentration nonradiolabeled ASDN in substrate solution

Total g substrate solution	8.1715 g
Mass of dilution B used in substrate prep	4.5901 g
Concentration of nonradiolabeled ASDN in substrate soln.	0.567338 µg/g

Calculation of Substrate Solution Specific Activity

1) Calculate µg [^{3}H]ASDN/g soln. = 0.00924 µg/g soln.
µg/g soln.

a. µCi/g soln 0.816
b. Specific activity of [H]ASDN (µCi/mmol) 25300000
c. Molecular wt of ASDN (mg/mmol) 286.4

Formula=a/b*c

2) Calculate total µg ASDN/g soln.

µg ASDN/g soln.= µg cold ASDN/g soln. + µg [^{3}H]ASDN/g soln.

= 0.567338 + 0.00924
= 0.576574 µg ASDN/g soln.

3) Calculate Solution Specific Activity

= (µCi/g soln.)/(µg ASDN/g soln.)
= 1.415 µCi/µg ASDN

899707 dpm/nmol

Assay Date	4/1/2005	Test Chemical ID	RC 4	tested	8		
ID	JG	Replicate #	5	Microsome type	Placental	Microsome ID	MET-0275A #4

Standards:

												BSA)	Total volume of stock (mL)	Protein stock ID
concentration (mg/mL)	Volume of stock used	Std	mg Protein per µL		µL Standard Used		mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results		
0			#DIV/0!		25		#DIV/0!				m, b			
0			#DIV/0!		25		#DIV/0!				se_m, se_b			
0			#DIV/0!		25		#DIV/0!				r , se_y			
0			#DIV/0!		25		#DIV/0!				F, df			
0			#DIV/0!		25		#DIV/0!				ss_{reg}, ss_{resid}			
0			#DIV/0!		25		#DIV/0!							
				Blank			r^2=					Regression results are calculated using the function LINEST		
							m=							
							b=							

Samples:

									Final vol.				
			A_{raw}	A_{adj}	mg protein measured	µL diluted µSOMES	prep. (µL)	Diluted usomes (µL)		mg protein/µL Prep.	average mg/µL	mg/mL	

Assay Date	4/1/2005	Test Chemical ID	RC 4		tested		8		
ID	JG	Replicate #		5	Microsome type	Placental	Microsome ID	MET-0275A #4	
								Protein stock (mg/10 mL)	Protein stock ID
Standards:	1.5	1	0.75	0.5	0.25	0.13	Blk	25	431007-3-16
	0.393	0.292	0.223	0.170	0.103	0.071	0.042		
	0.415	0.300	0.237	0.171	0.098	0.064	0.036		
	0.410	0.302	0.225	0.177	0.095	0.069	0.034		
Samples:	microsomes	0.125	0.5	1					
	0.137	0.069	0.185	0.306					
	0.122	0.070	0.185	0.318					
	0.139								

mg Protein per µL	µL Standard Used	mg Protein Measured	A_{raw}	A_{adj}	Curve Output	Variables	Regression results	
0.00150	25	0.0375	0.406	0.369	0.0367	m, b	0.100	0.000
0.00100	25	0.0250	0.298	0.261	0.0259	se_m, se_b	0.003	0.001
0.00075	25	0.0188	0.228	0.191	0.0188	r , se_y	0.997	0.001
0.00050	25	0.0125	0.173	0.135	0.0133	F, df	1374	4
0.00025	25	0.0063	0.099	0.061	0.0059	ss_{reg}, ss_{resid}	0.001	0.000
0.00013	25	0.0033	0.068	0.030	0.0028			
						Regression results are calculated using the function LINEST		
Blank	0.037	r^2=	0.997					
		m=	0.100					
		b=	0.000					

	A_{raw}	$A_{adj.}$	mg protein measured	µL diluted µSOMES	prep. (µL)	Final vol. Diluted usomes (µL)	mg protein/µL Prep.	average mg/µL	mg/mL
	0.137	0.099	0.010	25	140	5140	0.014	0.014	13.559
	0.122	0.084	0.008	25	140	5140	0.012		
	0.139	0.102	0.010	25	140	5140	0.015		
0.125	0.069	0.032	0.003	25	1	1	0.000	0.000	0.117
0.125	0.070	0.033	0.003	25	1	1	0.000		
0.125									
0.5	0.185	0.148	0.015	25	1	1	0.001	0.001	0.580
0.5	0.185	0.147	0.014	25	1	1	0.001		
0.5									
1	0.306	0.269	0.027	25	1	1	0.001	0.001	1.090
1	0.318	0.281	0.028	25	1	1	0.001		
1									
mg/mL BSA									
mg/mL BSA									
mg/mL BSA									

Assay Date	4/1/2005	Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	5

Microsome Dilution Details

Dilution A	0.14	mL microsome Stock used
	5.14	mL total volume
	36.71429	dilution factor
Dilution B	3.1	mL microsome Dilution A used
	45	mL total volume
	14.51613	dilution factor
Dilution C (if applicable)		mL microsome Dilution B used
		mL total volume
	NA	dilution factor
	532.9493	total dilution factor

Protein Concentration (stock microsomes, mg/mL):	13.559
Protein Concentration (dilution added to assay, mg/mL):	0.025441

Test Chemical Concentrations

Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	1.00E-08
4	6.50E-09
5	3.00E-09
6	1.00E-09
7	5.00E-10
8	1.00E-10

Assay Date	3/1/2005	Test Chemical ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID	MET-0275A #4	Technician ID	JG	Replicate #	5

Sample = 0			Calculate DPM in aqueous portion after extraction:						Calculate % turnover			Calculate nmol H_2O formed					
Sample type	Replicate/Level	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/mL	Total DPM	Volume of substrate (mL)	total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Background Tubes)	nmol ^3H_2O formed	microsomes used in assay tube (mL)	assay (mg/mL)	incubation time (min)	cytosol formed/mg protein/min
Full activity control	1	2	0.5	1	159.21	318.42	303.69	607.38	0.1	181127	0.34	390	0.0004	1	0.013	15	0.0011
			0.5	2	144.66	289.32			0.1					1		15	
	2	2	0.5	1	473.1	946.2	967.53	1934.86	0.1	181127	1.07	1717	0.0019	1	0.013	15	0.0050
			0.5	2	494.23	988.46			0.1					1		15	
	3	2	0.5	1	2475.03	4950.06	5001.25	10002.5	0.1	181127	5.52	9785	0.0109	1	0.013	15	0.0285
			0.5	2	2526.22	5052.44			0.1					1		15	
	4	2	0.5	1	2173.27	4346.54	4349.86	8699.72	0.1	181127	4.80	8482	0.0094	1	0.013	15	0.0247
			0.5	2	2176.59	4353.18			0.1					1		15	
Background control	1	2	0.5	1	56.06	112.12	113.85	227.7	0.1	181127	0.13	10	0.0000	1	0.013	15	0.0000
			0.5	2	55.79	111.58			0.1					1		15	
	2	2	0.5	1	48.77	97.54	101.74	203.48	0.1	181127	0.11	-14	0.0000	1	0.013	15	0.0000
			0.5	2	52.97	105.94			0.1					1		15	
	3	2	0.5	1	58.08	116.16	105.95	211.9	0.1	181127	0.12	-5	0.0000	1	0.013	15	0.0000
			0.5	2	47.87	95.74			0.1					1		15	
	4	2	0.5	1	62.51	125.02	113.22	226.44	0.1	181127	0.13	9	0.0000	1	0.013	15	0.0000
			0.5	2	50.71	101.42			0.1					1		15	
Positive control	1	2	0.5	1	1437.75	2875.5	2871.47	5742.94	0.1	181127	3.17	5526	0.0061	1	0.013	15	0.0161
			0.5	2	1433.72	2867.44			0.1					1		15	
	2	2	0.5	1	1587.12	3174.24	3193.7	6387.4	0.1	181127	3.53	6170	0.0069	1	0.013	15	0.0180
			0.5	2	1606.56	3213.16			0.1					1		15	
	3	2	0.5	1	1858.76	3717.52	3718.05	7436.1	0.1	181127	4.11	7219	0.0080	1	0.013	15	0.0210
			0.5	2	1859.29	3718.58			0.1					1		15	
	4	2	0.5	1	2094.97	4189.94	4256.79	8513.58	0.1	181127	4.70	8296	0.0092	1	0.013	15	0.0242
			0.5	2	2161.82	4323.64			0.1					1		15	
Negative Control	1	2	0.5	1	5170.86	10341.72	10321.63	20643.26	0.1	181127	11.40	20426	0.0227	1	0.013	15	0.0595
			0.5	2	5160.77	10301.54			0.1					1		15	
	2	2	0.5	1	5559.75	11119.5	11120.72	22241.44	0.1	181127	12.28	22024	0.0245	1	0.013	15	0.0641
			0.5	2	5560.97	11121.94			0.1					1		15	
	3	2	0.5	1	5233.07	10466.14	10467.33	20934.66	0.1	181127	11.56	20717	0.0230	1	0.013	15	0.0603
			0.5	2	5234.26	10468.52			0.1					1		15	
	4	2	0.5	1	5568.33	11136.66	11038.11	22076.22	0.1	181127	12.19	21859	0.0243	1	0.013	15	0.0637
			0.5	2	5469.79	10927.58			0.1					1		15	
RC 4	1-1	2	0.5	1	133.63	267.26	247.76	495.52	0.1	181127	0.27	275	0.0003	1	0.013	15	0.0008
			0.5	2	114.13	228.26			0.1					1		15	
	1-2	2	0.5	1	54.04	108.08	115.34	230.68	0.1	181127	0.13	13	0.0000	1	0.013	15	0.0000
			0.5	2	61.3	122.6			0.1					1		15	
	1-3	2	0.5	1	63.03	126.06	123.57	247.14	0.1	181127	0.14	30	0.0000	1	0.013	15	0.0001
			0.5	2	60.54	121.08			0.1					1		15	
	2-1	2	0.5	1	185.77	371.54	342.66	685.32	0.1	181127	0.38	466	0.0005	1	0.013	15	0.0014
			0.5	2	156.89	313.78			0.1					1		15	
	2-2	2	0.5	1	131.73	263.46	262.31	524.62	0.1	181127	0.29	307	0.0003	1	0.013	15	0.0009
			0.5	2	130.58	261.16			0.1					1		15	
	2-3	2	0.5	1	138.4	276.8	280.18	560.36	0.1	181127	0.31	343	0.0004	1	0.013	15	0.0010
			0.5	2	141.78	283.56			0.1					1		15	
	3-1	2	0.5	1	829.4	1658.8	1649.56	3299.12	0.1	181127	1.82	3082	0.0034	1	0.013	15	0.0090
			0.5	2	820.16	1640.32			0.1					1		15	
	3-2	2	0.5	1	776.63	1553.26	1591.47	3182.94	0.1	181127	1.76	2966	0.0033	1	0.013	15	0.0086
			0.5	2	814.84	1629.68			0.1					1		15	
	3-3	2	0.5	1	806.24	1612.48	1630.04	3260.08	0.1	181127	1.80	3043	0.0034	1	0.013	15	0.0089
			0.5	2	823.8	1647.6			0.1					1		15	
	4-1	2	0.5	1	1166.96	2333.92	2311.87	4623.74	0.1	181127	2.55	4406	0.0049	1	0.013	15	0.0126
			0.5	2	1144.91	2289.82			0.1					1		15	
	4-2	2	0.5	1	1138.55	2277.1	2280.74	4561.48	0.1	181127	2.52	4344	0.0048	1	0.013	15	0.0127
			0.5	2	1142.19	2284.38			0.1					1		15	
	4-3	2	0.5	1	1146.25	2292.5	2276.33	4552.66	0.1	181127	2.51	4335	0.0048	1	0.013	15	0.0126
			0.5	2	1130.08	2260.16			0.1					1		15	
	5-1	2	0.5	1	1797.3	3594.6	3626.84	7253.68	0.1	181127	4.00	7036	0.0075	1	0.013	15	0.0205
			0.5	2	1829.54	3659.08			0.1					1		15	
	5-2	2	0.5	1	1951.96	3903.92	3864.85	7729.3	0.1	181127	4.27	7512	0.0083	1	0.013	15	0.0219
			0.5	2	1912.89	3825.34			0.1					1		15	
	5-3	2	0.5	1	1846.46	3692.92	3686.26	7352.58	0.1	181127	4.05	7115	0.0079	1	0.013	15	0.0207
			0.5	2	1819.8	3639.6			0.1					1		15	
	6-1	2	0.5	1	3324.46	6648.92	6650.54	13301.08	0.1	181127	7.34	13084	0.0145	1	0.013	15	0.0381
			0.5	2	3326.08	6652.16			0.1					1		15	
	6-2	2	0.5	1	3190.54	6381.08	6384.34	12768.68	0.1	181127	7.05	12551	0.0140	1	0.013	15	0.0365
			0.5	2	3187.8	6375.6			0.1					1		15	
	6-3	2	0.5	1	3497.46	6994.92	7050.77	14101.54	0.1	181127	7.79	13864	0.0154	1	0.013	15	0.0404
			0.5	2	3553.31	7106.62			0.1					1		15	
	7-1	2	0.5	1	3596.26	7192.52	7254.71	14509.42	0.1	181127	8.01	14292	0.0159	1	0.013	15	0.0416
			0.5	2	3658.45	7316.9			0.1					1		15	
	7-2	2	0.5	1	3867.12	7734.24	7715.14	15430.28	0.1	181127	8.52	15213	0.0169	1	0.013	15	0.0443
			0.5	2	3848.02	7696.04			0.1					1		15	
	7-3	2	0.5	1	4233.46	8466.92	8498.04	16999.08	0.1	181127	9.38	16779	0.0186	1	0.013	15	0.0489
			0.5	2	4264.58	8529.16			0.1					1		15	
	8-1	2	0.5	1	5260.5	10521	10499.41	20998.82	0.1	181127	11.59	20781	0.0231	1	0.013	15	0.0605
			0.5	2	5238.91	10477.82			0.1					1		15	
	8-2	2	0.5	1	5492.22	10984.44	10970.12	21940.24	0.1	181127	12.11	21723	0.0241	1	0.013	15	0.0633
			0.5	2	5477.8	10955.6			0.1					1		15	
	8-3	2	0.5	1	5243.82	10487.64	10515.9	21031.8	0.1	181127	11.61	20814	0.0231	1	0.013	15	0.0606
			0.5	2	5272.08	10544.16			0.1					1		15	

Assay Date	4/1/2005	ID	RC 4	# Concentrations tested	8	Microsome type	Placental	Microsome ID		Technician ID	JG	Replicate #	5

Control Type	Portion	Average	SD
Full activity	Beginning	0.0031	0.0027
Full activity	End	0.0266	0.0027
Full activity	Overall	0.0148	0.0138
Background	Beginning	0.0000	4.98798E-05
Background	End	0.0000	2.99444E-05
Background	Overall	0.0000	3.41242E-05
Positive	Beginning	0.0170	0.0013
Positive	End	0.0226	0.0022
Positive	Overall	0.0198	0.0035
Negative	Beginning	0.0618	0.0033
Negative	End	0.0620	0.0024
Negative	Overall	0.0619	0.0023

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
RC 4	1	1	1.00E-06	-6.00	0.0008
RC 4	1	2	1.00E-06	-6.00	0.0000
RC 4	1	3	1.00E-06	-6.00	0.0001
RC 4	2	1	1.00E-07	-7.00	0.0014
RC 4	2	2	1.00E-07	-7.00	0.0009
RC 4	2	3	1.00E-07	-7.00	0.0010
RC 4	3	1	1.00E-08	-8.00	0.0090
RC 4	3	2	1.00E-08	-8.00	0.0086
RC 4	3	3	1.00E-08	-8.00	0.0089
RC 4	4	1	6.50E-09	-8.19	0.0128
RC 4	4	2	6.50E-09	-8.19	0.0127
RC 4	4	3	6.50E-09	-8.19	0.0126
RC 4	5	1	3.00E-09	-8.52	0.0205
RC 4	5	2	3.00E-09	-8.52	0.0219
RC 4	5	3	3.00E-09	-8.52	0.0207
RC 4	6	1	1.00E-09	-9.00	0.0381
RC 4	6	2	1.00E-09	-9.00	0.0366
RC 4	6	3	1.00E-09	-9.00	0.0404
RC 4	7	1	5.00E-10	-9.30	0.0416
RC 4	7	2	5.00E-10	-9.30	0.0443
RC 4	7	3	5.00E-10	-9.30	0.0489
RC 4	8	1	1.00E-10	-10.00	0.0605
RC 4	8	2	1.00E-10	-10.00	0.0633
RC 4	8	3	1.00E-10	-10.00	0.0606

Percent of control values				
	Log[test	Replicate		
.evel	ostance]	1	2	3
1	-6.00	5.46	0.26	0.58
2	-7.00	9.19	6.03	6.73
3	-8.00	60.50	58.22	59.73
4	-8.19	86.50	85.28	85.11
5	-8.52	138.14	147.47	139.68
6	-9.00	256.86	246.40	272.57
7	-9.30	280.58	298.66	329.40
8	-10.00	407.98	426.46	408.62

Appendix G

Statistical Analysis Report (BioSTAT Consultants, Inc.)

Statistical Analysis Summary
WIL Research Laboratories, LLC
Study Protocol – 431007

1 Statistical Methodology
1.1 Analysis Methodology
2 Results
3 Statistical Analysis Summaries
Table 3.1: Analysis of Variance Comparisons Among Concentration Response Curve Fits
Figure 3.2: Estimates for Slope and Confidence Intervals for all Replicates – Reference Chemical 1
Figure 3.3: Estimates for Slope and Confidence Intervals for all Replicates – Reference Chemical 2
Figure 3.4: Estimates for Slope and Confidence Intervals for all Replicates – Reference Chemical 3
Figure 3.5: Estimates for Log10IC50 and Confidence Intervals for all Replicates – Reference Chemical 1
Figure 3.6: Estimates for Log10IC50 and Confidence Intervals for all Replicates – Reference Chemical 2
Figure 3.7: Estimates for Log10IC50 and Confidence Intervals for all Replicates – Reference Chemical 3
Table 3.8: ANOVA for Background Activity Percent of Control Values
Table 3.9: ANOVA for Full Enzyme Activity Percent of Control Values
Table 3.10: ANOVA for Negative Control Tube Percent of Control Values
Table 3.11: ANOVA for Postive Control Tube Percent of Control Values
Table 3.12: ANOVA for Background Activity Percent of Control Values – Excluding Chemical 4
Table 3.13: ANOVA for Full Enzyme Activity Percent of Control Values – Excluding Chemical 4
Table 3.14: ANOVA for Negative Control Tube Percent of Control Values – Excluding Chemical 4
Table 3.15: ANOVA for Postive Control Tube Percent of Control Values – Excluding Chemical 4

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BioSTAT Consultants, Inc.
Statement of Quality Control

This report was quality checked in accordance with BioSTAT Procedural Guideline 2.0 (Quality Control Process for Tables and Reports). The statistical methodology and results of inferential statistics were verified by an independent quality control statistician. Based on these documented quality control activities, it is concluded that the statistical results incorporated in this report accurately reflect the statistical analysis of data received by BioSTAT.

1 Statistical Methodology

1.1 Analysis Methodology

Analysis of Variance Comparisons Among Concentration Response Curve Fits

Slope (β) and log10IC50 (μ) were subjected to a random effects analysis of variance. Response variable was β or μ and replicates were treated as random effects.

The SAS procedure Proc Mixed was used for analysis using the following statements:

```
proc mixed covtest;
class replicate;
model mean = /solution cl;
random replicate;
repeated/group=replicate;
parms (1) (STD1) (STD2) (STD3)/hold=2,3,4;
run;
```

where STD1, STD2 and STD3 are the standard error for each replicate provided by PRISM.

β and μ were estimated, separately within each reference chemical and replicate, and plotted along with the average across replicates (LSMean) and associated 95% confidence interval across replicates.

Full Enzyme Activity and Background Activity Percent of Control Values Across Replicates

Three factor mixed effects analysis of variance models were fit separately for the FEAC control, the BAC control and the positive and negative control tubes. The fixed effect factors in the analysis of variance were reference chemical, portion (beginning or end) and the portion by reference chemical interaction. The random effects were replicated nested within reference chemical and portion by replicate within reference chemical interaction.

The SAS procedure Proc Mixed was used for analysis using the following statements:

```
proc mixed covtest;
class chemical portion replicate;
model value = chemical portion chemical*portion;
random replicate(chemical) portion*replicate(chemical);
lsmeans chemical portion chemical*portion/cl;
run;
```

If the portion by replicate within reference chemical interaction was significant (at the 0.05 level) the nature of the effect was assessed by comparing the portion effect within each replicate within reference chemical to the portion effect averaged across replicates within reference chemical, adjusting for simultaneity by Bonferroni's method.

P-values from the ANOVA are presented in the tables. In addition, estimates for the LSMeans, standard errors and 95% confidence intervals are presented.

The analysis of variance methods described above for full enzyme activity and background activity percent of control values across replicates was conducted with all four chemicals and then repeated with chemicals 1, 2 and 3 only.

2 Results

Results of statistical analyses are summarized in Section 3.

References

SAS® Proprietary Software, Version 8.2; SAS Institute Inc.: Cary, NC, 1999-2001.

3 Statistical Analysis Summaries

Table 3.1: Analysis of Variance Comparisons Among Concentration Response Curve Fits

Reference Chemical	Parameter	Statistic	Replicate 1	Replicate 2	Replicate 3	Overall
1	Slope (ß)	Prism Best Fit Value (S.E.)	-0.965 (0.031)	-0.922 (0.019)	-0.931 (0.114)	--
		Prism 95% CI (lower, upper)	(-1.030, -0.900)	(-0.962, -0.881)	(-1.168, -0.694)	--
		LSM (LSM s.e.)	--	--	--	-0.936 (0.019)
		95% CI (lower, upper)	--	--	--	(-1.017, -0.854)
	log10IC50 (µ)	Prism Best Fit Value (S.E.)	-5.369 (0.022)	-5.479 (0.011)	-5.522 (0.063)	--
		Prism 95% CI (lower, upper)	(-5.414, -5.324)	(-5.503, -5.456)	(-5.653, -5.391)	--
		LSM (LSM s.e.)	--	--	--	-5.448 (0.045)
		95% CI (lower, upper)	--	--	--	(-5.641, -5.255)
2	Slope (ß)	Prism Best Fit Value (S.E.)	-1.011 (0.023)	-0.920 (0.047)	-0.998 (0.015)	--
		Prism 95% CI (lower, upper)	(-1.059, -0.964)	(-1.018, -0.823)	(-1.030, -0.966)	--
		LSM (LSM s.e.)	--	--	--	-0.997 (0.012)
		95% CI (lower, upper)	--	--	--	(-1.050, -0.944)
	log10IC50 (µ)	Prism Best Fit Value (S.E.)	-5.400 (0.017)	-5.407 (0.031)	-5.307 (0.009)	--
		Prism 95% CI (lower, upper)	(-5.436, -5.364)	(-5.471, -5.343)	(-5.326, -5.289)	--
		LSM (LSM s.e.)	--	--	--	-5.368 (0.034)
		95% CI (lower, upper)	--	--	--	(-5.513, -5.223)
3	Slope (ß)	Prism Best Fit Value (S.E.)	-1.001 (0.037)	-0.995 (0.025)	-1.009 (0.049)	--
		Prism 95% CI (lower, upper)	(-1.079, -0.923)	(-1.046, -0.944)	(-1.112, -0.907)	--
		LSM (LSM s.e.)	--	--	--	-0.998 (0.019)
		95% CI (lower, upper)	--	--	--	(-1.080, -0.917)
	log10IC50 (µ)	Prism Best Fit Value (S.E.)	-5.055 (0.022)	-4.968 (0.014)	-5.191 (0.030)	--
		Prism 95% CI (lower, upper)	(-5.099, -5.010)	(-4.997, -4.939)	(-5.254, -5.129)	--
		LSM (LSM s.e.)	--	--	--	-5.069 (0.064)
		95% CI (lower, upper)	--	--	--	(-5.347, -4.792)
4	Slope (ß)	Prism Best Fit Value (S.E.)	-0.976 (0.044)	--	--	--
		Prism 95% CI (lower, upper)	(-1.066, -0.885)	--	--	--
		LSM (LSM s.e.)	--	--	--	--
		95% CI (lower, upper)	--	--	--	--
	log10IC50 (µ)	Prism Best Fit Value (S.E.)	-8.711 (0.037)	--	--	--
		Prism 95% CI (lower, upper)	(-8.788, -8.635)	--	--	--
		LSM (LSM s.e.)	--	--	--	--
		95% CI (lower, upper)	--	--	--	--

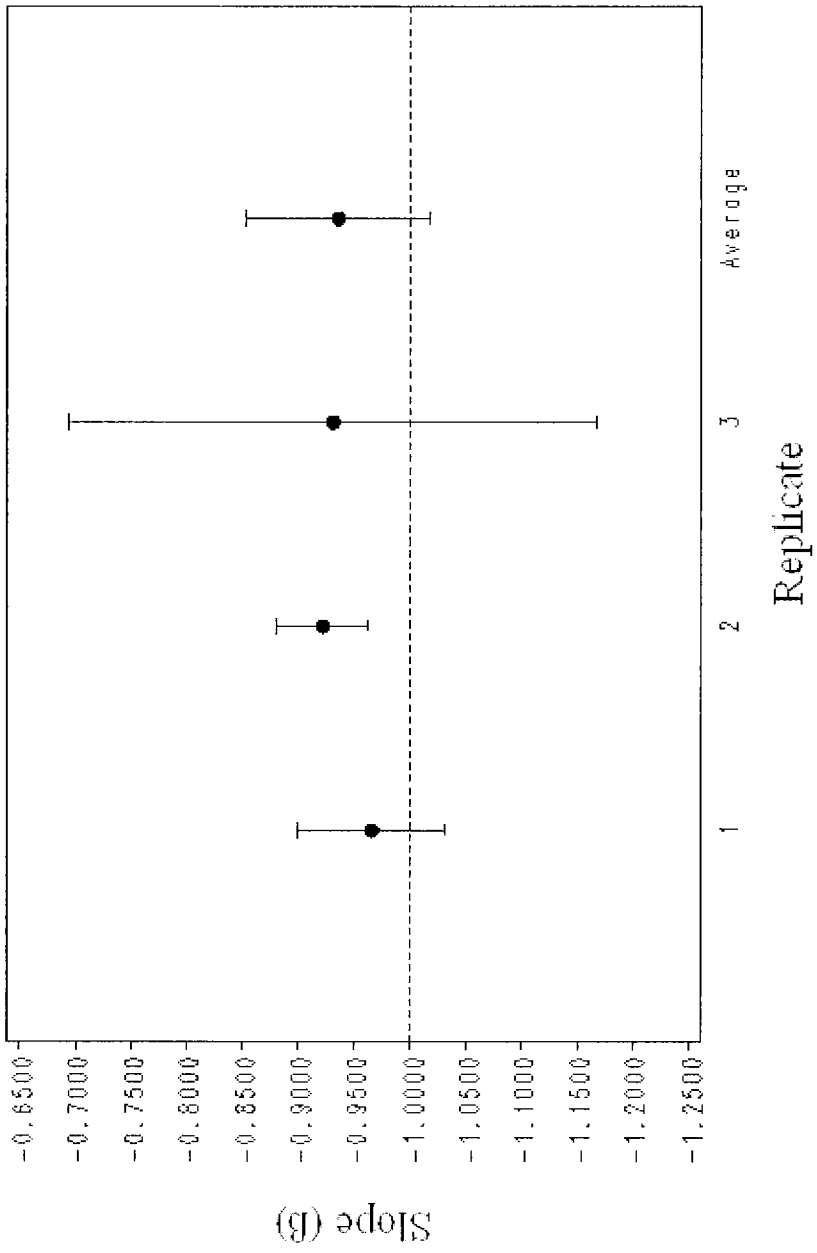

Figure 3.2: Estimates for Slope and Confidence Intervals for all Replicates – Reference Chemical 1

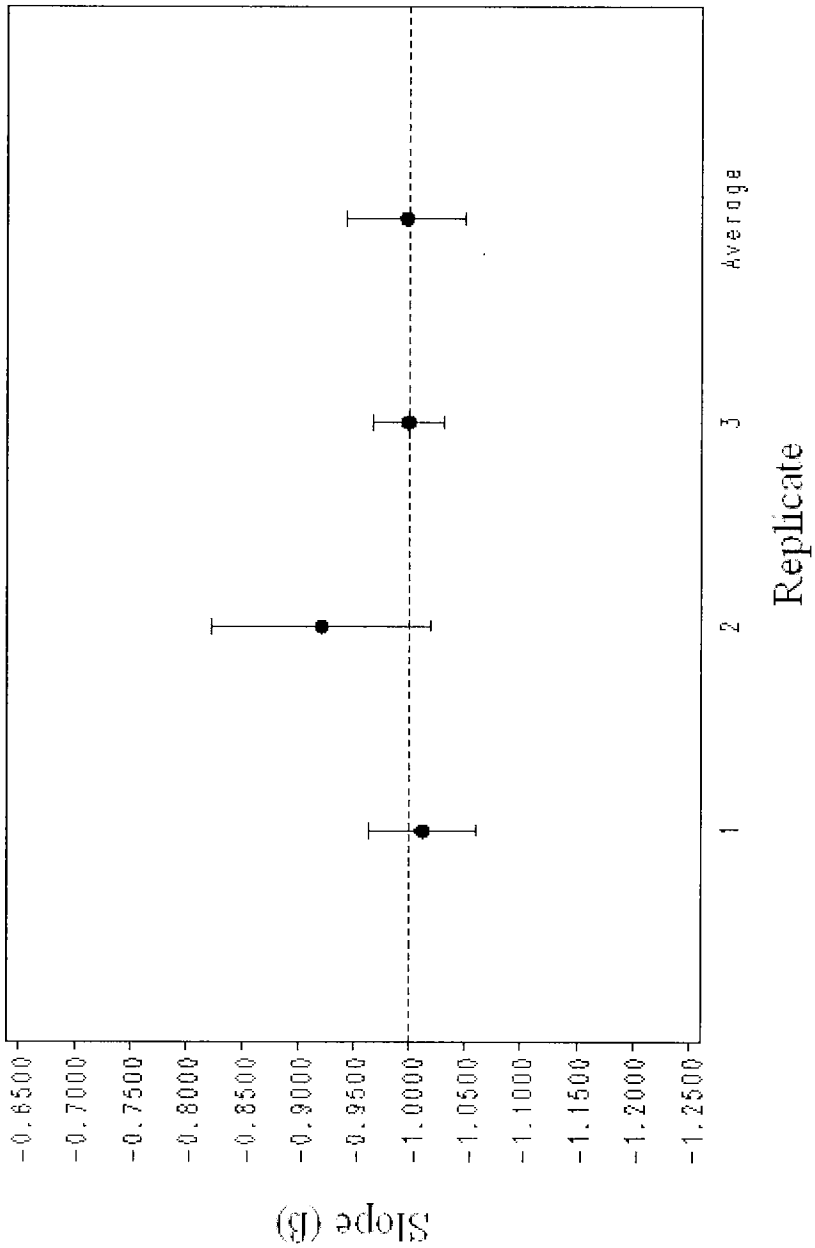

Figure 3.3: Estimates for Slope and Confidence Intervals for all Replicates – Reference Chemical 2

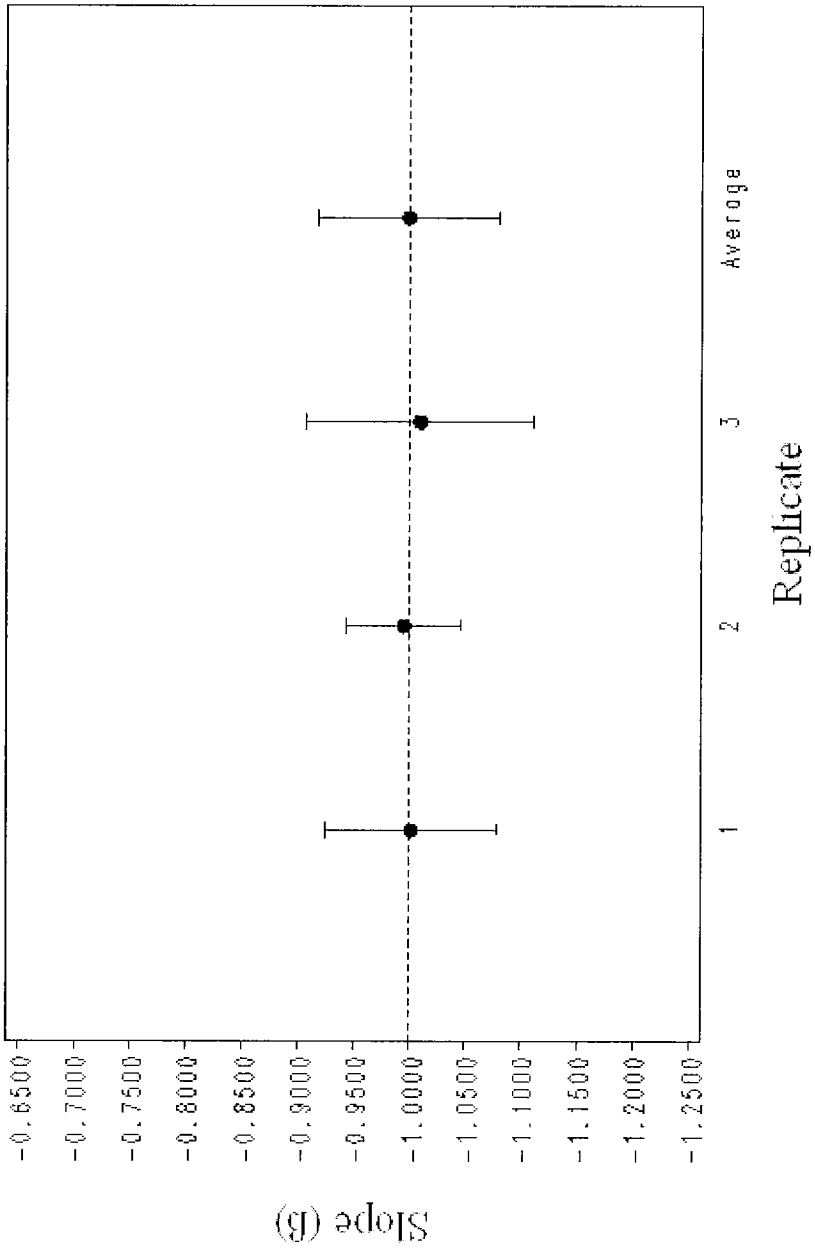

Figure 3.4: Estimates for Slope and Confidence Intervals for all Replicates – Reference Chemical 3

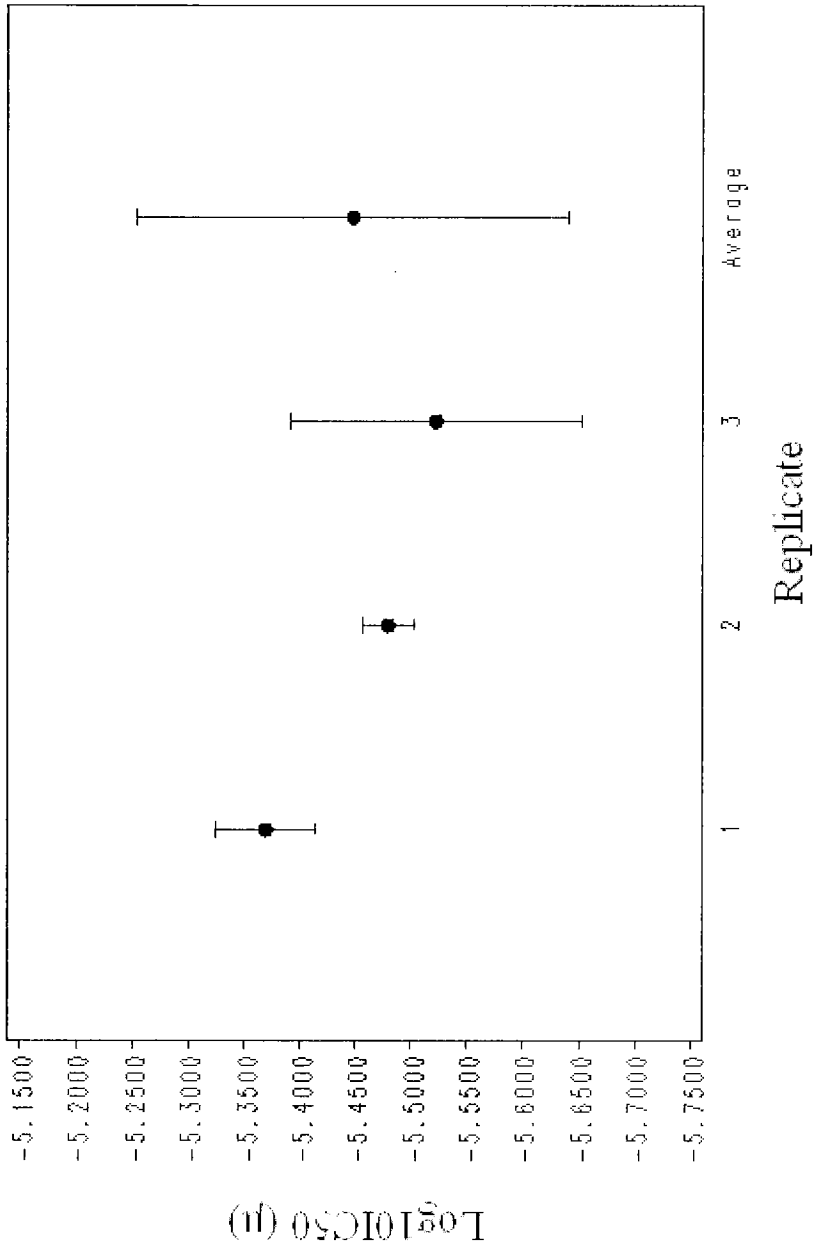

Figure 3.5: Estimates for Log10IC50 and Confidence Intervals for all Replicates – Reference Chemical 1

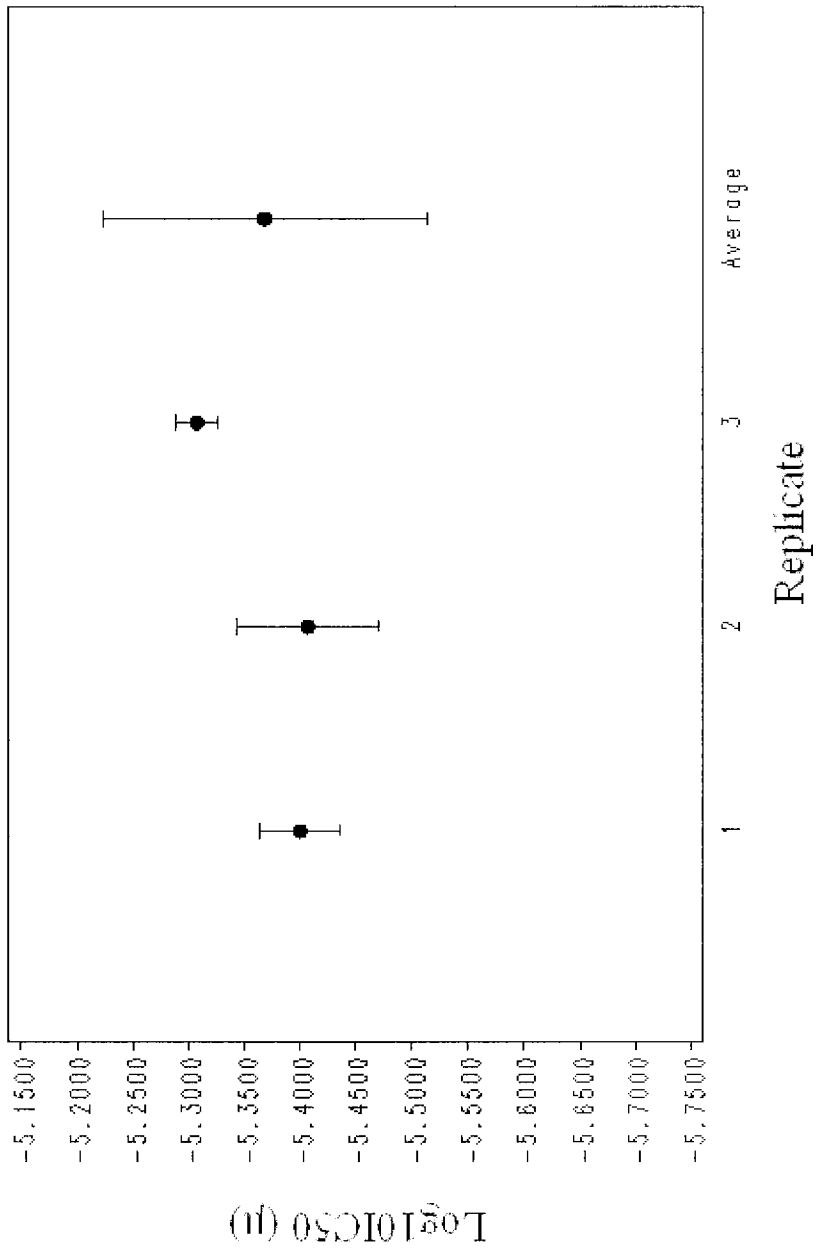

Figure 3.6: Estimates for Log10IC50 and Confidence Intervals for all Replicates – Reference Chemical 2

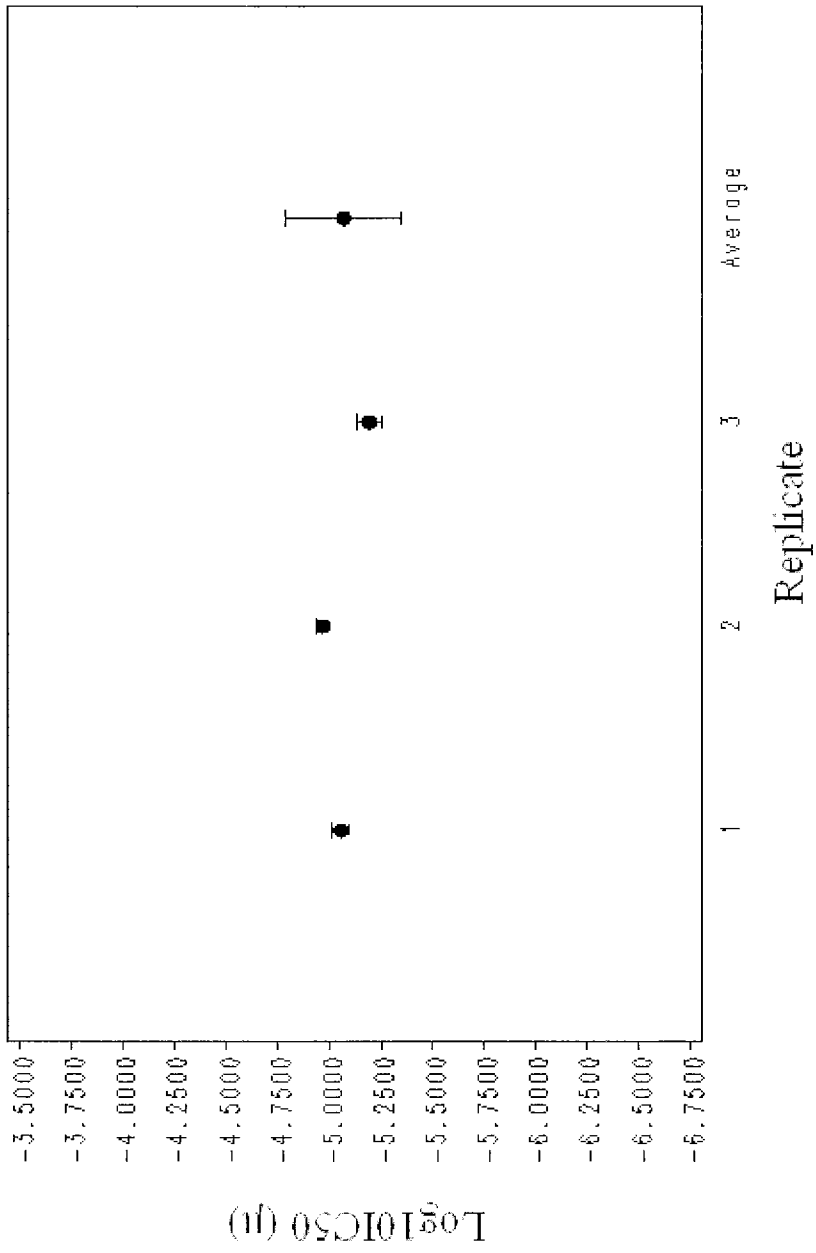

Figure 3.7: Estimates for Log10IC50 and Confidence Intervals for all Replicates – Reference Chemical 3

Table 3.8: ANOVA for Background Activity Percent of Control Values

Activity	Portion	Statistic	Chemical 1	2	3	4	Overall
Background	Beginning	LSM	0.113	0.558	0.009	-0.206	0.119
		LSM s.e.	0.394	0.394	0.394	0.306	0.187
		95% CI - lower	-0.766	-0.321	-0.870	-0.887	-0.298
		95% CI - upper	0.992	1.437	0.888	0.475	0.535
	End	LSM	-0.113	-0.558	-0.009	0.206	-0.119
		LSM s.e.	0.394	0.394	0.394	0.306	0.187
		95% CI - lower	-0.992	-1.437	-0.888	-0.475	-0.535
		95% CI - upper	0.766	0.321	0.870	0.887	0.298
	Overall	LSM	0.000	-0.000	0.000	0.000	
		LSM s.e.	0.279	0.279	0.279	0.216	
		95% CI - lower	-0.622	-0.622	-0.622	-0.481	
		95% CI - upper	0.622	0.622	0.622	0.481	
	ANOVA P-Values	Chemical = 1.0000					
		Portion = 0.3912					
		Chemical*Portion = 0.2529					
		Replicate(Chemical) = .					
		Portion*Replicate(Chemical) = .					

Table 3.9: ANOVA for Full Enzyme Activity Percent of Control Values

Activity	Portion	Statistic	Chemical 1	2	3	4	Overall
Full Enzyme	Beginning	LSM	103.307	102.698	100.569	45.129	87.926
		LSM s.e.	12.160	12.160	12.160	9.419	5.768
		95% CI - lower	76.213	75.604	73.475	24.142	75.074
		95% CI - upper	130.401	129.793	127.663	66.116	100.778
	End	LSM	96.693	97.302	99.431	154.869	112.074
		LSM s.e.	12.160	12.160	12.160	9.419	5.768
		95% CI - lower	69.599	70.207	72.337	133.882	99.222
		95% CI - upper	123.787	124.396	126.525	175.856	124.926
	Overall	LSM	100.000	100.000	100.000	99.999	
		LSM s.e.	8.598	8.598	8.598	6.660	
		95% CI - lower	80.841	80.841	80.841	85.159	
		95% CI - upper	119.159	119.159	119.159	114.839	
	ANOVA P-Values	Chemical = 1.0000					
		Portion = 0.0143					
		Chemical*Portion = 0.0004					
		Replicate(Chemical) = .					
		Portion*Replicate(Chemical) = 0.0579					

Table 3.10: ANOVA for Negative Control Tube Percent of Control Values

Activity	Portion	Statistic	Chemical 1	2	3	4	Overall
Negative	Beginning	LSM	110.559	101.723	98.887	217.294	132.116
		LSM s.e.	57.595	47.026	47.026	36.426	23.805
		95% CI - lower	-19.730	-4.658	-7.494	134.892	78.265
		95% CI - upper	240.849	208.104	205.268	299.696	185.967
	End	LSM	88.923	91.833	92.327	205.804	119.722
		LSM s.e.	57.595	47.026	47.026	36.426	23.805
		95% CI - lower	-41.367	-14.548	-14.054	123.402	65.870
		95% CI - upper	219.212	198.214	198.708	288.206	173.573
	Overall	LSM	99.741	96.778	95.607	211.549	
		LSM s.e.	56.978	46.523	46.523	36.036	
		95% CI - lower	-29.153	-8.464	-9.634	130.029	
		95% CI - upper	228.635	202.020	200.849	293.069	
	ANOVA P-Values	Chemical = 0.1745					
		Portion = 0.1082					
		Chemical*Portion = 0.9147					
		Replicate(Chemical) = 0.0190					
		Portion*Replicate(Chemical) = .					

Table 3.11: ANOVA for Postive Control Tube Percent of Control Values

Activity	Portion	Statistic	Chemical 1	2	3	4	Overall
Positive	Beginning	LSM	56.311	51.944	49.890	81.299	59.861
		LSM s.e.	12.684	12.684	12.684	9.825	6.016
		95% CI - lower	28.050	23.684	21.629	59.408	46.456
		95% CI - upper	84.572	80.205	78.150	103.190	73.266
	End	LSM	43.485	43.428	45.237	88.234	55.096
		LSM s.e.	12.684	12.684	12.684	9.825	6.016
		95% CI - lower	15.224	15.168	16.976	66.343	41.691
		95% CI - upper	71.745	71.689	73.498	110.125	68.501
	Overall	LSM	49.898	47.686	47.563	84.766	
		LSM s.e.	11.988	11.988	11.988	9.286	
		95% CI - lower	23.187	20.976	20.852	64.076	
		95% CI - upper	76.609	74.397	74.274	105.457	
	ANOVA P-Values	Chemical = 0.0661					
		Portion = 0.2532					
		Chemical*Portion = 0.2918					
		Replicate(Chemical) = 0.0253					
		Portion*Replicate(Chemical) = 0.2223					

Table 3.12: ANOVA for Background Activity Percent of Control Values – Excluding Chemical 4

Activity	Portion	Statistic	Chemical 1	2	3	Overall
Background	Beginning	LSM	0.113	0.558	0.009	0.227
		LSM s.e.	0.333	0.333	0.333	0.192
		95% CI - lower	-0.702	-0.257	-0.807	-0.244
		95% CI - upper	0.929	1.374	0.824	0.698
	End	LSM	-0.113	-0.558	-0.009	-0.227
		LSM s.e.	0.333	0.333	0.333	0.192
		95% CI - lower	-0.929	-1.374	-0.824	-0.698
		95% CI - upper	0.702	0.257	0.807	0.244
	Overall	LSM	0.000	-0.000	0.000	
		LSM s.e.	0.236	0.236	0.236	
		95% CI - lower	-0.577	-0.577	-0.577	
		95% CI - upper	0.577	0.577	0.577	
	ANOVA P-Values	Chemical = 1.0000				
		Portion = 0.1467				
		Chemical*Portion = 0.2897				
		Replicate(Chemical) = .				
		Portion*Replicate(Chemical) = .				

Table 3.13: ANOVA for Full Enzyme Activity Percent of Control Values – Excluding Chemical 4

Activity	Portion	Statistic	Chemical 1	Chemical 2	Chemical 3	Overall
Full Enzyme	Beginning	LSM	103.307	102.698	100.569	102.191
		LSM s.e.	2.187	2.187	2.187	1.263
		95% CI lower	97.955	97.346	95.217	99.101
		95% CI upper	108.660	108.051	105.921	105.282
	End	LSM	96.693	97.302	99.431	97.809
		LSM s.e.	2.187	2.187	2.187	1.263
		95% CI - lower	91.340	91.949	94.079	94.718
		95% CI - upper	102.045	102.654	104.783	100.899
	Overall	LSM	100.000	100.000	100.000	
		LSM s.e.	1.547	1.547	1.547	
		95% CI - lower	96.215	96.215	96.215	
		95% CI - upper	103.785	103.785	103.785	
	ANOVA P-Values	Chemical = 1.0000				
		Portion = 0.0495				
		Chemical*Portion = 0.4679				
		Replicate(Chemical) = .				
		Portion*Replicate(Chemical) = 0.4606				

Table 3.14: ANOVA for Negative Control Tube Percent of Control Values – Excluding Chemical 4

Activity	Portion	Statistic	Chemical 1	Chemical 2	Chemical 3	Overall
Negative	Beginning	LSM	110.559	101.723	98.887	103.723
		LSM s.e.	2.386	1.948	1.948	1.215
		95% CI - lower	104.426	96.715	93.879	100.600
		95% CI - upper	116.693	106.731	103.895	106.846
	End	LSM	88.923	91.833	92.327	91.028
		LSM s.e.	2.386	1.948	1.948	1.215
		95% CI - lower	82.789	86.825	87.319	87.904
		95% CI - upper	95.056	96.841	97.335	94.151
	Overall	LSM	99.741	96.778	95.607	
		LSM s.e.	1.687	1.378	1.378	
		95% CI - lower	95.404	93.237	92.066	
		95% CI - upper	104.078	100.319	99.149	
	ANOVA P-Values	Chemical = 0.2520				
		Portion = 0.0007				
		Chemical*Portion = 0.0432				
		Replicate(Chemical) = .				
		Portion*Replicate(Chemical) = 0.4810				

Table 3.15: ANOVA for Postive Control Tube Percent of Control Values – Excluding Chemical 4

Activity	Portion	Statistic	Chemical 1	2	3	Overall
Positive	Beginning	LSM	56.311	51.944	49.890	52.715
		LSM s.e.	4.657	4.657	4.657	2.688
		95% CI - lower	44.917	40.550	38.495	46.137
		95% CI - upper	67.705	63.339	61.284	59.293
	End	LSM	43.485	43.428	45.237	44.050
		LSM s.e.	4.657	4.657	4.657	2.688
		95% CI - lower	32.090	32.034	33.843	37.471
		95% CI - upper	54.879	54.823	56.631	50.628
	Overall	LSM	49.898	47.686	47.563	
		LSM s.e.	3.560	3.560	3.560	
		95% CI - lower	41.186	38.975	38.852	
		95% CI - upper	58.609	56.398	56.274	
	ANOVA P-Values	Chemical = 0.8753				
		Portion = 0.0465				
		Chemical*Portion = 0.6496				
		Replicate(Chemical) = 0.3415				
		Portion*Replicate(Chemical) = 0.3127				

APPENDIX D

BATTELLE INTERLABORATORY STATISTICAL ANALYSIS REPORT

DRAFT REPORT

CONDUCT MULTIPLE CHEMICAL STUDIES WITH CENTRALLY PREPARED MICROSOMES

INTERLABORATORY STATISTICAL ANALYSIS

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 5

February 9, 2006

Prepared for

U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

Prepared by

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505 King Avenue
Columbus, Ohio 43201

Placental Aromatase Validation Study

Conduct Studies with Centrally Prepared Microsomes
Interlaboratory Statistical Analysis

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 5

Zhenxu J. Ma, Author _____ Date _____

Paul I. Feder, Reviewer _____ Date _____

Quality Assurance Statement

Study Number: G608316

This study was inspected by the Quality Assurance Unit and reports were submitted to the Study Director and Management as follows:

Phase Inspected	Inspection Date	Date Reported to Principal Investigator/Management
Audit interlaboratory statistical analysis report	2/9/2006	2/9/2006

Quality Assurance Unit **Date**

Terri Pollock 2-13-06

Terri L Pollock

Summary and Conclusions

The principal results of the inter-laboratory analysis are summarized below.

Concentration Response $\log_{10}IC_{50}$ and Slope

The $\log_{10}IC_{50}$ estimates were similar among the three laboratories for all four reference chemicals. The among laboratory variance were zero or near zero (p-value=1). Coefficients of variation were calculated for variation in the IC_{50} ($\equiv 10^{\log_{10}IC_{50}}$) estimates across laboratories. The coefficient of variation among laboratories ranged from 2.6% to 8.6%.

The slope estimates for aminoglutethimide, ketoconazole, and econazole were similar among the three laboratories. The among laboratory variances were zero or near zero and the among laboratories CVs were between 1.4% and 2.3%.

For chrysin the estimated slope for Laboratory B was more than 59% smaller than those for Laboratories A and C (-0.94 versus -0.59 and-0.56). The among laboratory variance was 29 times larger than the within laboratory variances and among laboratories coefficient of variation was about 25%.

Control Activity

Comparisons among laboratories were made including and excluding the econazole control responses from Laboratory B. When the Laboratory B results were reported excluding econazole the end portion of the replicate was significantly lower than the beginning for each laboratory and averaged across laboratories, for full enzyme activity, negative, and positive controls. When the Laboratory B results were reported including econazole the significant difference between end and beginning no longer held for the positive and negative controls within Laboratory B. The end was significantly greater than the beginning for the full enzyme activity controls within Laboratory B. Averaged across laboratories the end was significantly lower than the beginning for the negative and positive controls but not for the full enzyme activity controls.

There were no significant differences between end and beginning for the background activity controls in Laboratories A and B and averaged across laboratories, whether econazole was included in or excluded from the Laboratory B results. In Laboratory C the end was significantly lower than the beginning.

Introduction and Background

Task 5 of Work Assignment 4-16, Placental Aromatase Validation Study, Conduct Multiple Chemical Studies with Centrally Prepared Microsomes, involved three individual laboratories (labeled as A, B, and C) that independently carried out the placental aromatase assay with four reference chemicals and with centrally prepared placental microsomes according to a common protocol. Intra-laboratory statistical analyses were carried out at each laboratory based

on each laboratory's test data, according to a common statistical analysis plan. The inter-laboratory statistical analysis discussed in this report combines summary values developed in the intra-laboratory analyses and assesses the relationships among them, the extent of inter-laboratory variation, and overall consensus estimates. This report discusses the methods used and the results obtained from combining the intra-laboratory statistical analysis results.

The inter-laboratory analysis is based on the $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined in the intra-laboratory analyses for each reference chemical. The inter-laboratory analysis also compares across the three laboratories the full enzyme activity, background activity, negative, and positive control responses at the beginnings and the ends of the replicates. It determines whether the control activity differed between the beginning and the end of each replicate.

Test Organization

Placental aromatase assay activity levels were determined for graded concentrations of each of the four reference chemicals: aminoglutethimide, chrysin, ketoconazole, and econazole. One to five replicates of the positive control study were carried out at each laboratory for each reference chemical (Table NR). Within each replicate three repetitions were run at each of the chemical concentrations. In addition, for each reference chemical, two repeat tubes of the full enzyme activity, background activity, negative, and positive controls were run prior to the concentration response runs and two repeat tubes of the full enzyme activity, background activity, negative, and positive controls were run following the concentration response runs.

Table NR. Number of Replicates at Each Laboratory for Each Reference Chemical

Chemical	Laboratory A	Laboratory B	Laboratory C
aminoglutethimide	3	3	5
chrysin	3	3	4
ketoconazole	4[1]	3	3
econazole	4[1]	1	3

Intra-laboratory statistical analyses were carried out on the percent of control activity responses. Percent of control activity is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the average background adjusted aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four background adjusted full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the four background adjusted background activity control tubes is necessarily 0 percent within each replicate.

[1] Three replicates were used in the analyses

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high chemical concentrations and approximately 100% near the low chemical concentrations, but this may vary with the inhibitor.

Intra-laboratory statistical analyses were performed based on a common statistical analysis plan. The following results were reported in each intra-laboratory analysis.

- Concentration curve fits within each replicate of each reference chemical to describe the trend in the percent of control activity across varying concentrations of each reference chemical.

- Estimates of the $\log_{10}IC_{50}$ concentration, slope, and associated standard errors within each replicate for each reference chemical.

- Average $\log_{10}IC_{50}$ concentration, average slope, and associated standard errors across replicates for each chemical.

- Results of analysis of variance applied to the data for the full enzyme activity, background activity, negative, and positive activity controls tested at the beginning and those tested at the end of each replicate. Laboratory A reported least squares means results at the end and at the beginning, separately for each chemical, Laboratory B reported least squares means results at the end and at the beginning combined across chemicals, and Laboratory C reported the differences between the beginning and the end combined across chemicals. In addition, laboratory B reported results both including and excluding econazole (i.e., results across all four chemicals and results across three chemicals only).

The inter-laboratory statistical analysis combines summary results from each of the intra-laboratory analyses to assess relationships among the results at each laboratory, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories with associated variability estimates (incorporating laboratory-to-laboratory variability). The inter-laboratory analysis is based on the average $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined by each of the test laboratories, as reported in the intra-laboratory analyses. It also compares among laboratories the differences between results obtained at the end of each replicate and those obtained at the beginning for full enzyme activity, background activity, negative, and positive controls.

Objectives

The objectives of the inter-laboratory statistical analysis are to:

- Determine the average values and the variabilities among laboratories for the above parameters.

- Determine the coefficients of variation among laboratories for the $\log_{10}IC_{50}$ and the slope parameters[2].

- Estimate the ratio of the among laboratory variation to the average within laboratory variation for the parameters mentioned above.

The inter-laboratory analyses for $\log_{10}IC_{50}$ and the slope parameters were carried out separately for each reference chemical, including the data from each laboratory for each chemical. The inter-laboratory analyses for the control activity responses were carried out on the results combined across reference chemicals, based on two versions of the data:

- Including the data from each laboratory for each reference chemical.
- Excluding the econazole results from Laboratory B.

Statistical Analysis Methods

Statistical analyses were carried out for each of the six endpoints discussed above: $\log_{10}IC_{50}$, slope, portion effects (beginning minus end) for background activity, full enzyme activity, negative, and positive controls. The analyses for $\log_{10}IC_{50}$ and slope were carried out separately for each reference chemical, while the analyses for the control activity responses were carried out combining all chemicals.

For each endpoint a one-way random effects analysis of variance model with heterogeneous variances among the participating laboratories was fitted to the summary responses within laboratories. Laboratory was treated as a random effect. The within laboratory variances were based on the squares of the standard errors associated with the endpoint estimates in each of the intra-laboratory analyses. The analysis of variance resulted in a weighted average across all the laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The weights included in the weighted averages incorporated both laboratory-to-laboratory variation and within laboratory variation. The degrees of freedom associated with the overall weighted averages were calculated based on Satterthwaite's approximation as

$$2*[((1/K)*\sum(S_L^2 + S_i^2))^2]/[(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))]$$

where S_L^2 is the random laboratory to laboratory variance, S_i^2 and df_i are the reported within laboratory variance and degrees of freedom for the i^{th} laboratory, $var(S_L^2)$ is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).

For each endpoint, the estimated overall average and its associated standard error (incorporating both within laboratory and among laboratory components of variation) and

[2] Coefficient of variation was not calculated for the control results because differences between the beginning and the end portions of replicates were reported and the distributions of these differences were anticipated to straddle 0.

associated degrees of freedom were used to construct a 95% confidence interval based on the t-distribution. For each laboratory the individual effect and associated 95% confidence interval (based on the within laboratory standard error) were also determined. These were plotted side-by-side to provide a graphical comparison among the laboratories.

When calculating the within laboratory mean $\log_{10}IC_{50}$ and slope across replicates, all three laboratories incorporated the replicate-to-replicate component of variation into the standard errors of the averages.

The three laboratories reported different summary statistics for the control activity responses, (Table A-1 through Table A-3). The differences between the beginning and the end results (i.e., end minus beginning) were therefore determined differently for each laboratory:

- Laboratory A reported least squares means and associated standard errors for the beginning and the end portions, separately for each reference chemical. For Laboratory A, the least squares means were first calculated for each position, beginning or end, by averaging the values for the position across the reference chemicals. The differences were calculated as end minus beginning. The associated standard errors for the differences between beginning and end (beginning minus end) were calculated as (LS Mean)/ $(\text{F-value})^{1/2}$, where the F-values and associated p-values were reported for the portion effects in the intra-laboratory analysis report.

- Laboratory B reported least squares means and associated standard errors for the beginning and the end portions, combined across reference chemicals. The difference was calculated as end minus beginning. The associated standard errors for the differences between beginning and end (beginning minus end) were calculated as (LS Mean)/$(\text{F-value})^{1/2}$, where the F-values and associated p-values were reported for the portion effects in the intra-laboratory analysis report.

- Results for Laboratory C are as reported in the intra-laboratory analysis report.

To describe the variability among the individual laboratory values relative to the overall average value, coefficients of variation (CV) and their associated 95% confidence intervals (95% CIs) were calculated for the $\log_{10}IC_{50}$ and the slope parameters. The coefficient of variation is defined as the standard deviation of the effect response divided by its mean. The methods for calculating the CV and the associated 95% CI were different depending on the underlying assumption about the distribution of the endpoint parameter.

For $\log_{10}IC_{50}$, the CV was determined for the variation of the IC_{50} among laboratories because this is the physically meaningful parameter. $IC_{50} \equiv 10^{\log_{10}IC_{50}}$ and the distribution of $\log_{10}IC_{50}$ was assumed to be approximately normally distributed, so the individual IC_{50} values were assumed to be approximately log normally distributed. The CV therefore is expressed as

$$CV = [10^{(S2)}-1]^{1/2} \times 100\%$$

where S^2 is the total variance (of $\log_{10}IC_{50}$) among the three laboratories. S^2 is approximated by $3(se)^2$ where se is the standard error of the pooled mean estimate of $\log_{10}IC_{50}$. This would be exact if the within laboratory variances were equal across laboratories.

The 95% confidence interval is based on the chi square distribution and is calculated as

$$[(10^{(df*S2/(\chi 2_{df,\,0.975}))} - 1)^{1/2} \times 100\%,\ (10^{(df*S2/(\chi 2_{df,\,0.025}))} - 1)^{1/2} \times 100\%]$$

where df is the estimated degree of freedom among the three laboratories.

For slope (β), the measurements are assumed to be approximately normal. The CV therefore is expressed as

$$CV=(S/\beta_{avg}) \times 100\%$$

where S^2 is the total variance among the three laboratories, defined as above and $S = \sqrt{S^2}$. The endpoints of the confidence interval for CV are based on the noncentral t distribution (Lehmann, 1986).

To describe the variability among laboratories relative to variability within laboratories the ratio of the variance among laboratories to the average variance within laboratories was calculated as

$$R=S^2_{lab}\,[1/3(s_1^2 + s_2^2 + s_3^2)]$$

where S^2_{lab} is the component of variance among the three laboratories and (s_1^2, s_2^2, s_3^2) are the squares of the within laboratory standard errors at the three laboratories. A confidence interval for this ratio

$$[R/F^{-1}(0.975),\ R/F^{-1}(0.025)]$$

was based on the F-distribution with (ν_{lab}, ν_{wi}) degree of freedom, where $\nu_{lab}=2$ and ν_{wi} is based on Satterthwaite's approximation

$$\nu_{wi} \approx [(s_1^2 + s_2^2 + s_3^2)^2]/[s_1^4/\nu_1 + s_2^4/\nu_2 + s_3^4/\nu_3].$$

This ratio was calculated for each of the six endpoint parameters.

In several places entries in the tables in the inter-laboratory analysis report tables may differ from corresponding entries in the intra-laboratory analysis report tables by one or a small number of trailing digits in the last decimal place. This is often due to differences between the intra-laboratory analyses and the inter-laboratory analysis in rounding in intermediate calculations.

Statistical Analysis Results

The inter-laboratory analyses for $\log_{10}IC_{50}$ and the slope parameters were carried out separately for each reference chemical. The inter-laboratory analyses for background activity full enzyme activity, negative, and positive activity controls were carried out combined across chemicals, with or without the results for the econazole controls in Laboratory B.

Concentration Response Relations. $\log_{10}IC_{50}$ and Slope

Table 1 displays the estimated parameter values and the associated within laboratory 95% confidence intervals about these values.[3]

Table 1 also displays the overall mean values across laboratories and their associated 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. These means and confidence intervals are graphically displayed in Figures 1 through 8. Each figure includes reference lines corresponding to the overall average. The estimated among laboratories CVs and their associated 95% confidence intervals for the overall means for the IC_{50} and slope parameters are also presented in Table 1.

Table 2 displays the within laboratory variances and their associated degrees of freedom for each laboratory.[4] These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 2 also displays the laboratory to laboratory random variation and the p-values, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95% confidence intervals.

The estimates for $\log_{10}IC_{50}$ were similar among the three laboratories for all four chemicals (Table 1). The among laboratory variances were zero or near zero. The p-values were 1.00 for each of the four reference chemicals (Table 2). The CVs for the IC_{50} ranged from 2.6% to 8.6%.

For chrysin the estimated slope for Laboratory B was more than 59% smaller than those for Laboratories A or C (-0.94 versus -0.59 or -0.56). For chrysin, the among laboratory variance (p-value=0.12) was about 29 times the average within laboratory variance. The

[3] The confidence intervals in Table 1 were calculated for the inter-laboratory analysis based on the least squares means, standard errors, and degrees of freedom reported in the intra-laboratory analyses within each laboratory. The confidence intervals in Table 1 thus may differ in the low significant digits from those displayed in the intra-laboratory analysis reports due to round off error in intermediate calculations.

[4] Degrees of freedom (Table 2) for Laboratory C were based on those specified in the Laboratory C inter-laboratory analysis report. Degrees of freedom for Laboratory C were based on the number of replicates (3) minus 1, except for econazole for which there was just one replicate. One degree of freedom was assigned for this situation. The degrees of freedom for Laboratory A were based on an analysis of variance model for all four chemicals combined. There were 12 observations (4 chemicals × 3 replicates per chemical) and four effects estimated, leaving 8 degrees of freedom for residual.

coefficient of variation among laboratories was about 25%. For the other chemicals, the slope estimates were similar among the three laboratories (Table 1). The among laboratory variances were zero or near zero (Table 2). The p-values for ketoconazole and econazole were 1.00. The p-value for aminoglutethimide was 0.37. The CV results ranged from 1.4% to 2.3%.

Control Activity

Table 3 displays the estimated parameter values and the associated within laboratory 95% confidence intervals about these values[5]. It also displays the overall mean values across laboratories and their associated 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. These mean values and confidence intervals are graphically displayed in Figures 9 to 16. Each figure includes reference lines corresponding to the overall average. Figures 9 to 12 display results corresponding to when the Laboratory B results excluded econazole. Figure 13 to 16 display results corresponding to when the Laboratory B results included econazole.

Table 4 displays the within laboratory variances and their associated degrees of freedom for each laboratory. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 4 also displays the laboratory to laboratory random variation and the p-values, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95% confidence intervals.

When the Laboratory B results excluded econazole, the end portion was statistically significantly lower than the beginning portion for the full enzyme activity, negative, and positive controls, for each individual laboratory and for the average across laboratories. When the Laboratory B results included econazole, statistical significances between the beginning and the end portions no longer existed for the full enzyme activity controls for the average across laboratories. The estimated variance among the laboratories was more than 7.4 times higher than the average within-laboratory variance. Statistical significance between beginning and end no longer existed for Laboratory B for the negative and positive controls. For the full enzyme activity controls for Laboratory B the end portion was significantly greater than the beginning.

References

Hartung, J. and Makambi, K.H. *Simple non-iterative t-distribution based tests for meta-analysis.* South African Statistical Journal, 2001, Vol. 35, p. 1-17.

Lehmann, E. *Testing Statistical Hypotheses.* John Wiley & Sons, Inc., 1986, p. 352-3

[5] The confidence intervals are based on the least squares means, standard errors, and degrees of freedom shown in Table A-4. The degrees of freedom in Table A-4 are based on those in Tables A-1 to A-3, which in turn are based on those reported in the intra-laboratory analyses.

Table 1. Parameter Estimates and 95% Confidence Intervals for $Log_{10}IC_{50}$ and Slope Parameter of Concentration Response Curves for the Placental Aromatase Assay. By Chemical.

Chemical	Parameter	Estimate and 95% Confidence Interval				
		Lab A[1]	Lab B[1]	Lab C[1]	Average[2]	CV(%) and 95% CI[3]
aminoglutethimide	$Log_{10}IC_{50}$	-5.370(-5.397,-5.342)	-5.368(-5.514,-5.222)	-5.387(-5.656,-5.118)	-5.370(-5.398,-5.341)	2.966(1.867,7.063)
	Slope	-0.975(-1.002,-0.948)	-0.997(-1.049,-0.945)	-0.963(-0.987,-0.939)	-0.978(-1.006,-0.951)	1.416(0.780,6.158)
chrysin	$Log_{10}IC_{50}$	-5.354(-5.467,-5.242)	-5.448(-5.642,-5.254)	-5.276(-5.980,-4.572)	-5.402(-5.498,-5.306)	8.612(5.025,28.130)
	Slope	-0.589(-0.673,-0.504)	-0.936(-1.018,-0.854)	-0.558(-0.687,-0.429)	-0.698(-1.013,-0.384)	24.899(13.846,119.132)
ketoconazole	$Log_{10}IC_{50}$	-5.143(-5.167,-5.120)	-5.069(-5.344,-4.794)	-5.218(-5.531,-4.905)	-5.143(-5.171,-5.116)	2.581(1.542,7.503)
	Slope	-1.010(-1.056,-0.964)	-0.998(-1.080,-0.916)	-0.927(-1.238,-0.616)	-1.001(-1.048,-0.953)	2.330(1.274,10.580)
econazole	$Log_{10}IC_{50}$	-8.679(-8.762,-8.595)	-8.711(-9.181,-8.241)	-8.681(-8.794,-8.568)	-8.687(-8.733,-8.641)	4.631(2.856,11.889)
	Slope	-1.029(-1.052,-1.006)	-0.976(-1.535,-0.417)	-1.058(-1.110,-1.006)	-1.034(-1.087,-0.981)	1.444(0.703,16.025)

1. The estimates and 95% confidence intervals are based on the intra-laboratory analyses for the three participating laboratories. The intra-laboratory analyses were carried out separately for each reference chemical. The confidence intervals were calculated based on the least squares means, standard errors, and degrees of freedom from the intra-laboratory analysis reports. They may thus differ from those in the intra-laboratory analysis reports in the low significant digits due to round off error.
2. The overall eestimates and confidence intervals were estimated based on one-way random effects analysis of variance with heterogeneous within laboratory variances among the three laboratories. The within laboratory variances are the squares of the within laboratory standard errors.
3. CV was calculated for the overall average results for the IC_{50} and slope parameters.

Table 2. Variance Components and Ratio of Between and Within Laboratories Variances for $Log_{10}IC_{50}$ and Slope Parameters of Concentration Response Curves for the Placental Aromatase Assay. By Chemical.

Chemical	Parameter	Within Laboratory Variance[1]				Among Laboratory Variance[3] and (p-value) (df=2)	Mean Variance[4,5]	Ratio[6] and 95% CI[6]
		Lab A[7]	Lab B[8]	Lab C	Pooled Average[2]			
aminoglutethimide	Log_IC50	0.000/df=8.0	0.001/df=2.0	0.010/df=4.2	0.004/df=5.3	0.000 (1.00)	0.000/df=5.3	0.000 (0.000, 0.000)
aminoglutethimide	Slope	0.000/df=8.0	0.000/df=2.0	0.000/df=53.2	0.000/df=13.7	0.000 (0.37)	0.000/df=2.6	0.360 (0.074, 14.206)
chrysin	Log_IC50	0.002/df=8.0	0.002/df=2.0	0.048/df=3.0	0.017/df=3.5	0.000 (1.00)	0.001/df=3.5	0.000 (0.000, 0.000)
chrysin	Slope	0.001/df=8.0	0.000/df=2.0	0.002/df=4.1	0.001/df=10.3	0.029 (0.12)	0.010/df=3.1	22.163 (4.113, 873.261)
econazole	Log_IC50	0.001/df=8.0	0.001/df=1.0	0.001/df=1.8	0.001/df=4.7	0.000 (1.00)	0.000/df=4.7	0.000 (0.000, 0.000)
econazole	Slope	0.000/df=8.0	0.002/df=1.0	0.000/df=3.7	0.001/df=1.5	0.000 (1.00)	0.000/df=1.5	0.000 (0.000, 0.000)
ketoconazole	Log_IC50	0.000/df=8.0	0.004/df=2.0	0.005/df=1.9	0.003/df=3.9	0.000 (1.00)	0.000/df=3.9	0.000 (0.000, 0.000)
ketoconazole	Slope	0.000/df=8.0	0.000/df=2.0	0.005/df=1.9	0.002/df=2.5	0.000 (1.00)	0.000/df=2.5	0.000 (0.000, 0.000)

1. The within laboratory variance for each laboratory is the square of the standard error associated with the parameter estimate, as reported in the intra-laboratory analyses for the three participating laboratories.
2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the three laboratories. Associated degrees of freedom were based on Satterthwaite's approximation
3. Among laboratories variance is based on a one-way random effects analysis of variance model with heterogeneous within laboratory variances, equal to the squares of the within laboratory standard errors.
4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2+S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where S_L^2 is the among laboratory variance, S_i^2 and df_i are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).
6. Ratio of the among-laboratory variance and the pooled average within laboratory variance.
7. Degrees of freedom for Laboratory A were based on a mixed effects analysis of variance for the four chemicals combined.
8. 1 degree of freedom for econazole for Laboratory B was specified because the results were based on one replicate.

Table 3. Parameter Estimates and 95% Confidence Intervals for Differences Between Beginning and End for the Percent of Control Responses for the Placental Aromatase Assay.

Parameter (End – Beginning)	Estimate and 95% Confidence Interval			
	Lab A[1,2]	Lab B[1,2]	Lab C[1,2]	Average[3]
Three Chemicals in Lab B[2]				
background Activity Control	0.023 (-0.157, 0.204)	-0.454 (-1.121, 0.213)	-0.165 (-0.247, -0.083)	-0.113 (-0.252, 0.025)
Full Enzyme Activity Control	-9.253 (-17.787, -0.718)	-4.382 (-8.751, -0.013)	-12.955 (-17.211, -8.699)	-8.683 (-15.116, -2.250)
Negative Control	-5.983 (-7.534, -4.431)	-12.695 (-17.568, -7.822)	-6.391 (-11.130, -1.652)	-8.033 (-13.405, -2.660)
Positive Control	-4.115 (-7.345, -0.885)	-8.665 (-17.144, -0.186)	-2.299 (-4.507, -0.091)	-3.200 (-4.978, -1.423)
Four Chemicals in Lab B[2]				
background Activity Control	0.023 (-0.157, 0.204)	-0.238 (-0.830, 0.354)	-0.165 (-0.247, -0.083)	-0.105 (-0.232, 0.023)
Full Enzyme Activity Control	-9.253 (-17.787, -0.718)	24.148 (5.968, 42.328)	-12.955 (-17.211, -8.699)	-1.145 (-32.436, 30.146)
Negative Control	-5.983 (-7.534, -4.431)	-12.394 (-28.049, 3.261)	-6.391 (-11.130, -1.652)	-6.070 (-7.465, -4.675)
Positive Control	-4.115 (-7.345, -0.885)	-4.765 (-13.522, 3.992)	-2.299 (-4.507, -0.091)	-2.979 (-4.678, -1.279)

1. The estimates and 95% confidence intervals are based on the intra-laboratory analyses for the three participating laboratories.
2. The results from the three laboratories are listed in Table A-4 (Appendix). The results from laboratories A and C are based on data from all four chemicals (aminoglutethimide, chrysin, econazole, and ketoconazole), while results for laboratory B are based on data with and without econazole (i.e., four chemicals and three chemicals respectively).
3. The overall effects and confidence intervals in this table were estimated using a one-way random effects analysis of variance, with heterogeneous variances among the three laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors.

Table 4. Variance Components and Ratios of Between to Within Laboratories Variances. Placental Aromatase Assay. Percent of Control.

Parameter (End – Beginning)	Within Laboratory Variance[1]				Among Laboratory Variance[4] and (p-value) (df=2)	Mean Variance[5,6]	Ratio and 95% CI[7]
	Lab A	Lab B	Lab C	Pooled Average[3]			
Three Chemicals in Lab B[2]							
background Activity Control	0.006/df=8.0	0.074/df=6.0	0.002/df=51.0	0.027/df=7.3	0.004 (0.3302)	0.003/df=6.9	0.142 (0.022, 5.584)
Full Enzyme Activity Control	13.698/df=8.0	3.188/df=6.0	4.207/df=21.8	7.031/df=17.1	10.591 (0.1889)	5.515/df=4.1	1.506 (0.327, 59.412)
Negative Control	0.453/df=8.0	3.965/df=6.0	5.058/df=17.3	3.159/df=21.8	5.896 (0.2104)	2.855/df=3.0	1.867 (0.425, 73.638)
Positive Control	1.962/df=8.0	12.009/df=6.0	0.978/df=9.8	4.983/df=9.1	0.000 (1.0)	0.619/df=9.1	0.000 (0.000, 0.000)
Four Chemicals in Lab B[2]							
background Activity Control	0.006/df=8.0	0.071/df=10.0	0.002/df=51.0	0.026/df=12.2	0.004 (0.3189)	0.003/df=10.3	0.140 (0.028, 5.508)
Full Enzyme Activity Control	13.698/df=8.0	66.577/df=10.0	4.207/df=21.8	28.160/df=15.3	209.170 (0.1603)	78.125/df=2.5	7.428 (1.566, 292.897)
Negative Control	0.453/df=8.0	49.366/df=10.0	5.058/df=17.3	18.292/df=12.3	0.000 (1.0)	0.412/df=12.3	0.000 (0.000, 0.000)
Positive Control	1.962/df=8.0	15.445/df=10.0	0.978/df=9.8	6.128/df=13.8	0.000 (1.0)	0.626/df=13.8	0.000 (0.000, 0.000)

1. The within laboratory variance for each laboratory is the square of the standard error associated with parameter estimate, which was reported in the intra-laboratory analyses for each of the three participating laboratories (see Appendix Table A-4).
2. The results from laboratories A and C are based on data from all four chemicals (aminoglutethimide, chrysin, econazole, and ketoconazole), while results for laboratory B are based on data with and without econazole (i.e., four chemicals and three chemicals respectively).
3. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the three laboratories. Associated degrees of freedom were based on Satterthwaite's approximation.
4. Variance among laboratories is based on a one-way random effects analysis of variance model with heterogeneous within laboratory variances among the three laboratories, equal to the squares of the within laboratory standard errors.
5. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
6. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2 + S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where S_L^2 is the among laboratory variance, S_i^2 and df_i are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).
7. Ratio of the among-laboratory variance and the pooled average within laboratory variance.

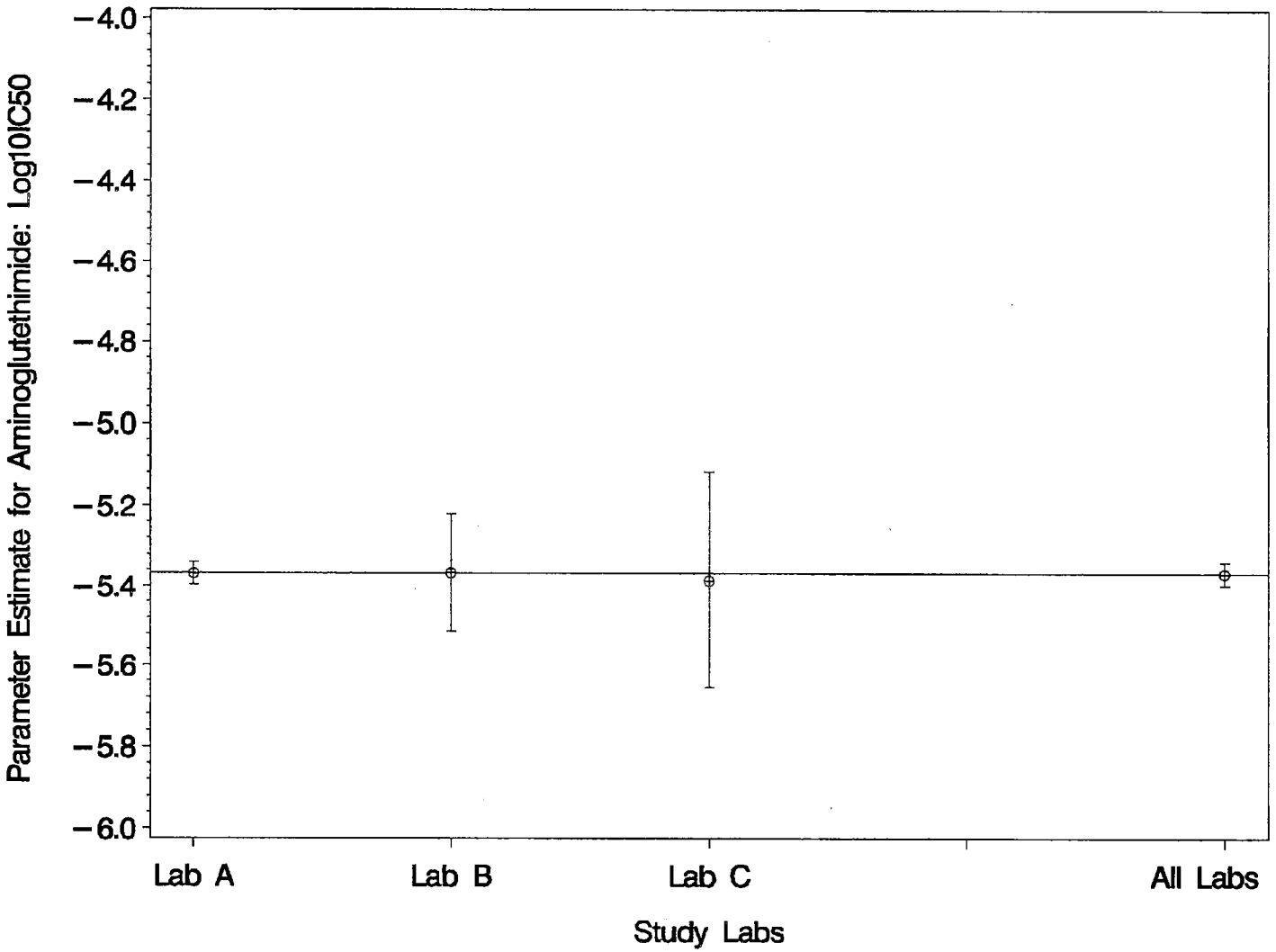

Figure 1. **Aminoglutethimide: Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

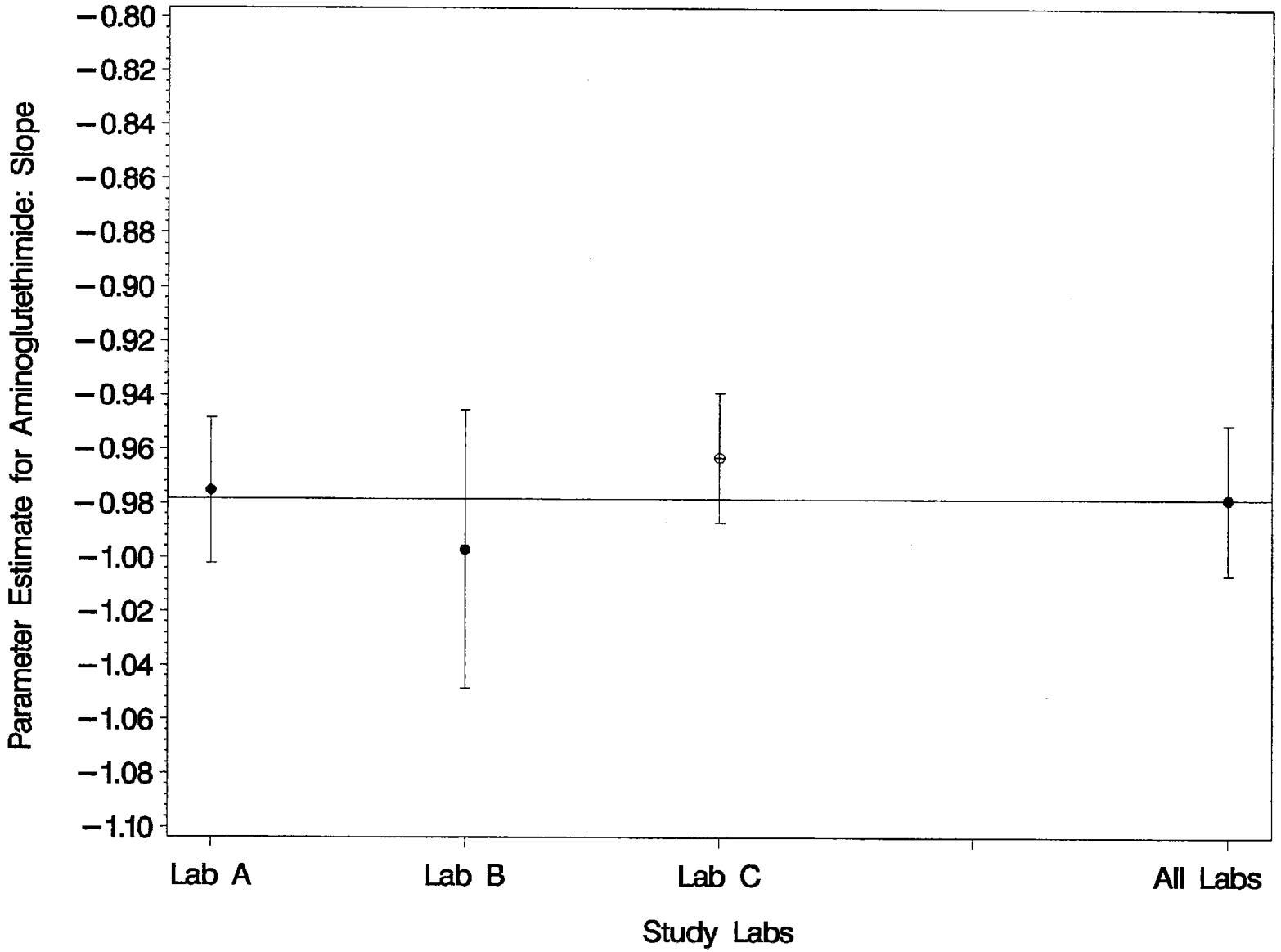

Figure 2. **Aminoglutethimide: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

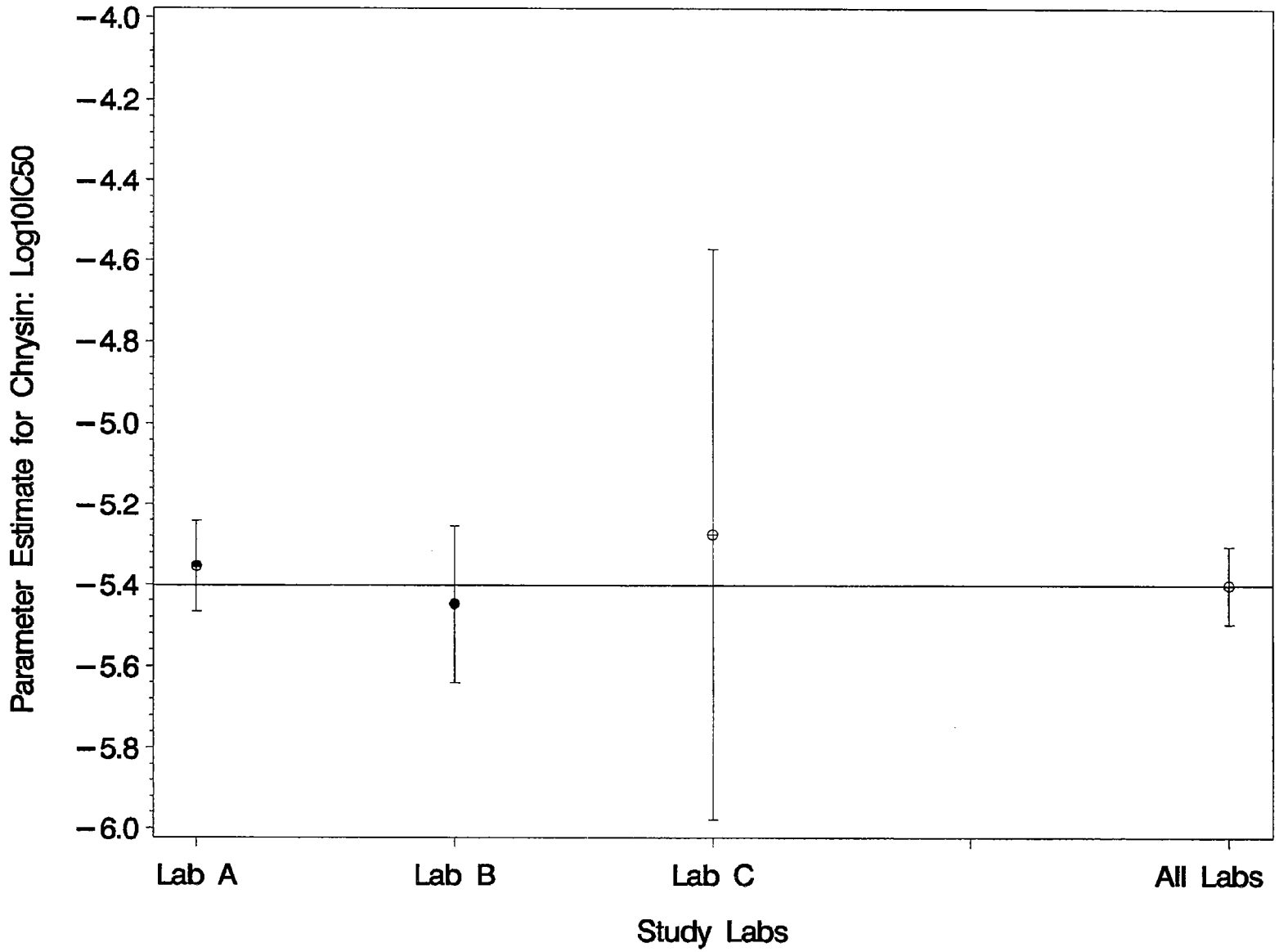

Figure 3. **Chrysin: Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

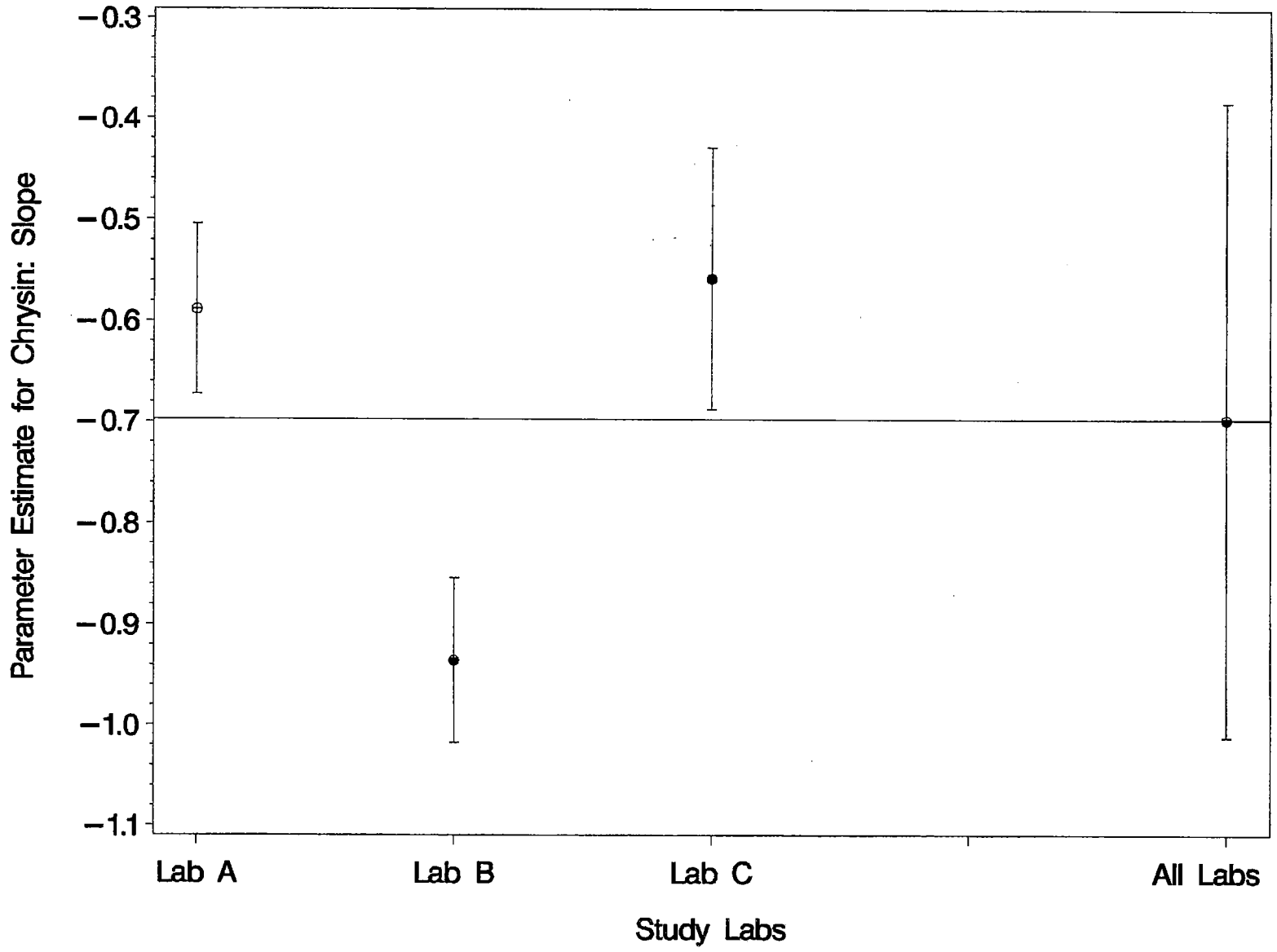

Figure 4. **Chrysin: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

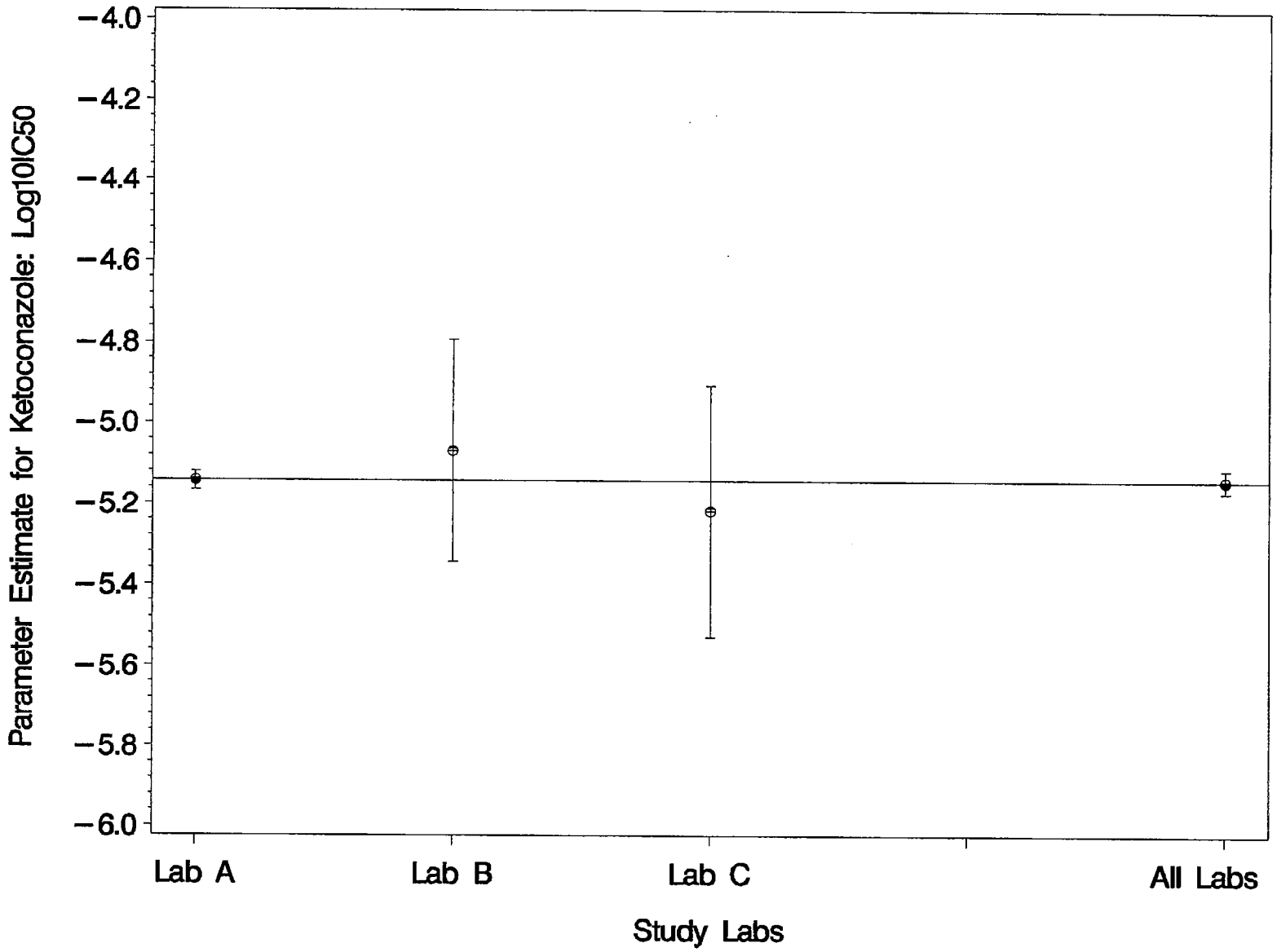

Figure 5. **Ketoconazole: Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

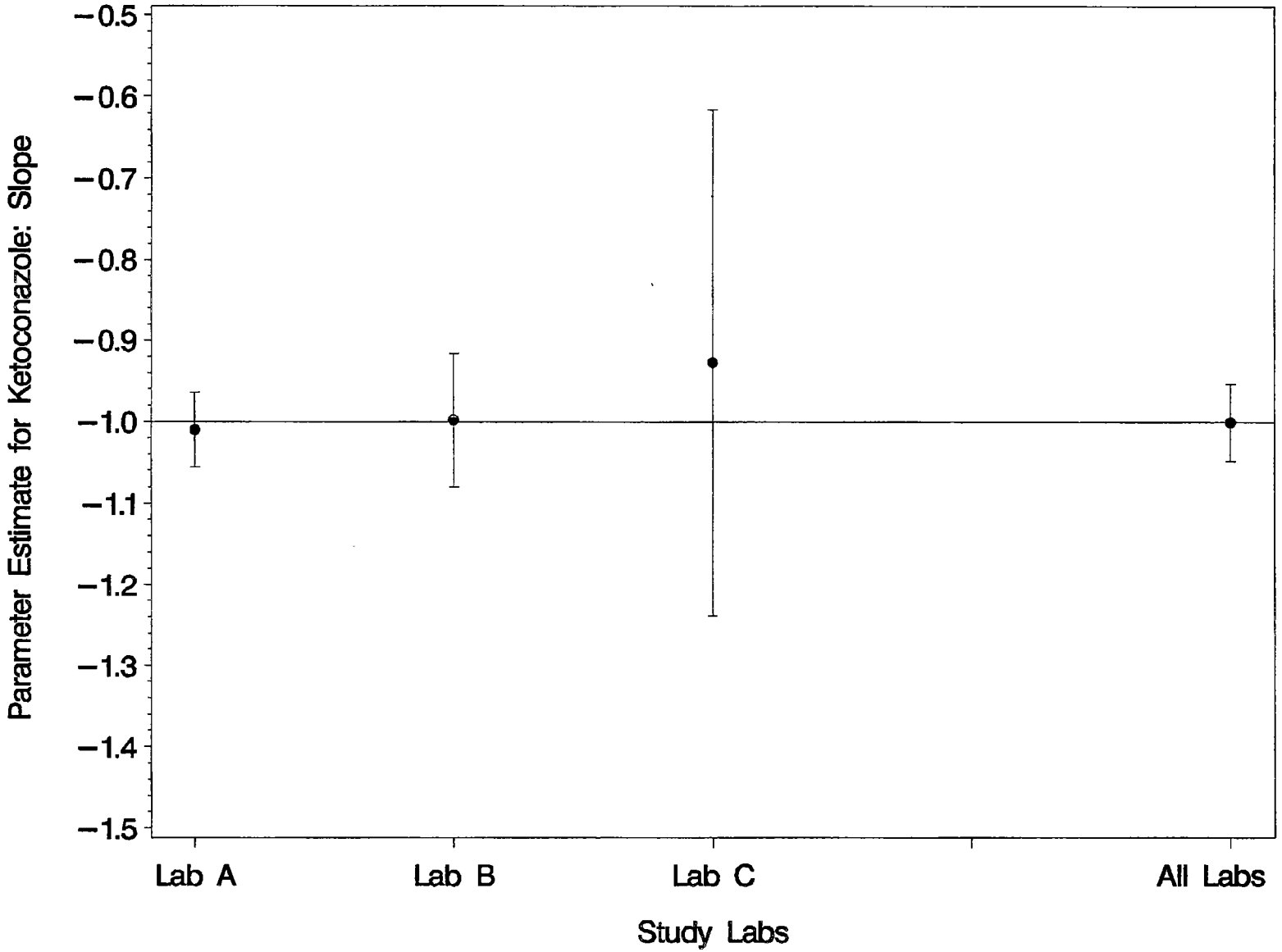

Figure 6. **Ketoconazole: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

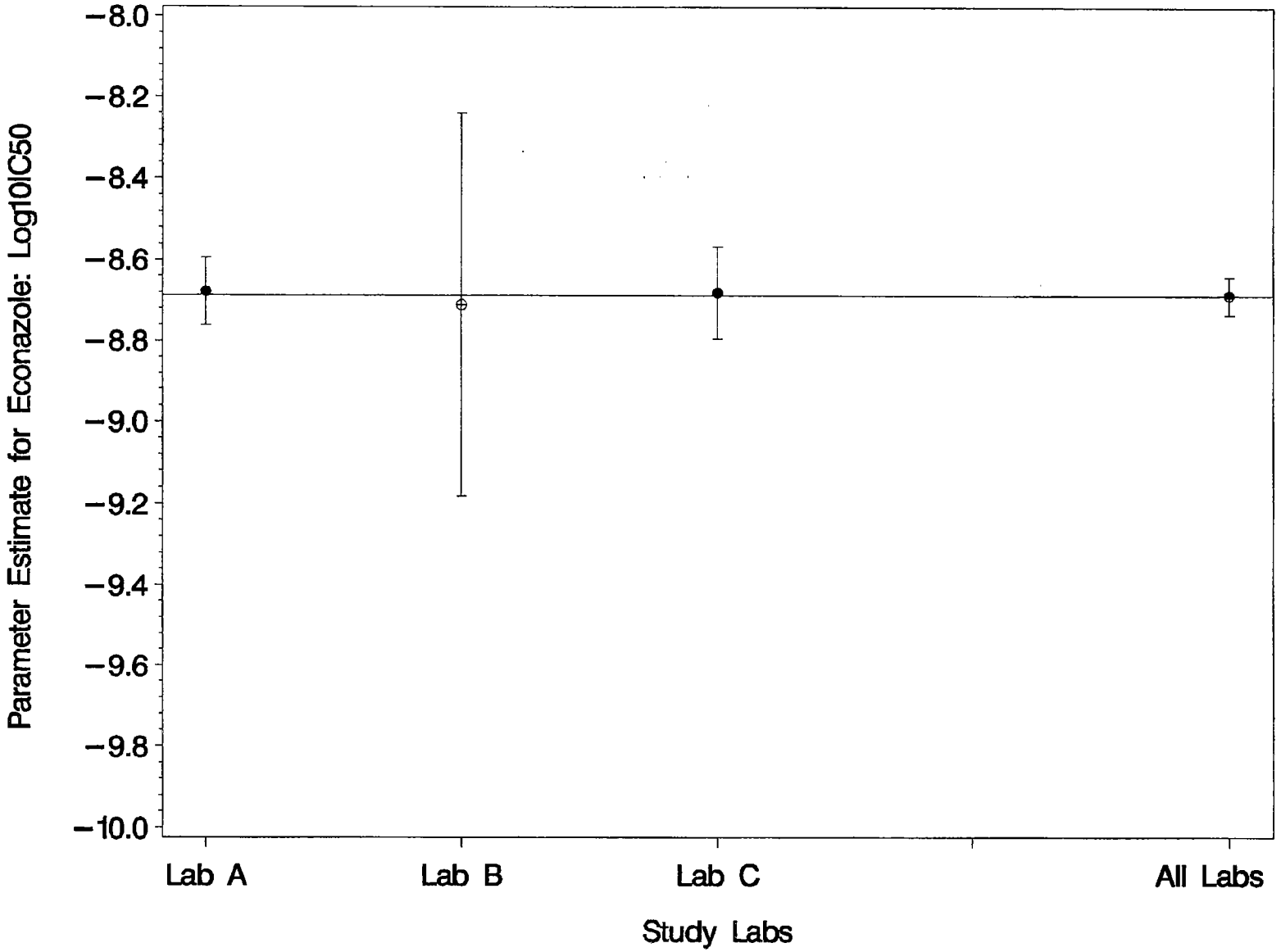

Figure 7. **Econazole: Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories.**

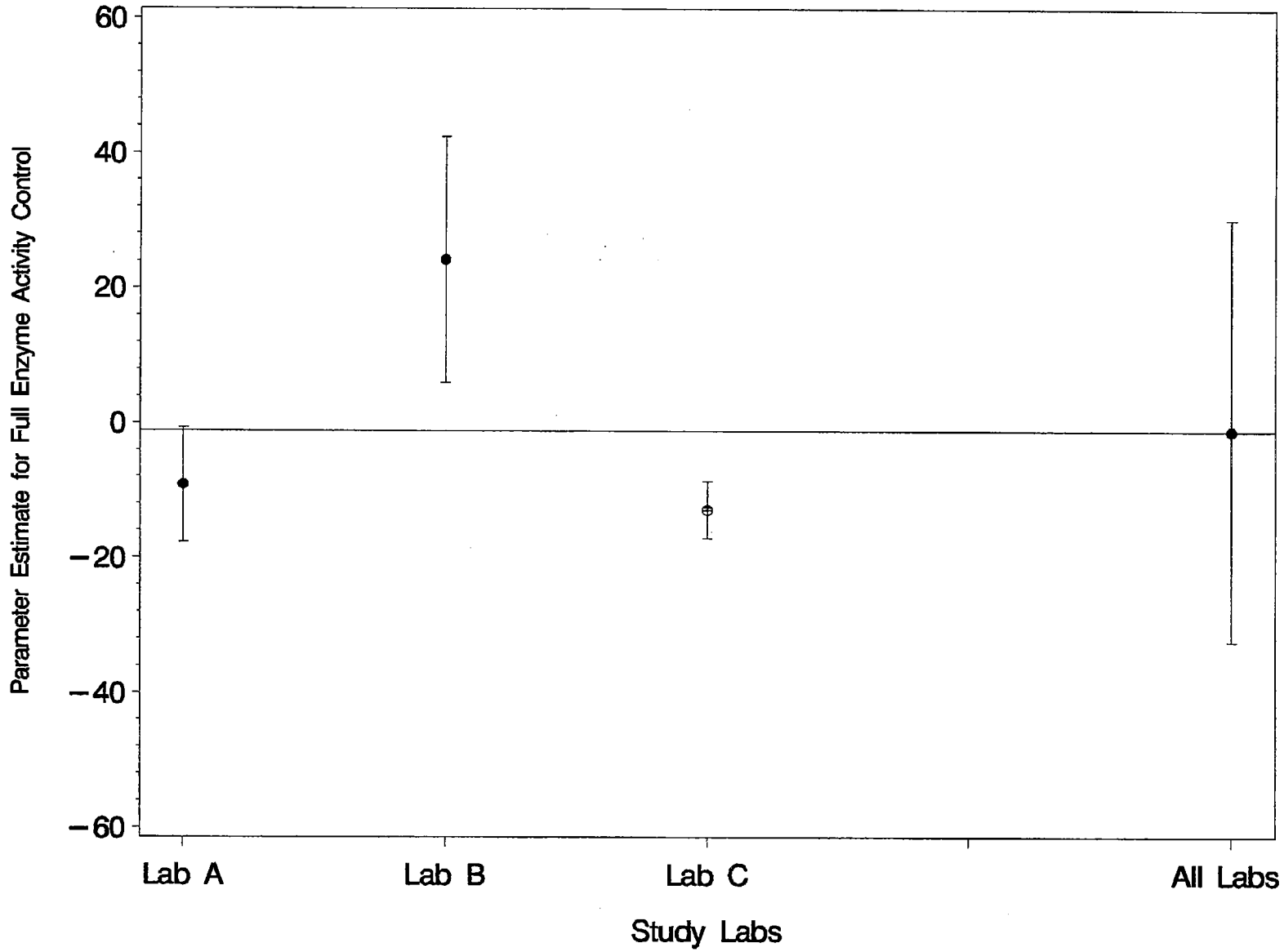

Figure 14. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Full Enzyme Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole.**

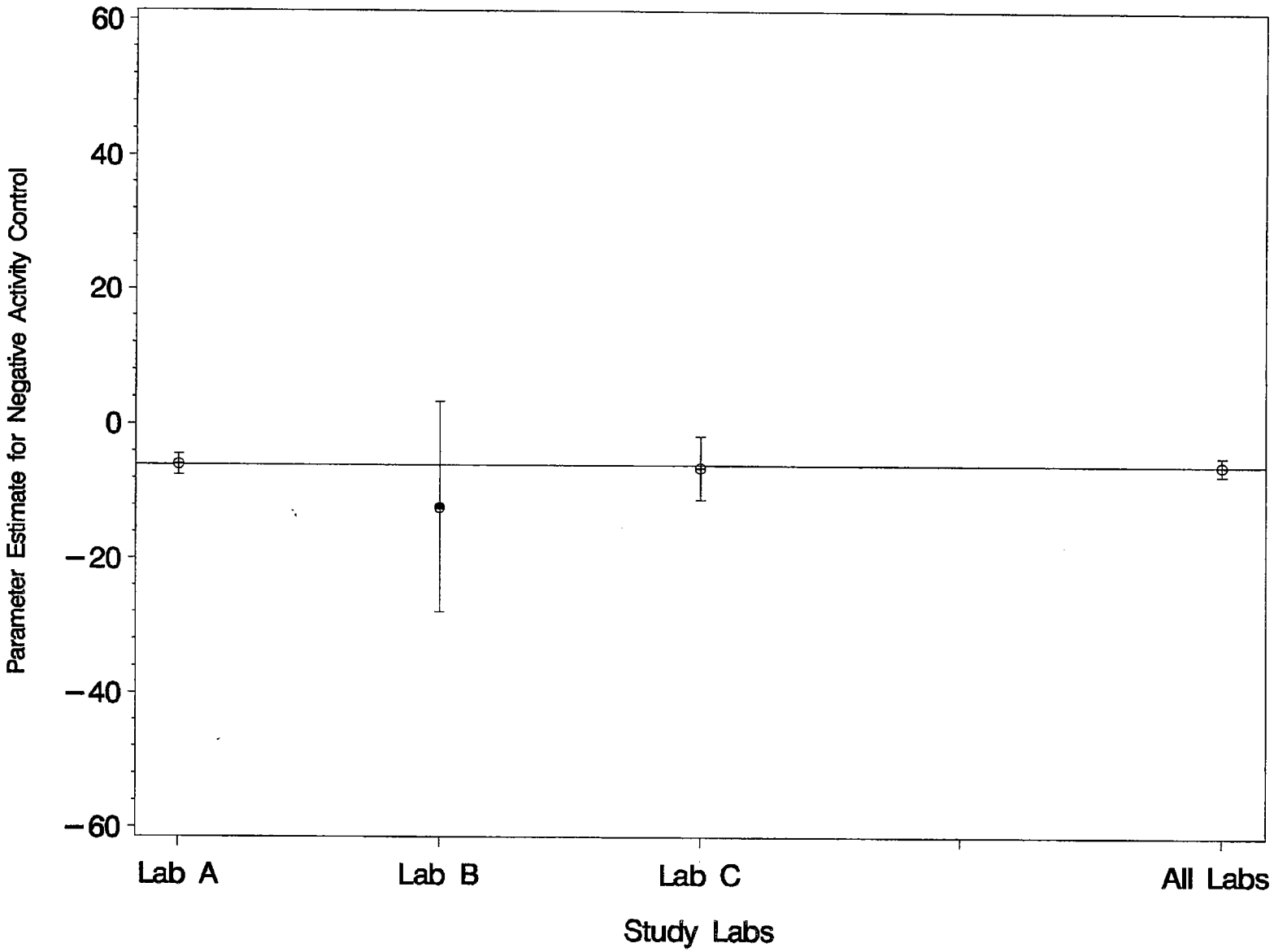

Figure 15. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Negative Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole.**

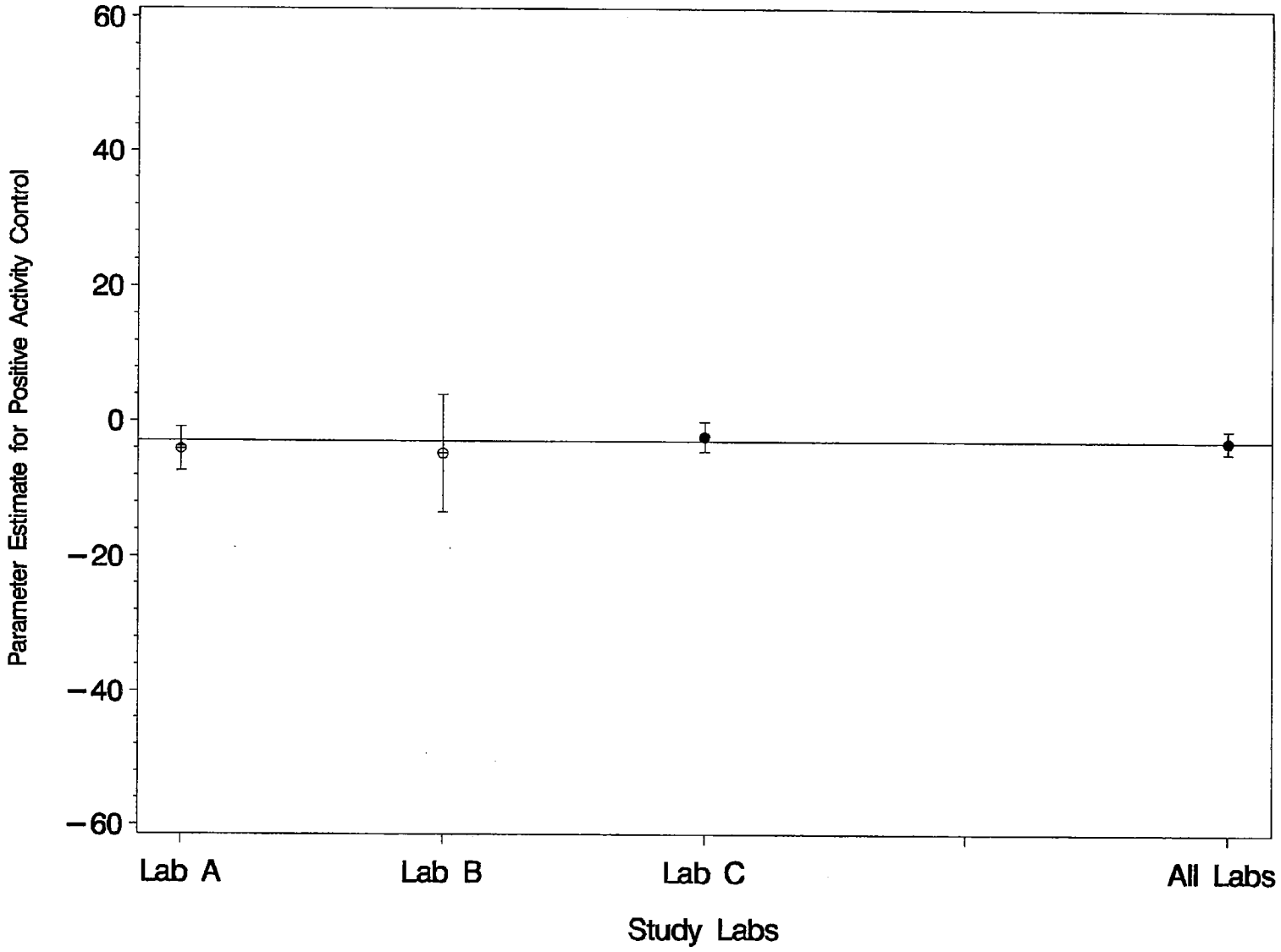

Figure 16. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Positive Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole.**

Appendix. Results of Analysis of Percentage of Control Data, as Reported by the Three Participating Laboratories.

Table A-1. Least Squares Means and Associated Standard Errors. Laboratory A. By Chemical and Portion.

Control Parameter	Chemical	Portion	LS Mean	Standard Error	Degree of Freedom
Background Activity Controls	aminoglutethimide	Begin	-0.030	0.1080	8
Background Activity Controls	chrysin	Begin	-0.010	0.1080	8
Background Activity Controls	econazole	Begin	0.120	0.1080	8
Background Activity Controls	ketoconazole	Begin	-0.127	0.1080	8
Background Activity Controls	aminoglutethimide	End	0.030	0.1080	8
Background Activity Controls	chrysin	End	0.010	0.1080	8
Background Activity Controls	econazole	End	-0.120	0.1080	8
Background Activity Controls	ketoconazole	End	0.127	0.1080	8
Full Enzyme Activity Controls	aminoglutethimide	Begin	103.360	5.2374	8
Full Enzyme Activity Controls	chrysin	Begin	102.150	5.2374	8
Full Enzyme Activity Controls	econazole	Begin	110.210	5.2374	8
Full Enzyme Activity Controls	ketoconazole	Begin	102.780	5.2374	8
Full Enzyme Activity Controls	aminoglutethimide	End	96.637	5.2374	8
Full Enzyme Activity Controls	chrysin	End	97.848	5.2374	8
Full Enzyme Activity Controls	econazole	End	89.787	5.2374	8

Control Parameter	Chemical	Portion	LS Mean	Standard Error	Degree of Freedom
Full Enzyme Activity Controls	ketoconazole	End	97.218	5.2374	8
Negative	aminoglutethimide	Begin	101.210	8.4439	8
Negative	chrysin	Begin	99.630	8.4439	8
Negative	econazole	Begin	84.751	8.4439	8
Negative	ketoconazole	Begin	101.820	8.4439	8
Negative	aminoglutethimide	End	93.865	8.4439	8
Negative	chrysin	End	95.456	8.4439	8
Negative	econazole	End	79.928	8.4439	8
Negative	ketoconazole	End	94.231	8.4439	8
Positive	aminoglutethimide	Begin	46.753	3.3152	8
Positive	chrysin	Begin	47.573	3.3152	8
Positive	econazole	Begin	55.491	3.3152	8
Positive	ketoconazole	Begin	46.745	3.3152	8
Positive	aminoglutethimide	End	43.083	3.3152	8
Positive	chrysin	End	47.559	3.3152	8
Positive	econazole	End	50.576	3.3152	8
Positive	ketoconazole	End	38.884	3.3152	8

Table A-2. Least Squares Means and Associated Standard Errors. Laboratory B[1]. By Portion.

Control Parameter	LS Mean Beginning	Standard Error Beginning	LS Mean End	Standard Error End	Degree of Freedom	Number of Chemicals Included
Background Activity Controls	0.227	0.192	-0.227	0.192	6	3
Full Enzyme Activity Controls	102.191	1.263	97.809	1.263	6	3
Negative	103.723	1.215	91.028	1.215	6	3
Positive	52.715	2.688	44.050	2.688	6	3
Background Activity Controls	0.119	0.187	-0.119	0.187	10	4
Full Enzyme Activity Controls	87.926	5.768	112.074	5.768	10	4
Negative	132.116	23.805	119.722	23.805	10	4
Positive	59.861	6.016	55.096	6.016	10	4

1. Laboratory B reported results for both with and without chemical econazole (i.e., including four chemicals and three chemicals respectively).

Table A-3. Least Squares Means and Associated Standard Errors. Laboratory C.

Control Parameter	Diff. LS Mean End-Beginning	Standard Error End-Beginning	Degree of Freedom
Background Activity Controls	-0.165	0.041	51.0
Full Enzyme Activity Controls	-12.955	2.051	21.8
Negative	-6.391	2.249	17.30
Positive	-2.299	0.989	9.84

Table A-4. Least Squares Means and Associated Standard Errors for Portion Effects (End Minus Beginning)

Laboratory	Control Parameter	LS Mean	Standard Error	Degree of Freedom	P-value[5]	Number of Chemicals Included[1]
A[2]	Background Activity Controls	0.0235	0.07822	8.00	0.7665	4
	Full Enzyme Activity Controls	-9.2527	3.70109	8.00	0.0370	4
	Negative	-5.9829	0.67287	8.00	<.0001	4
	Positive	-4.1149	1.40074	8.00	0.0188	4
B[3]	Background Activity Controls	-0.4540	0.27246	6.00	0.1467	3
	Full Enzyme Activity Controls	-4.3820	1.78543	6.00	0.0495	3
	Negative	-12.6950	1.99132	6.00	0.0007	3
	Positive	-8.6650	3.46538	6.00	0.0465	3
B[3]	Background Activity Controls	-0.2380	0.26556	10.00	0.3912	4
	Full Enzyme Activity Controls	24.1480	8.15945	10.00	0.0143	4
	Negative	-12.3940	7.02608	10.00	0.1082	4
	Positive	-4.7650	3.93000	10.00	0.2532	4
C[4]	Background Activity Controls	-0.1650	0.04100	51.00	0.0002	4
	Full Enzyme Activity Controls	-12.9550	2.05100	21.80	<0.0001	4
	Negative	-6.3910	2.24900	17.30	0.0111	4
	Positive	-2.2990	0.98900	9.84	0.043	4

1. All three laboratories reported results based on the control data from all four chemicals. In addition, Laboratory B reported results after excluding data for chemical econazole. (see Tables A-1, A-2, and A-3)
2. For Laboratory A, the least squares means were first calculated for each position (beginning or end) by averaging the values across the chemicals for the position, then the difference were calculated as the end minus the beginning. The associated standard errors for the differences between beginning and end (beginning minus end) were calculated as $(\text{LS Mean})/(\text{F-value})^{1/2}$.
3. For Laboratory B, the associated standard errors for the differences between beginning and end (beginning minus end) were calculated as $(\text{LS Mean})/(\text{F-value})^{1/2}$.
4. Results for Laboratory C are as reported in Table A-3.
5. p-values are as reported in the intra-laboratory analysis reports.