DRAFT REPORT

AROMATASE ASSAY VALIDATION
POSITIVE CONTROL STUDY: PLACENTAL MICROSOMES

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DRAFT REPORT

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QUALITY ASSURANCE STATEMENT

The Quality Assurance Unit inspected this study and reports were submitted to the study director and management as follows:

Phase Inspected	Inspection Date	Date Reported to Study Director /Management
Protocol review	12/2/2004	12/2/2004
Solution preparation	12/17/2004	12/20/2004
Dilution	12/17/2004	12/20/2004
Standard preparation	12/17/2004	12/20/2004
Test system preparation	12/17/2004	12/20/2004
Sample homogenization	12/17/2004	12/20/2004
Sample preparation	12/17/2004	12/20/2004
Centrifugation	12/17/2004	12/20/2004
Exaction	12/17/2004	12/20/2004
Protein analysis	12/17/2004	12/20/2004
Spectrophotometer absorbance reading	12/17/2004	12/20/2004
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Audit study file	3/31/2005	3/31/2005
Audit draft report	7/20/2005	7/20/2005
Audit draft report	10/18/05	10/18/05

This report provides an accurate record of the results obtained.

Quality Assurance Unit Date

COMPLIANCE STATEMENT

This study, Battelle Study Number G608316, 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 Battelle Memorial Institute; and the protocol as approved by the Sponsor.

Bozena D. Lusiak, Ph.D. Study Director	Date

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1.0 EXECUTIVE SUMMARY

The primary objective of this task was to demonstrate the responsiveness of the aromatase assay using the classical ^3H_2O method, 4-hydroxyandrostenedione (a known aromatase inhibitor), and a human placental microsomal preparation. A secondary objective was to determine intralaboratory variability estimates for the assay and, as one of three laboratories conducting this assay, provide data that could be used to determine interlaboratory variability. Briefly, 4-hydroxyandrostenedione (4-OH ASDN), at six different concentrations, was incubated with human placental microsomes in the presence of ^{3}H-androstenedione (substrate for aromatase), propylene glycol, and NADPH in a 0.1 M sodium phosphate buffer solution (pH 7.4) at 37 ± 1°C for 15 minutes. Controls included conducting the assay with all assay components except 4-OH ASDN (full enzyme activity control) and NADPH (background activity control). Within each replicate three repetitions were run at each graded concentration of 4-OH ASDN. Additionally two full enzyme activity control tubes and two background activity control tubes were run at the beginning of each replicate and two full enzyme activity and two background activity controls were run at the end. Concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration and extent of inhibition. Four independent replicates of the assay were conducted.

For the human placental microsomes, the overall mean (± sd, ± sem, and percent CV) full aromatase activity control value was 0.0520 nmol/mg protein/min (± 0.0156, ± 0.0039, 30.0%). The background activity control value was < 0.2% of the full enzyme activity control.

4-OH ASDN produced a concentration-dependent inhibition in aromatase activity. At the lowest (10^{-9} M) and highest (10^{-6} M) concentrations tested, the overall mean (± sem) percent of control aromatase activity values were 95.28 ± 0.74 and 7.98 ± 0.24%, respectively. The overall mean (± sem) IC_{50} value for 4-OH ASDN was 81.2 (± 5.5) nM.

Statistical analyses were carried out on the percent of control responses for aromatase activity in four independent replicates. Results were compared across replicates. In addition full enzyme activity and background activity control tube responses were compared between beginning and end of each replicate to identify differences within replicates and differences across replicates. Statistical analysis showed: a) the concentration response curves were similar across the four replicates; b) replicate 1 had a slightly lower estimated IC_{50} and a less negative slope than replicates 2 to 4; c) replicate 2 had a slightly higher estimated IC_{50} and a more negative slope than the other replicates; d) for both the background activity and the full enzyme activity controls averaged across replicates there were not significant differences between the beginning and the end portions; and e) the variability among repetitions within replicates was large compared to the variation of portion (end vs. beginning) effects among replicates.

2.0 INTRODUCTION

2.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the 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 P450 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 P450 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, with IC_{50} values for aromatase inhibition ranging from 0.04 μM to greater than 50 μM.

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 independent replicates (in triplicate) of the aromatase assay were performed using 4-hydroxyandrostenedione (4-OH ASDN) and human placental microsomes. Six different concentrations of 4-OH ASDN were tested, and the IC_{50} for each replicate was calculated using Prism software as specified in the protocol.

The objective of presented study was to conduct the aromatase assay using human placental microsomes and 4-OH ASDN (known aromatase inhibitor) to demonstrate the responsiveness of the assay to aromatase inhibitors. 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 Appendix 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 99%. 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 Results section.)

Preparing the substrate solution involved mixing of non-radiolabeled and radiolabeled [^{3}H]ASDN in order to achieve a100 nM final concentration of ASDN in the assay. The

amount of tritium added to each incubation was about 0.1 µCi. This substrate solution had a concentration of 2 µM with a radioactivity of about 1 µCi/mL.

The following describes 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 a 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 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 radioactivity content analysis.

3.2 Test Substance

The Sponsor' 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. (See Results section.)

3.2.1 4-Hydroxyandrostendione (4-OH ASDN)

Table 1 summarizes all information about used test substance.

Table 1. 4-Hydroxyandrostendione (4-OH ASDN)

Chemical Name	Chemical Code	Mfr. Purity	CAS No.	Molecular Formula	Molecular Weight (g/mol)	Stock Solution ID	Vehicle	Storage Conditions (°C)
4- hydroxyandrostenedione	4-OH ASDN	99%	566-48-3	$C_{19}H_{26}O_3$	302.4	1-ASDN-1	95% ethanol	2-8

3.2.2 Preparation of the Working Solutions of 4-Hydroxyandrostendione

Test substance stock solution was prepared (as described in Table 1) by Chemical Repository as a 0.01 M solution in 95% ethanol. Subsequent dilutions of the stock solution were prepared in 95% ethanol (supplied by CR) according to Table 2.

Table 2. Preparation of 4-Hydroxyandrostenedione Dilutions

Solution Name Concentration (mM)	Volume of Solution (µL)	Volume of Ethanol (µL)	Dilution Name Concentration (mM)	Final Concentration in the Assay (M)
Stock Sol (10 mM)	100	900	Sol.1 (1.0 mM)	N/A
Sol 1 (1 mM)	100	900	Sol 2 (0.1 mM)	1×10^{-6}
Sol 2 (0.1 mM)	100	900	Sol 3 (0.01 mM)	1×10^{-7}
Sol 2 (0.1 mM)	50	950	Sol 4 (0.005 mM)	5×10^{-8}
Sol 2 (0.1 mM)	25	975	Sol 5 (0.0025 mM)	2.5×10^{-8}
Sol 3 (0.01 mM)	100	900	Sol 6 (0.001 mM)	1×10^{-8}
Sol 6 (0.001 mM)	100	900	Sol 7 (0.0001 mM)	1×10^{-9}

3.3 Microsomes

Human placenta microsomes were provided by RTI International, Lot No. 11343-7 and were stored at approximately -70°C until the time of assay. On the day of use, microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter Elvejhem homogenizer (about five to ten passes) and then kept on ice until used (no longer than 2 hours).

The protein concentration in the 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 the microsomal stock suspension with 4.9 mL of buffer (total volume 5 mL). The second dilution (1:10) was obtained by gently mixing 3 mL of the first microsomal dilution with 27 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

The information about other assay components is provided in Table 3.

Table 3. Assay Components

Chemical	Supplier	Lot Number
NADPH	Sigma	103K7046
Propylene glycol	Spectrum Chemical	SQ0397
Sodium phosphate dibasic	Sigma	083K0120
Sodium phosphate monobasic	Sigma	100K0246
Ethanol, 95%	Sponsor	SW0045 and 04B10UB

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 by dissolving ca. 20 mg of NADPH in 4 mL of assay buffer.

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 Milli-Q water and one liter of 0.1 M solution of sodium phosphate dibasic (Na_2HPO_4) in Milli-Q water were prepared. The solutions were combined in the approximate ratio 80:20 (dibasic: monobasic sodium phosphate) to achieve a pH of 7.4.

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 the microsome solution (1:50 dilution in assay buffer) or a 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 transfer to disposable polystyrene cuvettes and the absorbance at 750 nm was measured using 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.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

The assays were performed in 13x100 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 final concentrations for the assay major components are presented in Table 4. 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. See Table 5 for the microsomal thaw times.

Table 4. Aromatase Assay Conditions using Human Placenta Microsomes

Assay Components	Component Volume Added to the Assay	Final Concentration in the Assay
Microsomal Protein	1.0 mL	0.0125 mg/mL
NADPH	100 µL	0.3 mM
[^{3}H]ASDN	100 µL	100 nM
Propylene glycol	100 µL	5 %(v/v)
4-OH ASDN	20 µL	Varies[a]
Assay buffer	680 µL	~ 0.094 M

[a] See Table 7 for details.

Table 5. Microsomal Thaw Times

Replicate	Removed from Freezer	End Time of 5 Min. Incubation Prior to Assay Start	Time Last Assay Tube Quenched	Time Elapsed Between Removal from Freezer to the Quenching of the Last Assay Tube
1	12:30 PM	1:25 PM	1:46 PM	1:16
2	11:20 AM	11:50 AM	12:11 PM	0:51
3	12:00 PM	12:45 PM	1:06 PM	1:06
4	11:20 AM	12:00 PM	12:21 PM	1:01

The total assay volume was 2.0 mL and the tubes were incubated for 15 minutes. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for ca. 5 seconds and placed on ice. The tubes were then vortex-mixed an additional 20-25 seconds, then centrifuged using a Beckman GS-6 centrifuge with 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 represents amount of formed ^3H_2O.

Results are presented as the activity (velocity) of the enzyme (aromatase). The amount of the estrogen product formed was determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate (expressed in 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 by the amount of microsomal protein used (in mg) times the incubation time (15 minutes).

Four independent replicates (each in triplicate) of the aromatase assay were performed as presented in Table 6. A fourth replicate was added to the study as per request of the Sponsor because the results from the second replicate appeared aberrant.

Table 6. Summary of the Assays by Dates and Technician

Replicate Number	Date of Assay	Technician
1	12-13-2004	BDL/TD
2	12-15-2004	BDL/TD
3	12-17-2004	BDL/TD
4	02-09-2005	BDL/TD

In each replicate/test run, full and background activity control samples were included. See Table 7 for a design of the assay groups. Full activity control contained substrate (ASDN), NADPH, propylene glycol, buffer, vehicle used for preparation of 4-OH ASDN solutions, and microsomes. Background activity controls contained all full activity control assay components expect aromatase co-factor NADPH and served as assay blanks. Four full activity and four background activity controls were included with each assay run and were processed in the same manner as the other samples. The controls sets were split, so that two tubes (for each full and background activity control sets) were run at the beginning, and two at the end of each assay.

Table 7. Positive Control Study Design

Sample Type	Repetitions (Test Tubes)	Description	Final 4-OH ASDN Concentration (M)
Full Activity Control	4	Complete assay[a] with inhibitor vehicle control	N/A
Background Activity Control	4	Complete assay with inhibitor vehicle control omitting NADPH	N/A
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

3.7 Data Analysis

The reported data include the following information: assay date and run number, technician, inhibitor, total dpm - background dpm, and % activity. The average background dpm values were subtracted from the assayed samples dpm values to provide dpm for specific aromatase activity. A spreadsheet developed by the RTI International was used to process the data into a final form for analysis and evaluation.

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.

IC_{50} was calculated using Prism version 4.0 (GraphPad, San Diego, CA). Percent of control activity data was exported to Prism for curve fitting of the percent of control activity versus log of 4-OH ASDN concentration data using the following equation:

$$Y=100/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

Where: X is the logarithm of 4-OH ASDN concentration (M)
Y is the percent activity.

The software incorporated a weighting factor for the percent of activity values of 1/Y. Observed individual percent activity values above 100% were set to 99.5%.
As shown in the above equation, the curve fitting equation uses the fixed value of 100 as the numerator. Fixing the top and bottom boundary allowed for estimation of the IC_{50} value on inhibition curves that may not span the entire inhibition range from 100% to 0%.

For each test substance 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 is displayed in Table 12 (also see Appendix G for full statistical analysis).

3.8 Retention of Records

All study records, including final report, are retained in the archives as specified in the study protocol.

4.0 RESULTS

4.1 Radiochemical Purity

The radiochemical purity for the ^{3}H-androstenedione was 97% as reported by RTI International (see Appendix C for RTI International report).

4.2 Stock Formulation Analysis

The 0.01 M solution of 4-hydroxyandrostenedione in 95 % ethanol was prepared by the Sponsor's Chemical Repository. The actual concentration was within 10 % of the target concentration (Appendix D).

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 a 1:50 microsomal dilution in the assay buffer was processed. The results of measuring the protein concentration are provided in Table 8.

Table 8. Protein Concentration

Replicate number	Measured Protein Concentration 1:50 dilution (mg/mL)	Stock Microsomes Protein Concentration (mg/mL)	Working Protein Concentration (mg/mL)	Final Protein Concentration in the Assay (mg/mL)
1	0.247	12.367	0.0247	0.0124
2	0.283	14.137	0.0283	0.0142
3	0.210	10.519	0.0210	0.0105
4	0.176	8.776	0.0176	0.0088

4.4 Aromatase Activity

Table 9 summarizes the full aromatase activity control values measured at the beginning and at the end of each assay run (in full control activity samples). Four independent measurements of the full aromatase activity (in duplicate, at the beginning and at the end of each assay) were performed. The overall full activity (mean ± sd, n=4) for all measurements was 0.0520 ± 0.0156 nmol mg^{-1} min^{-1}. Background aromatase activity in control samples (two at the beginning and two at the end of each assay) was very low (<0.2 % of full control aromatase activity) suggesting that there was no nonspecific product formation or unintentional contamination with NADPH (see Appendix E, Individual Replicate Spreadsheets for individual background activity values.)

Table 9. Full Aromatase Activity Controls (FAAC, nmoles/mg protein/min)

Replicate	FAAC Beginning	FAAC End	Within Replicates mean	Within Replicates sd	Within Replicates sem	% CV	Overall mean	Overall sd	Overall sem	Overall % CV
1	0.0444	0.0402	0.0410	0.0026	0.0013	6.41	0.0444[1]	0.0089	0.0026	20.04
1	0.0413	0.0381					0.0520[2]	0.0156	0.0039	29.96
2	0.0367	0.0371	0.0365	0.0007	0.0003	1.88	0.0557[3]	0.0164	0.0047	29.54
2	0.0355	0.0366								
3	0.0517	0.0552	0.0558	0.0035	0.0017	6.26				
3	0.0602	0.0559								
4	0.0766	0.0735	0.0748	0.0013	0.0007	1.80				
4	0.0748	0.0741								

[1] Calculated for first three replicates.
[2] Calculated for all four replicates.
[3] Calculated for 2-4 replicates.

4.5 Percent of Control

Table 10 summarizes aromatase activity (expressed as a present of full activity) detected in assays with various inhibitor (4-OH ASDN) concentrations. Increasing the 4-OH ASDN concentration affected the aromatase activity in a concentration-dependent manner. The highest applied concentration of 4-OH ASDN (1.0×10^{-6} M) inhibited aromatase activity to approximately 8% of full enzyme activity (92% inhibition); the lowest concentration of 4-OH ASDN (1.0×10^{-9}) inhibited aromatase activity approximately 5% (ca. 95% of aromatase activity remained intact, Table 11).

Table 10. Individual Percent of Control Values by Tube and Replicate

Test Substance	Replicate	Log [Test Substance]	Percent of Control Tube 1	Tube 2	Tube 3	mean	sd	sem	% CV
4-OH ASDN	1	-6.00	7.49	7.05	7.53	7.36	0.27	0.15	3.62
		-7.00	43.13	46.25	42.97	44.12	1.85	1.07	4.19
		-7.30	57.99	59.91	60.26	59.39	1.22	0.71	2.06
		-7.60	70.90	68.41	66.24	68.52	2.33	1.35	3.40
		-8.00	76.84	80.79	85.02	80.88	4.09	2.36	5.06
		-9.00	94.49	94.10	96.30	94.96	1.17	0.68	1.24
	2	-6.00	8.37	8.43	8.78	8.53	0.22	0.13	2.60
		-7.00	47.03	46.39	44.65	46.02	1.23	0.71	2.68
		-7.30	62.03	67.22	64.82	64.69	2.60	1.50	4.02
		-7.60	87.31	82.98	81.73	84.01	2.93	1.69	3.49
		-8.00	93.94	95.98	94.85	94.92	1.02	0.59	1.08
		-9.00	105.74	95.78	85.69	95.74	10.03	5.79	10.47

Table 10. Individual Percent of Control Values by Tube and Replicate (continued)

Test Substance	Replicate	Log [Test Substance]	Percent of Control			mean	sd	sem	% CV
			Tube 1	Tube 2	Tube 3				
4-OH ASDN	3	-6.00	7.90	8.22	8.20	8.11	0.18	0.10	2.21
		-7.00	42.84	42.57	43.25	42.89	0.34	0.20	0.80
		-7.30	58.40	62.52	64.48	61.80	3.10	1.79	5.02
		-7.60	72.08	73.62	77.11	74.27	2.58	1.49	3.47
		-8.00	90.07	86.88	87.20	88.05	1.76	1.01	2.00
		-9.00	94.93	92.05	93.32	93.43	1.44	0.83	1.54
	4	-6.00	8.36	7.72	7.67	7.92	0.38	0.22	4.86
		-7.00	46.42	46.78	47.45	46.88	0.52	0.30	1.11
		-7.30	65.23	62.38	57.74	61.78	3.78	2.18	6.12
		-7.60	80.52	76.30	75.38	77.40	2.74	1.58	3.54
		-8.00	92.36	87.58	91.00	90.31	2.46	1.42	2.73
		-9.00	99.93	92.19	98.82	96.98	4.19	2.42	4.32

Table 11. Replicate Mean and Overall Mean Percent of Control Values

Test Substance	Log [Test Substance]	Mean Percent of Control				Overall			
		Repl 1	Repl 2	Repl 3	Repl 4	mean	sd	sem	% CV
4-OH ASDN	-6.00	7.36	8.53	8.11	7.92	7.98	0.49	0.24	6.09
	-7.00	44.12	46.02	42.89	46.88	44.98	1.81	0.91	4.03
	-7.30	59.39	64.69	61.80	61.78	61.92	2.17	1.08	3.50
	-7.60	68.52	84.01	74.27	77.40	76.05	6.46	3.23	8.49
	-8.00	80.88	94.92	88.05	90.31	88.54	5.85	2.93	6.61
	-9.00	94.96	95.74	93.43	96.98	95.28	1.48	0.74	1.56

4.6 IC_{50}

Based on the curve-fit of the percent of control aromatase activity across six concentrations of 4-OH ASDN, the calculated IC_{50} values are presented in Table 12. The overall IC_{50} value based on calculations for all four replicates is 81.2 nM (13.4 % CV) and overall IC_{50} value based on calculation for three (2-4) replicates is 85.4 nM (9.7% CV).

Table 12. Calculated IC_{50} Values

Test Substance	Replicate	Log [IC_{50}]	SE Log [IC_{50}]	IC_{50} (nM)	Slope	SE Slope	Status *	Overall IC_{50}** (nM)	Overall sd	Overall sem	Overall % CV
4-OH ASDN	1	-7.166	0.02004	68.27	-0.8969	0.03032	C	81.2[1]	10.9[1]	5.5[1]	13.4[1]
	2	-7.028	0.02271	93.75	-1.0410	0.04470	C				
	3	-7.112	0.01416	77.25	-0.9511	0.02431	C	85.4[2]	8.3[2]	4.8[2]	9.7[2]
	4	-7.069	0.01339	85.31	-0.9933	0.02402	C				

* The Status of each response curve is indicated as "C" Complete (response curve ranging from essentially 0 to 100 percent of control).

** Arithmetic calculations.

[1] Calculated for all four replicates.

[2] Calculated for replicate 2, 3 and 4.

The following figures (Figures 1 through 3) present concentration response curves. Figure 1 presents average percent of control activity of each replicate (1-4), Figure 2 presents overall average concentration response curve across 1-4 replicates and average responses across repetitions and Figure 3 represents overall average concentration response curve across each replicate (2-4) and average responses across repetitions.

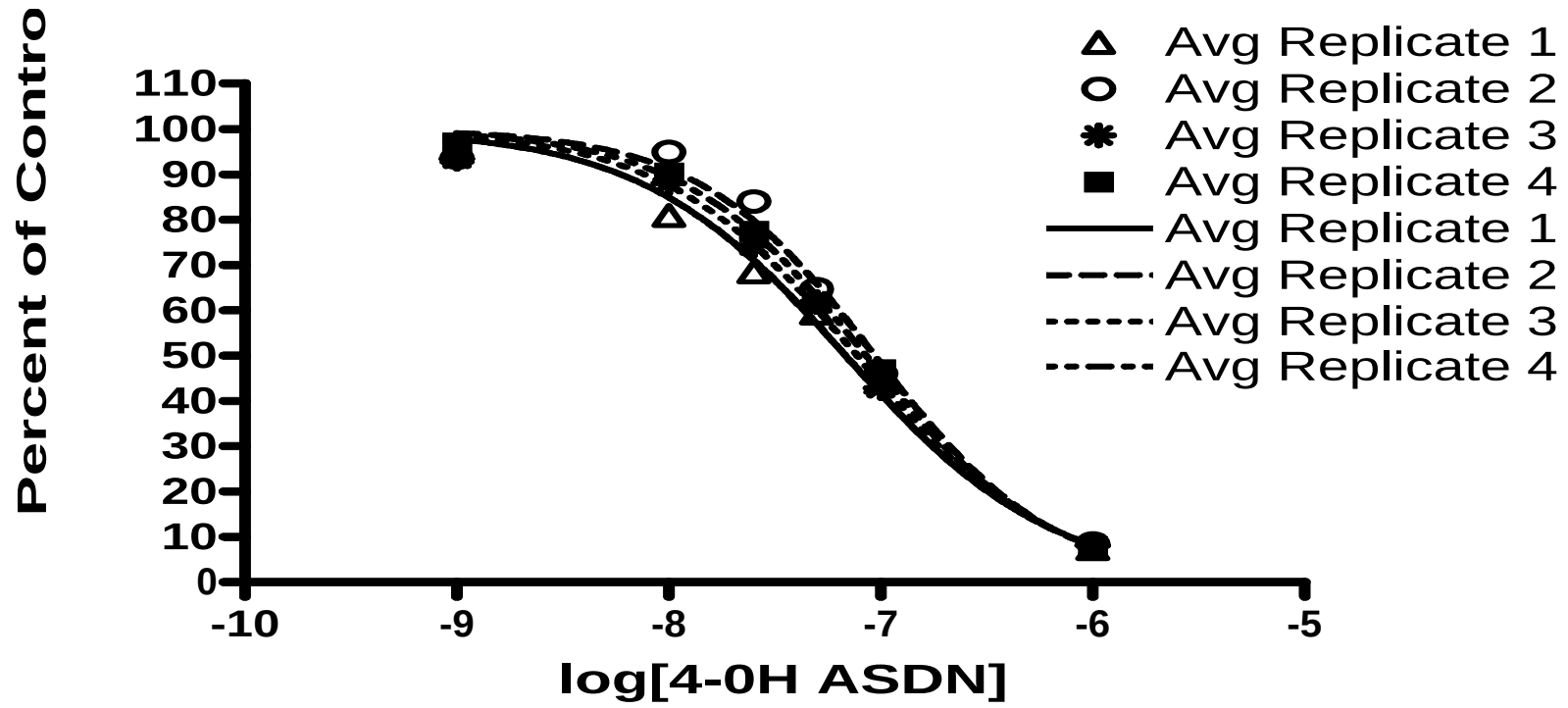

Figure 1. Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations.

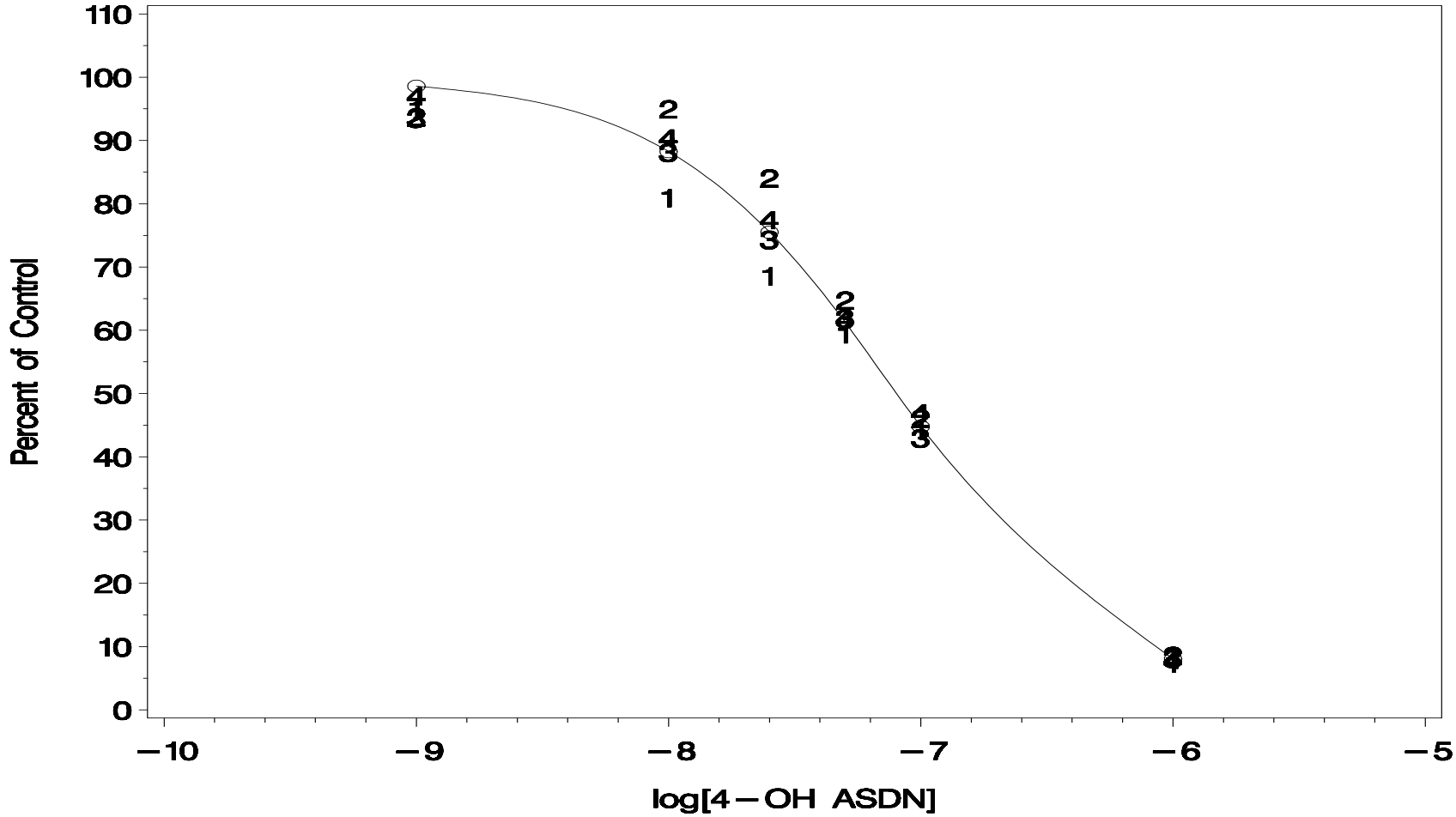

Figure 2. Overall Average Concentration Response Curve Across Replicates 1 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 1 to 4 Parameter Values.

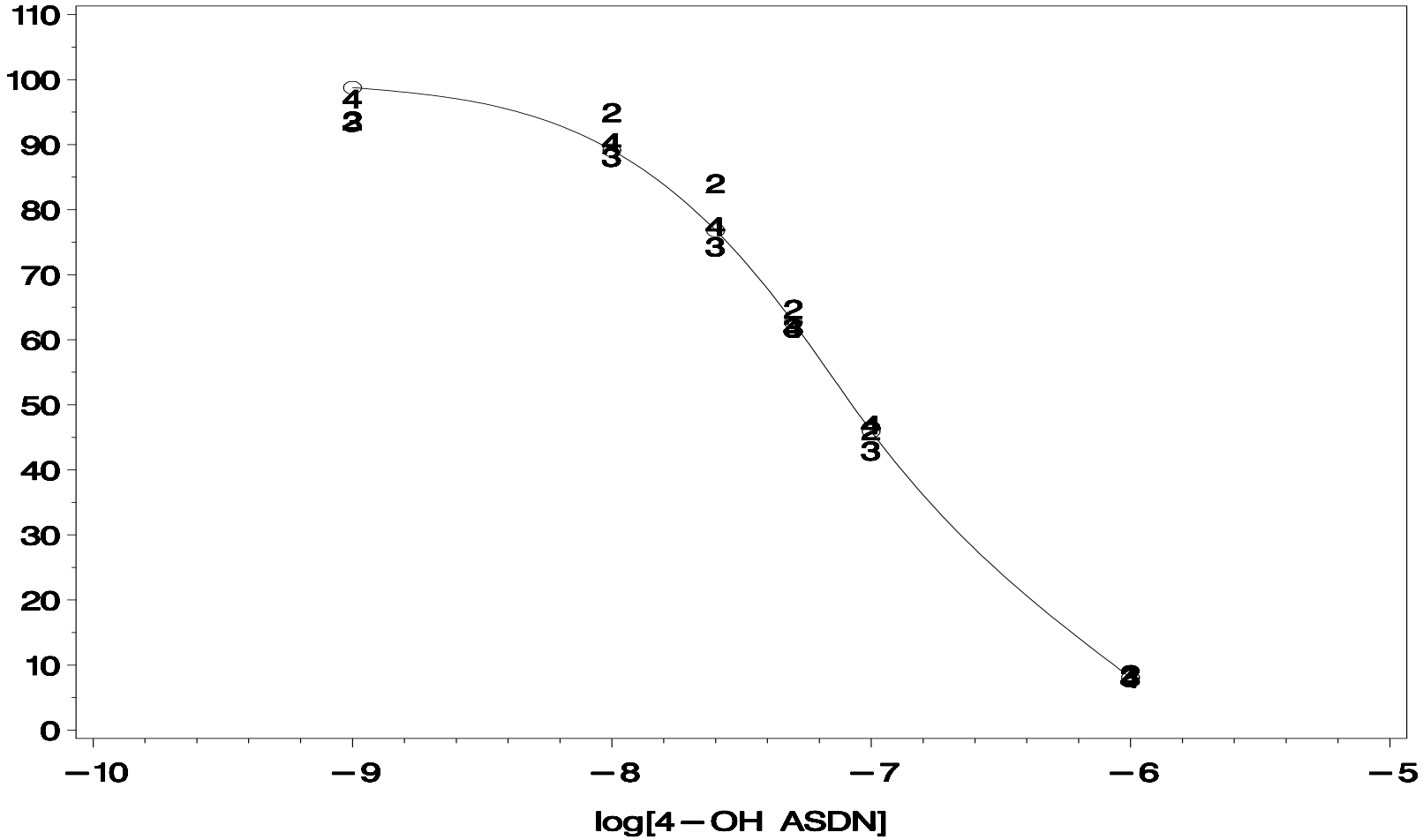

Figure 3. Overall Average Concentration Response Curve Across Replicates 2 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 2 to 4 Parameter Values.

4.7 Statistical Analysis

Full statistical analysis report is presented in Appendix G. There are some small differences in data obtained from Prism output and data presented in Statistical report obtained by applying SAS statistical analysis system. Mixed effects analysis of variance models were fitted to the background activity control and full enzyme activity control data with portion as a fixed effect and with replicate and replicate by portion interaction as random effect. The component of variation due to replicate is constrained to be zero (0) by the definitions of background activity and full enzyme activity control responses. The results are presented in Table 13. No significant differences between the beginning and the end, averaged across replicates, were observed for either background or full enzyme activity controls. The estimated variance for the portion by replicate interaction is considerably smaller than the residual variation, which is based on the variation between the two repetitions carried out within the same portion of the same replicate.

Table 13. Variance Components of Full Enzyme Activity Control and Background Activity Control Percent of Control Values. Position Effects and Variation Across Replicates of Portion Effects Within Replicates.

Parameter	Difference Between Beginning and End Portions		Variance Components			
	Estimate (%) (Std. Error)	p-Value/ Degree of Freedom	Replicate[a]	Portion Replicate	Residual (Repetition)	Total Variance
Replicates 1 to 4						
Background Activity	0.1340 (0.08399)	0.1617/df=6	0	0.0010	0.0263	0.0272
Full Enzyme Activity Control	2.5365 (2.0422)	0.2346/df=14	0	<0.000001	16.6823	16.6823
Replicates 2 to 4						
Background Activity	0.1787 (0.1101)	0.1798/df=4	0	0.0055	0.0253	0.0308
Full Enzyme Activity Control	0.3623 (2.1301)	0.8683/df=10	0	0	13.6123	13.6123

a. The replicate component of variation is constrained to be 0, by definition of background activity and full enzyme activity control responses

The average $\log_{10}IC_{50}$ (μ) and slope (β) estimates across replicates and associated 95% confidence intervals are presented in Table 14 (for graph see Appendix G). Since replicate 1 had a lower IC_{50} and more slowly decreasing slope (β), the average across replicates 2 to 4 had higher IC_{50} and more rapidly decreasing slope (β) than average across four replicates. However, the differences are slight.

The results of analyses of variance for $\log_{10}IC_{50}$ (μ) and slope (β) are presented in Table 15. For each replicate the squares of the standard errors associated with each parameter (μ and β) are given. The variance components across replicates 1 to 4 are greater than those across replicates 2 to 4. For $\log_{10}IC_{50}$, replicate to replicate variation is more than five times the individual replicate within replicate variances, when all four replicates are considered, and more than two times the individual replicate within-replicates variances when just replicates 2 to 4 are considered.

Table 14. 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 of Replicates 2-4 [b]	Mean of Replicates 1-4 [b]
$Log_{10}IC_{50}$	-7.166 (-7.208, -7.124)	-7.028 (-7.076, -6.980)	-7.112 (-7.142, -7.082)	-7.069 (-7.097, -7.041)	-7.072 (-7.178, -6.966)	-7.094 (-7.189, -7.000)
Slope	-0.897 (-0.961, -0.833)	-1.041 (-1.136, -0.947)	-0.951 (-1.003, -0.900)	-0.993 (-1.044, -0.942)	-0.985 (-1.079, -0.891)	-0.966 (-1.062, -0.871)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual 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 15. 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 for Replicates 2-4		Overall for Replicates 1-4	
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Random Replicate (p-value)[d]	Variance of Mean	Random Replicate (p-value)[d]	Variance of Mean
$Log_{10}IC_{50}$	0.000402 /df=16	0.000516 /df=16	0.000201 /df=16	0.000179 /df=16	0.001359 /df=2 (p=0.2123)	0.000548 /df=1.894	0.002962 /df=3 (p=0.1421)	0.000820 /df=2.823
Slope	0.000919 /df=16	0.001998 /df=16	0.000591 /df=16	0.000577 /df=16	0.000622 /df=2 (p=0.3526)	0.000490 /df=2.030	0.002153 /df=3 (p=0.2170)	0.000771 /df=2.646

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual 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 $2*((1/K)*(S_r^2 + S_i^2))^2/(var(S_r^2)+(2/K^2)*(S_i^4/d\ f_i))$, 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.

5.0 DISCUSSION

4- Hydroxyandrostendione (4-OH ASDN) is a known aromatase inhibitor. Six different concentrations of 4-OH ASDN ranging from 1 x 10^{-9} to 1 x 10^{-6} M were applied to create the dose response curve. Four independent replicates for the aromatase assay were performed (one replicate was added because the IC_{50} calculated for the second replicate was slightly out of range when compared to replicates 1 and 3). At an inhibitor concentration of 1 x 10^{-9} M, almost no inhibition was observed (95.28% of control activity) and at 1 x 10^{-6} M almost full inhibition (7.98% of control activity) was observed. The concentration response curves were similar across the four replicates.

The overall IC_{50} value for 1-4 replicates was 81.2 nM and for 2-4 replicates was 85.4 nM. The IC_{50} for the first replicate was significantly lower than the average across 2-4 replicates and the slope for first replicate was significantly higher than the average.

No background enzyme activity was detected for all four replicates. There was no significant difference between full enzyme activity control at the beginning and at the end of each assay within each replicate. A significant difference in full enzyme activity was observed between replicates. The highest full enzyme activity was detected for replicate number four; it was 68.5% higher than average full enzyme activity for remaining three replicates. This phenomenon could be explained by significantly different protein concentrations in 1-3 replicates and replicate 4. The average protein concentration in 1-3 replicates was 0.0124 mg/mL versus 0.0088 mg/mL in replicate 4.

6.0 CONCLUSION

The responsiveness (in concentration dependent manner) of the human placental microsomes aromatase assay to 4-OH ASDN was confirmed.

APPENDIX A

BATTELLE STUDY PROTOCOL

AROMATASE ASSAY VALIDATION
POSITIVE CONTROL STUDY: PLACENTAL MICROSOMES

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

Battelle
The Business *of* Innovation

Experiment Start Date: December 7, 2004
Experiment End Date: January 15, 2005

AROMATASE ASSAY VALIDATION
POSITIVE CONTROL STUDY: PLACENTAL MICROSOMES

APPROVED, BATTELLE:

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AROMATASE ASSAY VALIDATION
POSITIVE CONTROL STUDY: PLACENTAL MICROSOMES

1.0 OBJECTIVES

The objective of this protocol is to describe procedures for conduct of the aromatase assay using human placental microsomes. Positive Control Study refers to the use of 4-hydroxyandrostenedione (4-OH ASDN, a known aromatase inhibitor) in the aromatase assay to demonstrate the responsiveness of the assay to aromatase inhibitors.

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.

Route of administration and reason for its choice: The route of administration is not applicable since the test system uses human placental microsomes. The microsomes, reagents, and test substance will be incubated in a common reaction vessel so that the effect of test substance on aromatase enzymatic activity can be evaluated.

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 ([1β-^{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.

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 Test Substance

4-OH ASDN is a known aromatase inhibitor. Other known or potential inhibitors may be tested.

2.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

CAS No.: 566-48-3
Molecular Formula/Weight: $C_{19}H_{26}O_3$; 302.4 g/mol
Supplier: Sigma
Lot No: 063K4069
Purity: 99% (as per Sigma, assessed by TLC)
Storage Conditions: 2-8°C (for bulk chemical, solution storage conditions to be determined)

2.2.2 Test Substance Formulation and Analysis

Test substance stock solutions will be prepared and analyzed by the Chemical Repository (CR) for the EDSP and distributed to the laboratories. 4-OH ASDN will be formulated in 95% ethanol. The total volume of test 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. Dilutions of the stock solution will be prepared in ethanol on the day of use such that the target concentration of inhibitor can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

2.3 Microsomes

Placental microsomes will be supplied to each laboratory by the lead laboratory. The microsomes must be stored at approximately -70°C. Bulk microsomes (not diluted) could be thawed/freeze several times. The approximate protein content of the microsomes will be provided.

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.

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 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. Under no conditions should microsomes, that have been thawed and diluted for use, be refrozen and used again.

2.4 Other Assay Components

2.4.1 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).

2.4.2 Propylene Glycol

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

2.4.3 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, ranging from 0.13 to 1.5 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) 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. 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 1. 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 1. Optimized Aromatase Assay Conditions

	Assay Type
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.

Results will be presented as the activity (velocity) of the enzyme (aromatase). The amount of estrogen product formed will be determined by dividing the total amount of ^3H_2O formed by the specific activity of the [^{3}H]ASDN substrate (expressed in dpm/nmol). The activity of the enzyme will be expressed in nmol (mg protein)$^{-1}$min^{-1} and will be calculated by dividing the amount of estrogen formed by the amount of microsomal protein used (in mg) times the incubation time, e.g. 15 minutes.

5.0 USE OF THE AROMATASE ASSAY FOR MEASUREMENT OF IC_{50}

5.1 Positive Control Study

Each study will test the aromatase activity inhibition in the presence of 4-OH ASDN. Six different concentrations of 4-OH ASDN will be used. Each concentration of 4-OH ASDN will be run in triplicate. Three replicates of aromatase assay will be run independently. See Table 2 for the study design. Full and background activity control samples will be included in each assay run. Full activity controls will contain substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of 4-OH ASDN solutions), and microsomes. Background activity controls will contain all full activity control assay components except NADPH and will serve as assay blanks. Four full activity and four background activity controls will be included with each assay run and will be processed in the same manner as the other samples. The controls sets will be split so that two tubes (of each full and background activity control) will be run at the beginning and two at the end of each study set.

The assay will be conducted as described in Section 4.0 with the following modification. 4-OH ASDN 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.

Table 2. Positive Control Study Design

Sample type	Repetitions (test tubes)	Description	Final 4-OH ASDN concentration (M)
Full Activity Control	4	Complete assay[a] with inhibitor vehicle control	N/A
Background Activity Control	4	Complete assay with inhibitor vehicle control omitting NADPH	N/A
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, technician, inhibitor, total dpm - background dpm, and % activity. The average background dpm values should be subtracted from the assayed samples dpm values to provide dpm for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from dpm to nmol, as well as the actual methods for calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates 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) yields 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 yields the percent of the substrate that was converted to product. The total dpm remaining in the aqueous portion after extraction is 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 is then converted to nmol product formed by dividing by the substrate specific activity (dpm/nmol). The activity of the enzyme is expressed in nmol (mg protein)$^{-1}$min^{-1} and is 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 is calculated. Percent of activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given inhibitor concentration by the average full activity control and multiplying by 100.

IC_{50} will be calculated using Prism (Version 4.0) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

Where: X is the logarithm of concentration
Y is the percent activity
Bottom is the lower plateau
Top is the upper plateau.

The data will be formatted as follows:

- One spreadsheet or table will display the dpm for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.
- Another table will present the results of the analysis of variability of the assay and will include :
  (1) the variation between replicates within a single assay,
  (2) the day to day (study-to-study) variation.
- Graphs of activity versus log chemical concentration.
- Table of IC_{50}s by date, run, technician, assay method.

6.0 STATISTICAL ANALYSES

Statistical analysis of the data will be conducted by a statistical analysis plan developed by Battelle's EDSP Data Coordination Center. The salient aspects of the plan are described below.

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

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

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 response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1000/DAVG. Model fits will be carried out using Prism software (Version 3 or higher).

The concentration response fits will be carried out for each replicate test within each test compound. 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 an inhibitor compound will be the 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 can be calculated based on a one-way random effects analysis of variance model fit.

6.1 Graphical and Analysis of Variance Comparisons among Concentration Response Curve Fits.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}) and covariance $\Sigma_{(\beta, \mu)}$ across replicates. Let B_{avg}, T_{avg} denote the average bottom and top across the replicates. Let

$$Z / (Y- B_{avg})/(T_{avg} - B_{avg})$$

$$L \equiv \log_{10}(Z/(1 - Z)).$$

The average response curve is expressed as

$$L \equiv \beta_{avg}(\mu_{avg} - X)$$

with approximate standard errors of prediction of L at a given X based on $\Sigma_{(\beta, \mu)}$ and propagation of errors. These are used to calculate approximate confidence intervals for predictions at each X. The linearized response curve and associated confidence intervals are back transformed to yield the response curve in terms of percent of control, Y

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

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average and associated 95% confidence interval across replicates.

6.2 Negative and Positive Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full activity control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are constant 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 combined across replicates and expressed as percent of (full) control activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four full activity controls within a replicate must necessarily be 100. The two beginning controls and the two end controls will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (full activity control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Two-way analysis of variance will be carried out, separately for the full activity control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response

will be percent of control aromatase activity. If the daily replicates are in control the portion main effect and portion by replicate interaction should be nonsignificant. Note that the replicate effects will not be estimable because of the constrained totals within each replicate. For purposes of evaluation replicate will be treated as a fixed effect. If portion by replicate interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates, adjusting for simultaneity by Scheffe's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

6.3 Variability Assessment

For the inhibitor test compound variability among replicates and variability among repetitions within replicates will be estimated and assessed for statistical significance. The response will be aromatase activity. These analyses will treat inhibitor concentration as a classification variable and will include both the full and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including full and background activity groups), replicate, replicate by concentration interaction, and residual variation. Residual variation corresponds to repetition within replicate and concentration. Inhibitor concentration will be treated as a fixed effect. Replicate and replicate by concentration interaction will be treated as random effects. The analysis of variance fit will incorporate weights. The weight for responses in each concentration group will be based on the average of the dpm across all the replicates and repetitions within replicates associated with that concentration group. The weight for each concentration group will be 1000/[Average dpm].

Normal probability plots will be prepared to identify outlying replicates or repetitions. Deviations of average within replicate from average across replicates within that concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average dpm}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups. Deviations of repetitions from average across repetitions within replicate and concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average dpm}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups.

6.4 Statistical Software

Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS).

6.5 Inter-laboratory Statistical Analysis

The inter-laboratory statistical analysis will be carried out by Battelle's EDSP Data Coordination Unit.

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 is 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 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

PROTOCOL AMENDMENT NUMBER 1

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Positive Control Study: Placental Microsomes (WA 4-16, Task 4)**

PART TO BE CHANGED: The second sentence of the Section 3, page 7 is changed from: A 6-point standard curve will be prepared, ranging from 0.13-1.5 mg protein/mL.

CHANGED TO: A 6-point standard curve will be prepared, ranging from 0.14-1.0 mg protein/mL.

REASON FOR CHANGE: The protein standard (bovine serum albumin) stock solution from BioRad Laboratories is 1.4 mg/mL and not 2.5 mg/mL as was originally believed based on information obtained from the Lead Laboratory, Research Triangle Institute, International.

EFFECTIVE DATE: December 16, 2004

APPROVED BY:

Bozena D. Lusiak

Bozena D. Lusiak, Study Director

12-29-04

Date

Jerry D. Johnson

Sponsor Representative

12-29-04

Date

PROTOCOL AMENDMENT NUMBER 2

STUDY NUMBER: **G608316**

STUDY TITLE: **Aromatase Assay Validation: Positive Control Study: Placental Microsomes (WA 4-16, Task 4)**

PART TO BE CHANGED: Paragraph 5.2 (Data Analysis and Presentation) and Section 6.0 (Statistical Analyses); See Attachment 1.

CHANGE TO: See Attachment 2.

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

EFFECTIVE DATE: January 14, 2005

APPROVED BY:

Bozena D. Lusiak, Study Director — January-19-05 Date

Sponsor Representative — 1-20-05 Date

G608316

ATTACHMENT 1

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, technician, inhibitor, total dpm - background dpm, and % activity. The average background dpm values should be subtracted from the assayed samples dpm values to provide dpm for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from dpm to nmol, as well as the actual methods for calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates 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) yields 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 yields the percent of the substrate that was converted to product. The total dpm remaining in the aqueous portion after extraction is 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 is then converted to nmol product formed by dividing by the substrate specific activity (dpm/nmol). The activity of the enzyme is expressed in nmol (mg protein)$^{-1}$min^{-1} and is 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 is calculated. Percent of activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given inhibitor concentration by the average full activity control and multiplying by 100.

IC_{50} will be calculated using Prism (Version 4.0) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogIC_{50}-X)*HillSlope)})$$

Where: X is the logarithm of concentration
Y is the percent activity
Bottom is the lower plateau
Top is the upper plateau.

The data will be formatted as follows:

- One spreadsheet or table will display the dpm for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.
- Another table will present the results of the analysis of variability of the assay and will include :
  (1) the variation between replicates within a single assay,
  (2) the day to day (study-to-study) variation.

- Graphs of activity versus log chemical concentration.
- Table of IC_{50}s by date, run, technician, assay method.

6.0 STATISTICAL ANALYSES

Statistical analysis of the data will be conducted by a statistical analysis plan developed by Battelle's EDSP Data Coordination Center. The salient aspects of the plan are described below.

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

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

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 response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1000/DAVG. Model fits will be carried out using Prism software (Version 3 or higher).

The concentration response fits will be carried out for each replicate test within each test compound. 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 an inhibitor compound will be the 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 can be calculated based on a one-way random effects analysis of variance model fit.

6.1 Graphical and Analysis of Variance Comparisons among Concentration Response Curve Fits.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}) and covariance $\Sigma_{(\beta, \mu)}$ across replicates. Let B_{avg}, T_{avg} denote the average bottom and top across the replicates. Let

$$Z / (Y - B_{avg})/(T_{avg} - B_{avg})$$

$$L / \log_{10}(Z/(1 - Z)).$$

The average response curve is expressed as

$$L / \beta_{avg}(\mu_{avg} - X)$$

with approximate standard errors of prediction of L at a given X based on $\Sigma_{(\beta, \mu)}$ and propagation of errors. These are used to calculate approximate confidence intervals for predictions at each X. The linearized response curve and associated confidence intervals are back transformed to yield the response curve in terms of percent of control, Y

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

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average and associated 95% confidence interval across replicates.

6.2 Negative and Positive Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full activity control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are constant 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 combined across replicates and expressed as percent of (full) control activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four full activity controls within a replicate must necessarily be 100. The two beginning controls and the two end controls will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (full activity control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Two-way analysis of variance will be carried out, separately for the full activity control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response will be percent of control aromatase activity. If the daily replicates are in control the portion main effect and portion by replicate interaction should be nonsignificant. Note that the replicate effects will not be estimable because of the constrained totals within each replicate. For purposes of evaluation replicate will be treated as a fixed effect. If portion by replicate interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates, adjusting for simultaneity by Scheffe's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

6.3 Variability Assessment

For the inhibitor test compound variability among replicates and variability among repetitions within replicates will be estimated and assessed for statistical significance. The response will be aromatase activity. These analyses will treat inhibitor concentration as a classification variable and will include both the full and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including full and background activity groups), replicate, replicate by concentration interaction, and residual variation. Residual variation corresponds to repetition within replicate and concentration. Inhibitor concentration will be treated as a fixed effect. Replicate and replicate by concentration interaction will be treated as random effects. The analysis of variance fit will incorporate weights. The weight for responses in each concentration group will be based on the average of the dpm across all the replicates and repetitions within

replicates associated with that concentration group. The weight for each concentration group will be 1000/[Average dpm].

Normal probability plots will be prepared to identify outlying replicates or repetitions. Deviations of average within replicate from average across replicates within that concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average dpm}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups. Deviations of repetitions from average across repetitions within replicate and concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average dpm}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups.

6.4 Statistical Software

Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons will be carried out using the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS).

6.5 Inter-laboratory Statistical Analysis

The inter-laboratory statistical analysis will be carried out by Battelle's EDSP Data Coordination Unit.

G608316

ATTACHMENT 2

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, technician, chemical and log chemical concentration, total dpm-background dpm, and % activity. The average of the dpm for the background tubes should be subtracted from the tubes with Total dpm to provide dpm for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from dpm to nmol, as well as the actual methods for calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates 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) yields 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 yields the percent of the substrate that was converted to product. The total dpm remaining in the aqueous portion after extraction is corrected for background by subtracting the average dpm present in the aqueous portion of the background activity tubes (for that day/assay). 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)$^{-1}$min^{-1} and is calculated by dividing the amount of estrogen formed (nmol) by the product of mg microsomal protein used times the incubation time. Average activity in the full enzyme activity control samples for a given Study is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given concentration by the average full enzyme activity control and multiplying by 100.

IC_{50} will be calculated using Prism (Version 3 or higher) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=100/(1+10^{((LogIC50-X)*HillSlope)})$$

Where: X is the logarithm of concentration
Y is the percent activity.

The data will be formatted as follows:

- One spreadsheet or table will display the dpm for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.

- Another table will present the results of the analysis of variability of the assay and will include :

(1) the variation between repetitions within a single replicate of the assay,
(2) the day to day (replicate-to-replicate) variation, and
(3) technician variation

- Graphs of activity versus log chemical concentration.
- Table of IC_{50}s by date, run, technician, assay method.

6.0 STATISTICAL ANALYSES

6.1 Concentration Response Fits for the Test Substance

For the test substance multiple independent replicates of the concentration response curve fit will be carried out. The number of replicates will be three. Full enzyme activity and background activity control percent activity values will be compared across daily replicate tests for each test substance.

For each replicate two repeat tubes of the full enzyme activity controls and the background activity controls will be prepared prior to the preparation of the repetitions of the inhibitor compound and two repeat tubes of the full enzyme activity controls and the background activity controls will be prepared after the repetitions of the inhibitor compound are prepared. Three repetitions will be prepared for each level of the inhibitor compound (4-OH ASDN).

For each repetition at each level the Excel database spreadsheet will include total dpms per tube (corrected for background dpms) and total aromatase activity per tube. The aromatase activity is calculated as the (background corrected) dpm, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The aromatase activity is corrected for the background dpms, as measured by the average of the background activity tubes. Percent activity is the (background corrected) aromatase activity divided by the average of the aromatase activity in the full enzyme activity control tubes, multiplied by 100. Thus the average percent activity across the four background activity 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. The total dpm values are not corrected for background.

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 individual experimental percent of control activity 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 inhibitor 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 inhibitor compound (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 inhibitor 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 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%.[1]

The concentration response fits will be carried out for each replicate test. 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 the inhibitor 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 can be calculated based on a one-way random effects analysis of variance model fit.

For each test substance and replicate the estimated $\log_{10}IC_{50}$ (Φ), 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".

[1]This adjustment tacitly assumes an upper bound of 100% and a lower bound of 0%. Fixing these bounds rather than permitting PRISM to fit variable Top and Bottom parameters permits estimation of the IC_{50} concentration on inhibition curves that do not span the entire inhibition range from 100% to 0%.

6.2 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 inhibitor compound 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 inhibitor concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curve for each replicate will be superimposed on the plot. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of inhibitor compound 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 random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average across replicates and associated 95% confidence interval across replicates (including replicate-to-replicate variation).

6.3 Full Enzyme Activity and Background Activity Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the full enzyme activity control tubes. Half the repetitions will be carried out at the beginning of the replicate and half at the end. If the conditions are constant 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 combined across replicates and expressed as percent of full enzyme activity control activity. The average of the four background activity 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 will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (full enzyme activity control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate.

Two-way analysis of variance will be carried out, separately for the full enzyme activity control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response will be percent of control aromatase activity. If the daily replicates are in control the portion main effect and portion by replicate interaction should be non-significant. Note that the replicate effects will necessarily be zero because of the constrained totals within each replicate. For the purposes of evaluation, replicate will be treated as a fixed effect. If portion by replicate interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates, adjusting for simultaneity by Bonferroni's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

6.4 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 the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS).

6.5 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.

Appendix B

1.0 TITLE AND APPROVAL

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

Task 4 - Conduct Positive Control Studies
in the Participating Laboratories

for

EPA CONTRACT NUMBER 68-W-01-023

December 7, 2004

SIGNATURE PAGE

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

Concurrences and Approvals

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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 positive control 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 Tasks 4 through 7 of the work assignment.

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

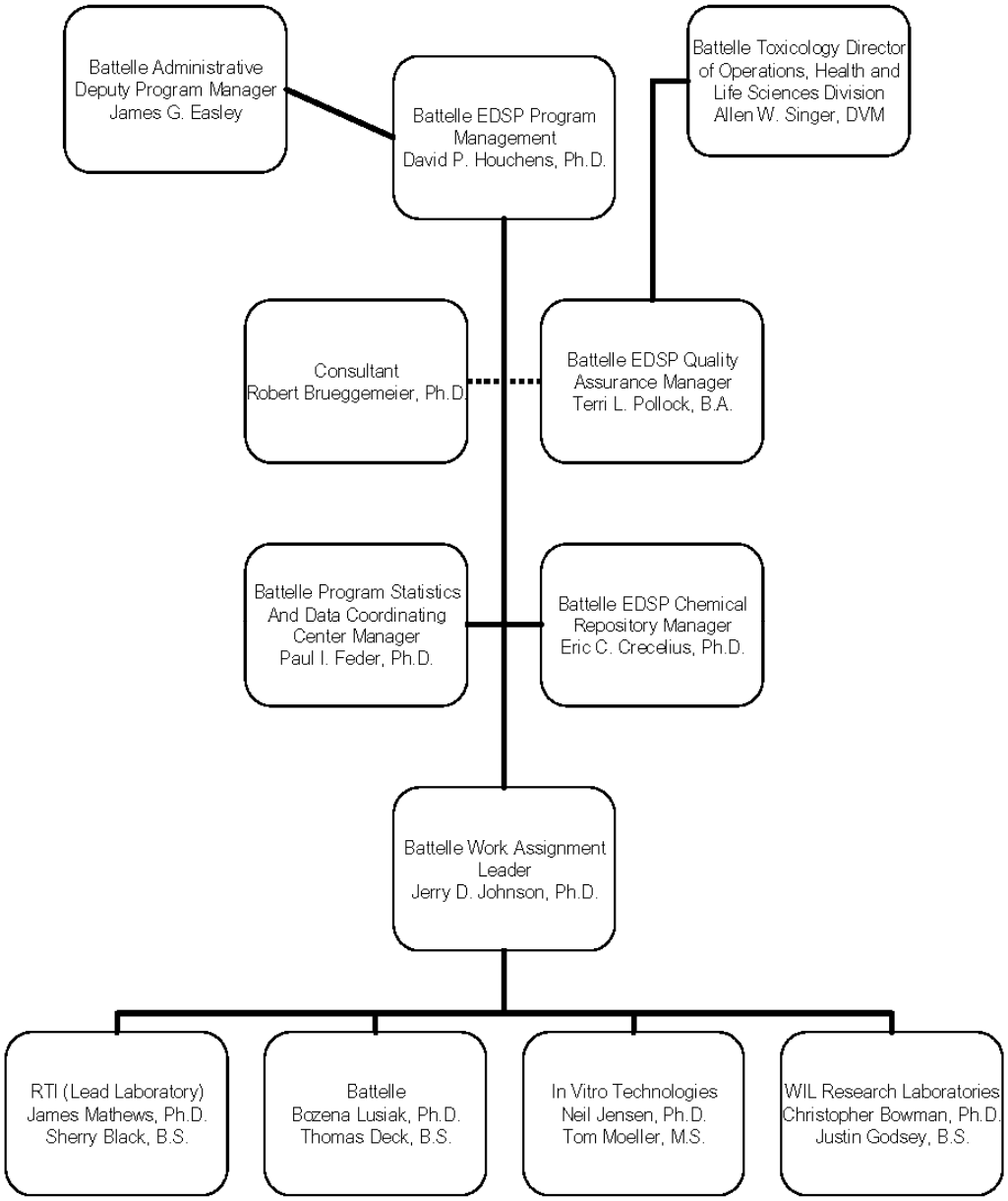

Figure 1. WA 4-16 Project Organization Overview

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 is 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 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 WA L/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 prevalidation 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 $P450_{arom}$ 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 $P450_{arom}$ 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 μM to greater than 50 μM.

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 4 is under the control by 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 protocol included as an attachment. The Task 4 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 will be completed by staff at Battelle, WIL and In Vitro. RTI staff will not conduct any experiments on this task but will be involved in the review of the data produced by the other laboratories. 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 replicates of a Positive Control Study. In this Study, 4-OH androstenedione (4-OH ASDN, a known aromatase inhibitor) will be 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 will be 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) will supply 4-OH ASDN to each laboratory as a stock solution and will conduct all necessary pre-assay chemistry activities for 4-OH ASDN.

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 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). Battelle's CR will supply the test chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test 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 are also included in each assay set to measure full aromatase activity (without any inhibitor added) and background activity (without NADPH co-factor). Battelle's CR will supply the test chemicals to each laboratory as individual stock solutions and will conduct all necessary pre-assay chemistry activities for the test 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 mean positive control activity for each assay/laboratory should be within the overall mean ± 15% 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 positive control activity between and within laboratories should be statistically equivalent at the $p > 0.1$ level. Any modifications to this criterion would 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 positive control and background activity samples that are run with each assay are 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.

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 will be 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 will conduct the aromatase assay including positive control and background activity samples and a series of samples containing varying amounts of a known aromatase inhibitor (4-OH ASDN). The resultant data will be 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, they will be 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 for freezing. 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, otherwise the samples should be frozen and stored at about -20°C.

Each test 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 Chemical Solutions

The test 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 (positive and negative) are run with each assay. 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.

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 manually into Prism data files for calculation of IC_{50} and undergo a 100% QC check. 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 are 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 are 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 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

DRAFT PROTOCOL FOR TASK 4

PROTOCOL		Page 1 of 15

EPA Contract No.:
EPA Work Assignment No.:

TITLE: Template Protocol for WA 4-16 Task 4: Conduct of the Positive Control Studies in the Participating Laboratories

SPONSOR:

TESTING FACILITY:

PROPOSED EXPERIMENTAL START DATE:

PROPOSED EXPERIMENTAL END DATE:

AMENDMENTS:

Number	Date	Section(s)	Page(s)
1			
2			
3			
4			
5			

Approved By:

Study Director Date

Jerry Johnson, Ph.D, DABT Date
Battelle Work Assignment Leader

David Houchens, Ph.D. Date
Battelle EDSP Program Manager

Reviewed By:

Quality Assurance Specialist Date

Terri Pollock, B.A. Date
EDSP Quality Assurance Manager

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1.0 OBJECTIVES

Task 4: Conduct of the Positive Control Studies in the Participating Laboratories

The objective of this protocol is to describe procedures for conduct of the aromatase assay using placental microsomes. Positive Control Study refers to the use of 4-hydroxyandrostenedione (4-OH ASDN, a known aromatase inhibitor) in the aromatase assay to demonstrate the responsiveness of the assay to aromatase inhibitors.

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.

Route of administration and reason for its choice: The route of administration is not applicable since the test system is a microsome. The method used for treating the microsomes will be to mix the microsomes, reagents, and test article in a common reaction vessel so that microsomal uptake of the test article can be used to evaluate the effect on enzymatic activity.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

A sufficient supply of chemical reagents, radiolabeled and non-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.

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 ([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 (of each lot that is used) will be assessed by the lead laboratory as described in Section 2.1.2

2.1.2 Radiochemical Purity (Lead Laboratory only)

The radiochemical purity of the [^{3}H]ASDN will be 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 will be collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method uses a Zorbax SB-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 will be monitored by UV absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions will be collected manually into vials containing ca. 10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS). A reference standard of nonradiolabeled ASDN will be analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN will be confirmed.

The radiochemical purity of the [^{3}H]ASDN will be greater than approximately 95 percent. If the radiochemical purity is less than 95 percent, then the Sponsor will be notified.

2.1.3 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 Test Substances

4-OH ASDN is a known aromatase inhibitor. Other known or potential inhibitors may be tested.

2.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

CAS No.: 566-48-3
Molecular Formula/Weight: $C_{19}H_{26}O_3$; 302.4 g/mol
Supplier: Sigma
Lot No: tbd
Purity: tbd
Storage Conditions: 2-8°C (for bulk chemical, solution storage conditions to be determined)

2.2.2 Test Substance Formulation and Analysis

Test substance stock solutions will be prepared and analyzed by the CR and distributed to the laboratories. 4-OH ASDN will be formulated in 95 percent ethanol. The total volume of test 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. Dilutions of the stock solution will be prepared in 95 percent ethanol on the day of use such that the target concentration of inhibitor can be achieved by the addition of 20 µL of the dilution to a 2 mL assay volume.

2.3 Microsomes

Placental microsomes will be supplied to each laboratory by the lead laboratory. The microsomes must be stored at -70 to -80°C. The approximate protein content of the microsomes will be provided.

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.

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 (about 5-10 passes) prior to use. The microsomes are diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.008 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.004 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. 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. Under no conditions should microsomes be thawed and refrozen for later use in the assay.

2.4 Other Assay Components

2.4.1 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).

2.4.2 Propylene Glycol

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

2.4.3 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. 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 1. 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 1. Optimized Aromatase Assay Conditions

Assay factor (units)	Assay Type	
	Human Placental	Human Recombinant
Microsomal Protein (mg/mL)[a]	0.0125	0.004
NADPH (mM)[a]	0.3	0.3
[^{3}H]ASDN (nM)[a]	100	100
Incubation Time (min)	15	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.

Results will be presented as the activity (velocity) of the enzyme reaction. 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). 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.

5.0 USE OF THE AROMATASE ASSAY FOR MEASUREMENT OF IC_{50}

5.1 Positive Control Study

Each study will test the response of aromatase activity to the presence of six concentrations of 4-OH ASDN. This study will be conducted in three independent replicates by each participating laboratory. Each concentration of 4-OH ASDN will be run in triplicate tubes in each Study. See Table 2 for the study design. Full enzyme activity control and background

activity samples will be included for each study. Full enzyme activity controls will contain substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of 4-OH ASDN solutions) and microsomes. Background activity samples contain all full enzyme activity control assay components except NADPH and serve as assay blanks. Four full enzyme activity control and four background activity samples are included with each Study and are treated the same as the other samples. The controls sets will be split so that two tubes (of each full enzyme activity control and background activity samples) are run at the beginning and two at the end of each study set.

The assay will be conducted as described in Section 4.0 with the following modification. 4-OH ASDN 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.

Table 2. Positive Control Study Design

Sample type	Repetitions (test tubes)	Description	4-OH ASDN concentration (M final)
Full enzyme activity control	4	Complete assay[a] with inhibitor vehicle control	N/A
Background Activity	4	Complete assay with inhibitor vehicle control omitting NADPH	N/A
4-OH ASDN Concentration 1	3	Complete assay with 4-OH ASDN added	1×10^{-6}
4-OH ASDN Concentration 2	3	Complete assay with 4-OH ASDN added	1×10^{-7}
4-OH ASDN Concentration 3	3	Complete assay with 4-OH ASDN added	5×10^{-8}
4-OH ASDN Concentration 4	3	Complete assay with 4-OH ASDN added	2.5×10^{-8}
4-OH ASDN Concentration 5	3	Complete assay with 4-OH ASDN added	1×10^{-8}
4-OH ASDN Concentration 6	3	Complete assay with 4-OH ASDN added	1×10^{-9}

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

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, technician, chemical and log chemical concentration, total DPM-background DPM, and % activity. The DPMs for the background tubes should be subtracted from the tubes with Total DPMs to provide DPMs for specific aromatase activity. A spreadsheet will be developed by the lead laboratory that will be used to process the data into a final form for analysis and evaluation. A working document detailing the conversion of the data from DPMs to nmol, as well as the actual methods for calculations of the final aromatase activity will be distributed to the laboratories. This process is briefly summarized below.

The spreadsheet calculates 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) yields 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 yields the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the background activity tubes (for that day/assay). 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)$^{-1}$min^{-1} and is calculated by dividing the amount of estrogen formed (nmol) by the product of mg microsomal protein used times the incubation time. Average activity in the positive control samples for a given Study is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given concentration by the average positive control activity and multiplying by 100.

IC_{50} will be calculated using Prism (Version 3.02) software to fit the percent of control activity and log concentration data to a curve using the following equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogIC50-X)*HillSlope))}$$

Where: X is the logarithm of concentration
Y is the percent activity
Bottom is the lower plateau
Top is the upper plateau.

The data will be formatted as follows:

- One spreadsheet or table will display the DPMs for all assay tubes, calculations of activity (nmol (mg protein)$^{-1}$min^{-1}) etc.

- Another table will present the results of the analysis of variability of the assay and will include :

(1) the variation between replicates within a single assay,
(2) the day to day (study-to-study) variation, and
(3) technician variation.

- Graphs of activity versus log chemical concentration.
- Table of IC_{50}s by date, run, technician, assay method.

6.0 STATISTICAL ANALYSES

Concentration-response curves will be fitted to describe trends in the aromatase activity percent of control responses. Full enzyme activity control and background activity values will be compared across daily replicate tests for each test substance.

6.1 Concentration Response Fits for the Test Substance

For the test substance 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 positive controls and the background activity samples will be prepared prior to the preparation of the repetitions of the inhibitor compound and two repeat tubes of the positive controls and the background activity samples will be prepared after the repetitions of the inhibitor compound are prepared. Three repetitions will be prepared for each level of the inhibitor compound (4-OH ASDN).

For each repetition at each level the Excel database spreadsheet will include total DPMs per tube and total aromatase activity per tube. The aromatase activity is calculated as the DPM, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The aromatase activity is corrected for the background DPMs, as measured by the average of the background activity tubes. Thus the average aromatase activity across the four background activity repeat tubes must necessarily equal 0 within each replicate. The total DPM values are not corrected for background.

For each repetition within each inhibitor concentration, percent of control activity is determined by dividing the aromatase activity for that tube by the average positive control activity 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 individual experimental percent of control activity values will sometimes extend below 0% or above 100%. Thus upper and lower response curve plateaus need to be included in the response curve models,

Concentration response trend curves will be fitted to the percent of control activity values within each of the repeat tubes at each inhibitor 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 inhibitor compound (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
T ≡ upper plateau of the concentration response curve
B ≡ lower plateau of the concentration response curve
DAVG ≡ average DPMs across the repeat tubes with the same inhibitor 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

$$Y = B + (T - B)/[1 + 10^{(\mu - X)\beta}] + \epsilon$$

where ϵ is the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts). The response curve will be fitted by weighted least squares nonlinear regression analysis with weights equal to 1000/DAVG. Model fits will be carried out using Prism software (Version 3 or higher).

The concentration response fits will be carried out for each replicate test within each test compound. 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 an inhibitor compound will be the 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 can be calculated based on a one-way random effects analysis of variance model fit.

6.2 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 inhibitor compound 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 inhibitor concentration on the same plot. Plotting symbols will distinguish among replicates. The fitted concentration response curve for each replicate will be superimposed on the plot. On a separate plot the average percent of control values for each replicate will be plotted versus logarithm of inhibitor compound concentration. The average concentration response curve across replicates will be superimposed on the same plot with 95 percent confidence intervals on average control values at each observed concentration. Replicate-to-

replicate variation will be treated as a random effect for purposes of calculating confidence intervals.

For each replicate treat (β, μ) as a random variable with mean (β_{avg}, μ_{avg}) and covariance $\Sigma_{(\beta, \mu)}$ across replicates. Let B_{avg}, T_{avg} denote the average bottom and top across the replicates. Let

$$Z \equiv (Y - B_{avg})/(T_{avg} - B_{avg})$$

$$L \equiv \log_{10}(Z/(1 - Z)).$$

The average response curve is expressed as

$$L \equiv \beta_{avg}(\mu_{avg} - X)$$

with approximate standard errors of prediction of L at a given X based on $\Sigma_{(\beta, \mu)}$ and propagation of errors. These are used to calculate approximate confidence intervals for predictions at each X. The linearized response curve and associated confidence intervals are back transformed to yield the response curve in terms of percent of control, Y

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

Slope (β) and $\log_{10}IC_{50}$ (μ) will also be compared across replicates based on random effects analysis of variance, treating the replicates as random effects. β and μ are estimated, separately within each replicate, and plotted along with the average and associated 95% confidence interval across replicates.

6.3 Negative and Positive Control Values Across Replicates

Within each replicate, quadruplicate repetitions will be made of the background activity tubes and the 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 constant 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 combined across replicates and expressed as percent of (positive) control activity. The average of the four background activity samples within a replicate must necessarily be 0 and the average of the four positive controls within a replicate must necessarily be 100. The two beginning controls and the two end controls will be plotted by replicate with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity) or 100% (positive control) respectively. These plots will display the extent of consistency across replicates with respect to average value and variability and will provide comparisons of beginning versus end of each replicate. Two-way analysis of variance will be carried out, separately for the positive control tubes and the background activity tubes. The factors in the analysis of variance will be replicate, portion (beginning or end), replicate by portion interaction. The error corresponds to repetition within replicate and portion. The response will be percent of control aromatase activity. If the daily replicates are in control the

portion main effect and portion by replicate interaction should be nonsignificant. Note that the replicate effects will not be estimable because of the constrained totals within each replicate. For purposes of evaluation replicate will be treated as a fixed effect. If portion by replicate interaction is significant the nature of the effect will be assessed by comparing the portion effect within each replicate to the portion effect averaged across replicates, adjusting for simultaneity by Scheffe's method. The portion effect within each replicate and the portion effect averaged across replicates, and associated 95% confidence intervals, will be presented graphically.

6.4 Variability Assessment

For the inhibitor test compound variability among replicates and variability among repetitions within replicates will be estimated and assessed for statistical significance. The response will be aromatase activity. These analyses will treat inhibitor concentration as a classification variable and will include both the positive and background activity groups. The factors in the mixed effects analysis of variance will be concentration group (including positive and background activity groups), replicate, replicate by concentration interaction, and residual variation. Residual variation corresponds to repetition within replicate and concentration. Inhibitor concentration will be treated as a fixed effect. Replicate and replicate by concentration interaction will be treated as random effects. The analysis of variance fit will incorporate weights. The weight for responses in each concentration group will be based on the average of the DPMs across all the replicates and repetitions within replicates associated with that concentration group. The weight for each concentration group will be 1000/[Average DPM].

Normal probability plots will be prepared to identify outlying replicates or repetitions. Deviations of average within replicate from average across replicates within that concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average DPM}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups. Deviations of repetitions from average across repetitions within replicate and concentration group will be ordered and plotted on a normal probability scale. The differences will be normalized by $[\text{Average DPM}]^{1/2}$ for their concentration group to adjust for differing variability across concentration groups.

6.5 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 the SAS statistical analysis system, Version 8 or higher, or other general purpose statistical packages (e.g. SPSS).

6.6 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 (e.g. outlying laboratories), the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories.

The results of the intra-laboratory analyses will be concentration response curve fits associated with the positive control inhibitor 4-OH-ASDN. For each inhibitor compound they will also characterize variability among replicates and variability among repetitions within replicates.

The inter-laboratory analysis will be based on the IC_{50} and slope parameters of the concentration response curve fits and the replicate-to-replicate and repetition within replicate components of variation. The objectives of the inter-laboratory statistical analysis are to:

- Determine the average values and variability among laboratories with respect to the within-laboratory parameters mentioned above
- Determine the coefficient of variation among laboratories for each of the within-laboratory parameters mentioned above
- Estimate the ratio of within laboratory variation to among laboratory variation for each of the parameters
- Identify outlying laboratories, if any
- Assess the extent of variation across the inhibitor compounds of the coefficients of variation among laboratories for each of the inhibitor compounds.

For each endpoint a one-way mixed effects analysis of variance with heterogeneous variances among the participating laboratories will be fitted to the summary responses within laboratories. Laboratory will be treated as a random effect. Weights will incorporate laboratory-to-laboratory variation and within laboratory variation. The within laboratory variation will be the square of the standard error reported by each laboratory. The analysis of variance will provide an estimated weighted average effect across all laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The mixed effects analysis of variance will be carried out using PROC MIXED in the SAS statistical analysis system.

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 will be prepared for this study. This study will be conducted in compliance with the Federal Register, 40 CFR Part 160, Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Good Laboratory Practice Standards.

9.0 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

Appendix C

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

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
Quality Assurance Specialist

9/28/2005
Date

Approval:

Carrie Ingalls
Quality Assurance Assistant Manager

09/28/2005
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).

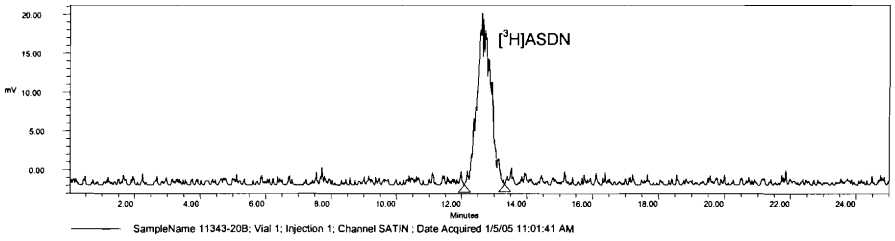
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through 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

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 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
Dispensing*	12/ 2/2004	12/ 2/2004
Formulation analysis*	12/ 2/2004	12/ 2/2004
Formulation preparation*	12/ 2/2004	12/ 2/2004
Audit analytical report	7/26/2005	7/26/2005
Audit study file	7/26/2005	7/26/2005
Audit analytical report		

* 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

EXECUTIVE SUMMARY

The title compound, 4-hydroxyandrostenedione, was analyzed in support of the 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.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

The purpose of this work was to provide all necessary chemistry support activities for 4-hydroxyandrostenedione on 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 20-mL amber glass bottle of 4-hydroxyandrostenedione, 063K4069, was received from the repository at Battelle's Marine Science 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).

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	$C_{19}H_{28}O_3$
Molecular Weight	302.41

TEST	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

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-hydroxyandrostenedione (0.30200 ± 0.0.03020 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 ~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 ± 0.0.00302 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 ~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).

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, 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 1 minutes, increase at 15°C/minute to 320°C
Detector Type	Flame Ionization
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 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 of benzophenone was added to a 25-mL volumetric flask. 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 25 ± 1.0 mg of 4-hydroxyandrostenedione (4-OH ASDN) each into 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.

4.3.1.3 Vehicle/Calibration Standards

Vehicle/calibration standards were prepared as shown in Table 2. 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 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 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 correct 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 at each concentration.

4.3.4 Results

Specificity is shown by representative overlaid chromatograms from high and low 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.

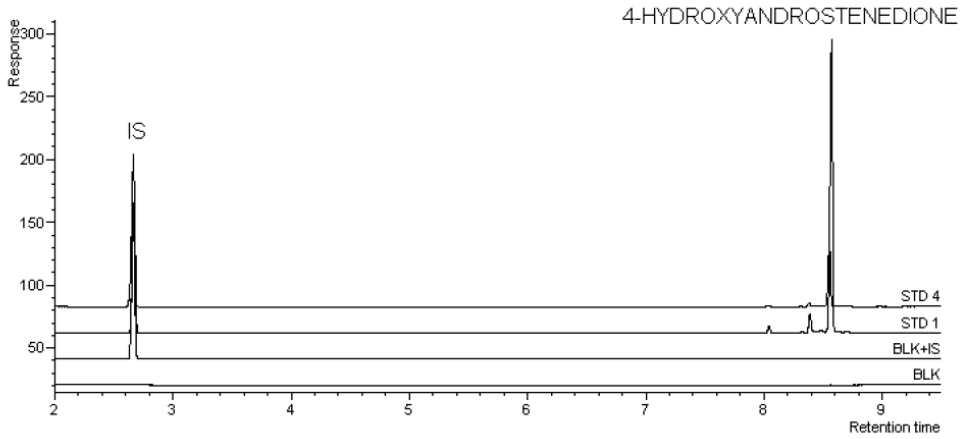


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with Internal Standard, and Blank from the Validation (Shown Top to Bottom)

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
506.4	496.8	509.6	24.2	4.7	-1.9	0.6
	494.5				-2.3	
	537.5				6.1	
298.1	298.4	NA	NA	NA	-2.9	NA
202.5	198.8	NA	NA	NA	-1.9	NA
99.38	100.7	100.4	0.4	0.4	1.3	1.0
	99.98				0.5	
	100.5				1.1	

The method validation sensitivity was 1.2 µg/mL, the limit of detection (LOD), which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 12 µg/mL when a formulation is diluted 1 to 10 for analysis. The limit of quantitation (LOQ), was 4.2 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), Day 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 mintues and allowed to cool to room temperature. 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 flask was diluted to ~80% volume with 95% ethanol, sealed and mixed well. The flask was 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.

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±0.3
11/10/04	11/24/04	14	3.080	3.085	3.149	3.080±0.071	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	3.216	3.125±0.095	103.8±3.1
12/2/04	5/24/05	173	3.126	3.142	3.129	3.133±0.008	104.1±0.03

For the sample prepared 11/10/04, the pooled relative standard deviation 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 12/2/04, the pooled relative standard deviation 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.

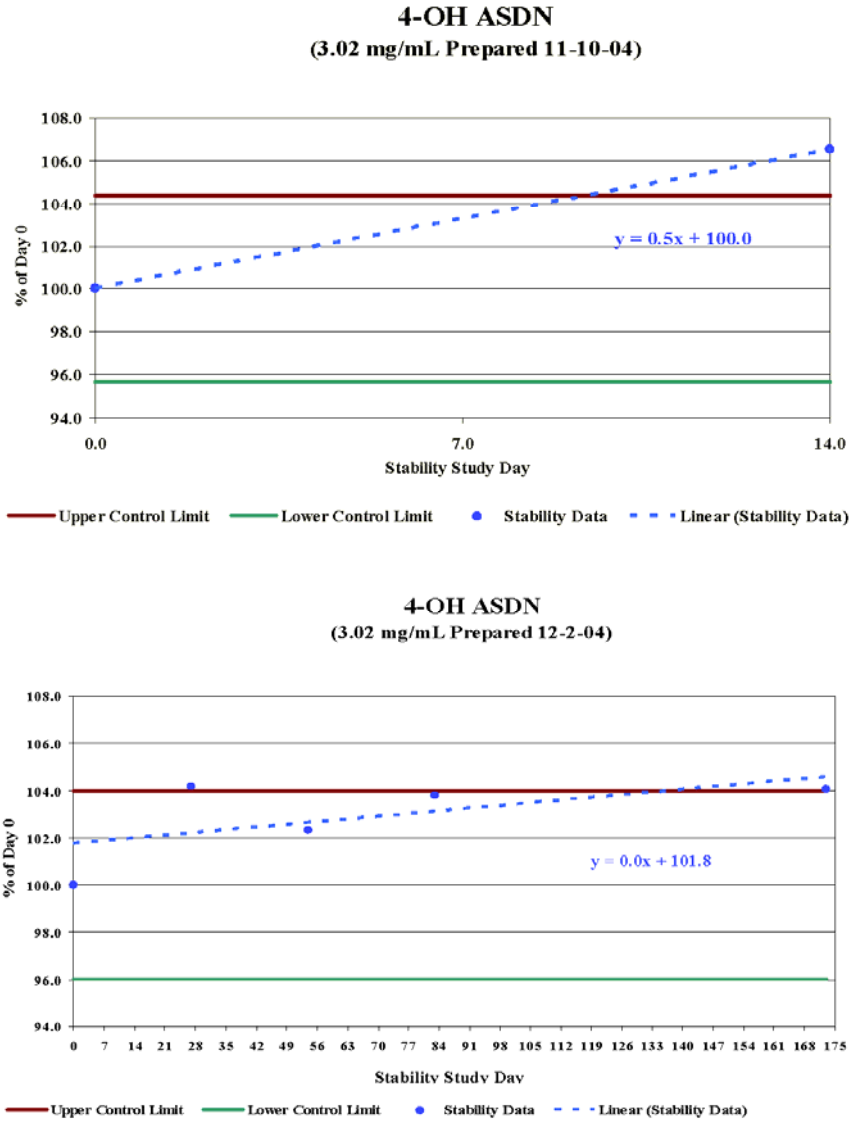


Figure 3 – Control Charts for the Storage Stability Studies

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 11/10/04). Concentrations for Day 54 and 83 samples were within the upper and lower significance levels and Day 27 and Day 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 12/2/04, 1/25/05, 3/21/05 and 6/27/05 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 flask was diluted to ~80% volume with 95% ethanol, sealed and mixed well. The flask was 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

Auto injector 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 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 relative error 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 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. The average determined concentration, standard deviation, and percent relative standard deviation 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 internal standard and a blank presented in Figure 4.

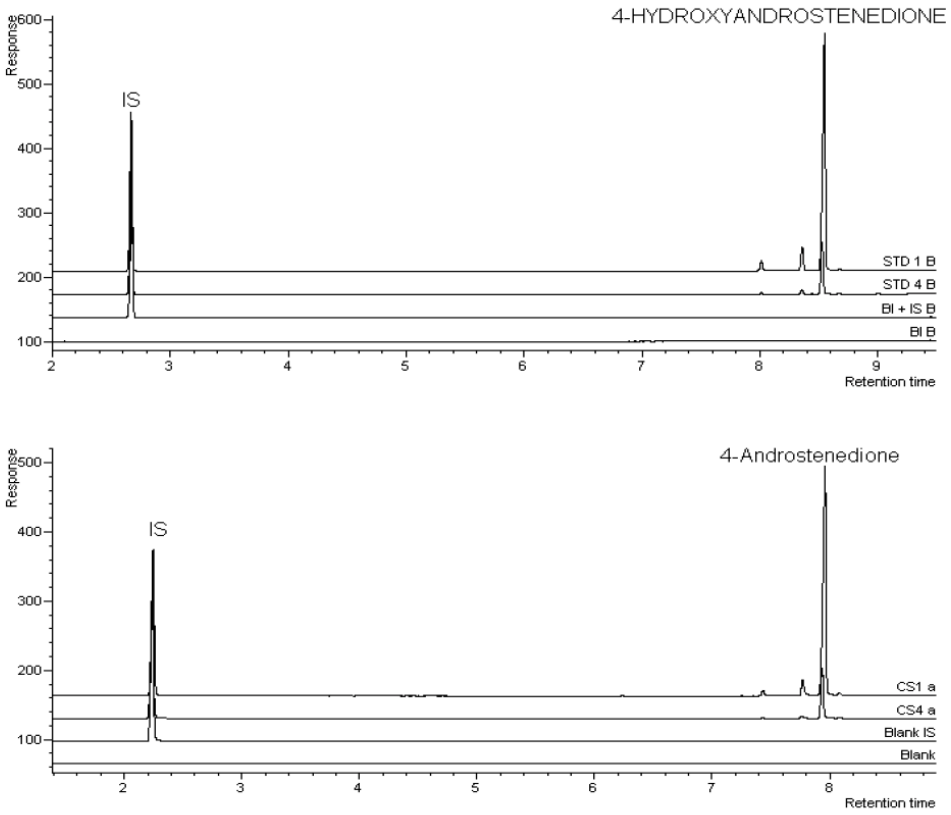


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis Batch 1-ASDN and Batch 2-ASDN (Shown Top to Bottom)

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	1.5	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 relative standard deviation were within acceptance criteria. Therefore the formulations were suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Sandy Runyon, Chris 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. Assessment of the overall quality of the data and report was performed by Hillary Flory.

Appendix E

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0196	21679	1106071		
2	0.0195	23065	1182821		
3	0.0197	24256	1231269		
4	0.0196	24565	1253316		
5	0.0196	25154	1283367		
				Average DPM/g soln	1211369
				SD	69358
				CV	5.73
				μCi/g soln	0.546

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	10.2	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	8.0338	g
Mass of dilution B used in substrate prep	4.5205	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.573939	μg/g

Calculation of Substrate Solution Specific Activity

1) Calculate μg [^{3}H]ASDN/g soln. = 0.00618 μg/g soln.
μg/g soln.

a. μCi/g soln 0.546
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.

= 0.573939 + 0.00618
= 0.580116 μg ASDN/g soln.

3) Calculate Solution Specific Activity

= (μCi/g soln.)/(μg ASDN/g soln.)
= 0.941 μCi/μg ASDN

598046 dpm/nmol

Assay Date	12/13/2004	Test Chemical ID	4OH ASDN		# Concentrations tested		6					
Technician ID	BDL/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	28	20	210000238
	0.305	0.222	0.180	0.115	0.084	0.057	0.000			
	0.303	0.233	0.189	0.134	0.079	0.041	0.000			
	0.277	0.243	0.172	0.150	0.074	0.056	0.000			
Samples:										
	0.081									
	0.094									
	0.085									

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	0.295	0.295	0.0253	m, b	0.092	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.233	0.233	0.0196	se_m, se_b	0.002	0.000
0.6	10.7	25	0.00060	25	0.0150	0.180	0.180	0.0148	r^2, se_y	0.997	0.001
0.4	7.1	25	0.00040	25	0.0099	0.133	0.133	0.0105	F, df	1416	4
0.2	3.6	25	0.00020	25	0.0050	0.079	0.079	0.0055	ss_{reg}, ss_{resid}	0.000	0.000
0.14	2.5	25	0.00014	25	0.0035	0.051	0.051	0.0030			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.997	
		m=	0.092	
		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	0.006	25	100	5000	0.011	0.012	12.367
0.094	0.094	0.007	25	100	5000	0.014		
0.085	0.085	0.006	25	100	5000	0.012		

Assay Date	########	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	1

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	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):	12.367
Protein Concentration (dilution added to assay, mg/mL):	0.024734

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	5.00E-08
4	2.50E-08
5	1.00E-08
6	1.00E-09

Assay Date	12/13/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	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 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	2522	5044	4978	9956	0.1	121137	8.22	9843	0.0165	1	0.012	15	0.0444
			0.5	2	2456	4912											
	2	2	0.5	1	2316	4632	4643	9286	0.1	121137	7.67	9173	0.0153	1	0.012	15	0.0413
			0.5	2	2327	4654											
	3	2	0.5	1	2259	4518	4517	9034	0.1	121137	7.46	8921	0.0149	1	0.012	15	0.0402
			0.5	2	2258	4516											
	4	2	0.5	1	2138	4276	4280	8560	0.1	121137	7.07	8447	0.0141	1	0.012	15	0.0381
			0.5	2	2142	4284											
Background control	1	2	0.5	1	25	50	51	102	0.1	121137	0.08	-11	0.0000	1	0.012	15	0.0000
			0.5	2	26	52											
	2	2	0.5	1	31	62	62	124	0.1	121137	0.10	11	0.0000	1	0.012	15	0.0000
			0.5	2	31	62											
	3	2	0.5	1	25	50	62	124	0.1	121137	0.10	11	0.0000	1	0.012	15	0.0000
			0.5	2	37	74											
	4	2	0.5	1	25	50	51	102	0.1	121137	0.08	-11	0.0000	1	0.012	15	0.0000
			0.5	2	26	52											
Positive control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
Negative Control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
4OH ASDN	1-1	2	0.5	1	204	408	397	794	0.1	121137	0.66	681	0.0011	1	0.012	15	0.0031
			0.5	2	193	386											
	1-2	2	0.5	1	186	372	377	754	0.1	121137	0.62	641	0.0011	1	0.012	15	0.0029
			0.5	2	191	382											
	1-3	2	0.5	1	208	416	399	798	0.1	121137	0.66	685	0.0011	1	0.012	15	0.0031
			0.5	2	191	382											
	2-1	2	0.5	1	1008	2016	2018	4036	0.1	121137	3.33	3923	0.0066	1	0.012	15	0.0177
			0.5	2	1010	2020											
	2-2	2	0.5	1	1087	2174	2160	4320	0.1	121137	3.57	4207	0.0070	1	0.012	15	0.0190
			0.5	2	1073	2146											
	2-3	2	0.5	1	1004	2008	2011	4022	0.1	121137	3.32	3909	0.0065	1	0.012	15	0.0176
			0.5	2	1007	2014											
	3-1	2	0.5	1	1335	2670	2694	5388	0.1	121137	4.45	5275	0.0088	1	0.012	15	0.0238
			0.5	2	1359	2718											
	3-2	2	0.5	1	1389	2778	2781	5562	0.1	121137	4.59	5449	0.0091	1	0.012	15	0.0246
			0.5	2	1392	2784											
	3-3	2	0.5	1	1399	2798	2797	5594	0.1	121137	4.62	5481	0.0092	1	0.012	15	0.0247
			0.5	2	1398	2796											
	4-1	2	0.5	1	1650	3300	3281	6562	0.1	121137	5.42	6449	0.0108	1	0.012	15	0.0291
			0.5	2	1631	3262											
	4-2	2	0.5	1	1602	3204	3168	6336	0.1	121137	5.23	6223	0.0104	1	0.012	15	0.0280
			0.5	2	1566	3132											
	4-3	2	0.5	1	1542	3084	3069	6138	0.1	121137	5.07	6025	0.0101	1	0.012	15	0.0272
			0.5	2	1527	3054											
	5-1	2	0.5	1	1683	3366	3551	7102	0.1	121137	5.86	6989	0.0117	1	0.012	15	0.0315
			0.5	2	1868	3736											
	5-2	2	0.5	1	1853	3706	3731	7462	0.1	121137	6.16	7349	0.0123	1	0.012	15	0.0331
			0.5	2	1878	3756											
	5-3	2	0.5	1	1968	3936	3923	7846	0.1	121137	6.48	7733	0.0129	1	0.012	15	0.0349
			0.5	2	1955	3910											
	6-1	2	0.5	1	2183	4366	4354	8708	0.1	121137	7.19	8595	0.0144	1	0.012	15	0.0387
			0.5	2	2171	4342											
	6-2	2	0.5	1	2173	4346	4336	8672	0.1	121137	7.16	8559	0.0143	1	0.012	15	0.0386
			0.5	2	2163	4326											
	6-3	2	0.5	1	2228	4456	4436	8872	0.1	121137	7.32	8759	0.0146	1	0.012	15	0.0395
			0.5	2	2208	4416											

Assay Date	12/13/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	1

Control Type	Portion	Average	SD
Full activity	Beginning	0.0429	0.0021
Full activity	End	0.0391	0.0015
Full activity	Overall	0.0410	0.0026
Background	Beginning	0.0000	7.01112E-05
Background	End	0.0000	7.01112E-05
Background	Overall	0.0000	5.72455E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4OH ASDN	1	1	1.00E-06	-6.00	0.0031
4OH ASDN	1	2	1.00E-06	-6.00	0.0029
4OH ASDN	1	3	1.00E-06	-6.00	0.0031
4OH ASDN	2	1	1.00E-07	-7.00	0.0177
4OH ASDN	2	2	1.00E-07	-7.00	0.0190
4OH ASDN	2	3	1.00E-07	-7.00	0.0176
4OH ASDN	3	1	5.00E-08	-7.30	0.0238
4OH ASDN	3	2	5.00E-08	-7.30	0.0246
4OH ASDN	3	3	5.00E-08	-7.30	0.0247
4OH ASDN	4	1	2.50E-08	-7.60	0.0291
4OH ASDN	4	2	2.50E-08	-7.60	0.0280
4OH ASDN	4	3	2.50E-08	-7.60	0.0272
4OH ASDN	5	1	1.00E-08	-8.00	0.0315
4OH ASDN	5	2	1.00E-08	-8.00	0.0331
4OH ASDN	5	3	1.00E-08	-8.00	0.0349
4OH ASDN	6	1	1.00E-09	-9.00	0.0387
4OH ASDN	6	2	1.00E-09	-9.00	0.0386
4OH ASDN	6	3	1.00E-09	-9.00	0.0395

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	7.49	7.05	7.53
2	-7.00	43.13	46.25	42.97
3	-7.30	57.99	59.91	60.26
4	-7.60	70.90	68.41	66.24
5	-8.00	76.84	80.79	85.02
6	-9.00	94.49	94.10	96.30

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0194	23740	1223711		
2	0.0194	25315	1304897		
3	0.0194	25863	1333144		
4	0.0194	27670	1426289		
5	0.0194	26786	1380722		
				Average DPM/g soln	1333753
				SD	76992
				CV	5.77
				μCi/g soln	0.601

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	8.035	g
Mass of dilution B used in substrate prep	4.517	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.590274	μg/g

Calculation of Substrate Solution Specific Activity

1) Calculate μg [^{3}H]ASDN/g soln. = 0.00680 μg/g soln.
μg/g soln.

a. μCi/g soln	0.601
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.

= 0.590274 + 0.00680
= 0.597075 μg ASDN/g soln.

3) Calculate Solution Specific Activity

= (μCi/g soln.)/(μg ASDN/g soln.)
= 1.006 μCi/μg ASDN

639764 dpm/nmol

Assay Date	12/15/2004	Test Chemical ID	4OH ASDN		# Concentrations tested		6				
Technician ID	BDL/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	0.14	Blank	28	20	210000238
	0.305	0.222	0.180	0.115	0.084	0.057	0.000			
	0.303	0.233	0.189	0.134	0.079	0.041	0.000			
	0.277	0.243	0.172	0.150	0.074	0.056	0.000			
Samples:										
	0.088									
	0.109									
	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	0.295	0.295	0.0253
0.8	14.3	25	0.00080	25	0.0200	0.233	0.233	0.0196
0.6	10.7	25	0.00060	25	0.0150	0.180	0.180	0.0148
0.4	7.1	25	0.00040	25	0.0099	0.133	0.133	0.0105
0.2	3.6	25	0.00020	25	0.0050	0.079	0.079	0.0055
0.14	2.5	25	0.00014	25	0.0035	0.051	0.051	0.0030
Blank				0.000				

Variables	Regression results	
m, b	0.092	-0.002
se_m, se_b	0.002	0.000
r^2, se_y	0.997	0.001
F, df	1416	4
ss_{reg}, ss_{resid}	0.000	0.000

Regression results are calculated using the function LINEST

r^2=	0.997	
m=	0.092	
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
0.088	0.088	0.006	25	100	5000	0.013	0.014	14.137
0.109	0.109	0.008	25	100	5000	0.017		
0.091	0.091	0.007	25	100	5000	0.013		

Assay Date	12/15/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	2

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	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):	14.137
Protein Concentration (dilution added to assay, mg/mL):	0.028274

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	5.00E-08
4	2.50E-08
5	1.00E-08
6	1.00E-09

Assay Date	12/15/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/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	2533	5066	5022	10044	0.1	133375	7.53	9949	0.0156	1	0.014	15	0.0367
			0.5	2	2489	4978											
	2	2	0.5	1	2417	4834	4864	9728	0.1	133375	7.29	9633	0.0151	1	0.014	15	0.0355
			0.5	2	2447	4894											
	3	2	0.5	1	2528	5056	5081	10162	0.1	133375	7.62	10067	0.0157	1	0.014	15	0.0371
			0.5	2	2553	5106											
	4	2	0.5	1	2483	4966	5017	10034	0.1	133375	7.52	9939	0.0155	1	0.014	15	0.0366
			0.5	2	2534	5068											
Background control	1	2	0.5	1	33	66	54	108	0.1	133375	0.08	13	0.0000	1	0.014	15	0.0000
			0.5	2	21	42											
	2	2	0.5	1	33	66	58	116	0.1	133375	0.09	21	0.0000	1	0.014	15	0.0001
			0.5	2	25	50											
	3	2	0.5	1	28	56	44	88	0.1	133375	0.07	-8	0.0000	1	0.014	15	0.0000
			0.5	2	16	32											
	4	2	0.5	1	15	30	35	70	0.1	133375	0.05	-26	0.0000	1	0.014	15	-0.0001
			0.5	2	20	40											
Positive control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
Negative Control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
4OH ASDN	1-1	2	0.5	1	235	470	462	924	0.1	133375	0.69	829	0.0013	1	0.014	15	0.0031
			0.5	2	227	454											
	1-2	2	0.5	1	236	472	465	930	0.1	133375	0.70	835	0.0013	1	0.014	15	0.0031
			0.5	2	229	458											
	1-3	2	0.5	1	245	490	482	964	0.1	133375	0.72	869	0.0014	1	0.014	15	0.0032
			0.5	2	237	474											
	2-1	2	0.5	1	1187	2374	2375	4750	0.1	133375	3.56	4655	0.0073	1	0.014	15	0.0172
			0.5	2	1188	2376											
	2-2	2	0.5	1	1170	2340	2343	4686	0.1	133375	3.51	4591	0.0072	1	0.014	15	0.0169
			0.5	2	1173	2346											
	2-3	2	0.5	1	1120	2240	2257	4514	0.1	133375	3.38	4419	0.0069	1	0.014	15	0.0163
			0.5	2	1137	2274											
	3-1	2	0.5	1	1572	3144	3117	6234	0.1	133375	4.67	6139	0.0096	1	0.014	15	0.0226
			0.5	2	1545	3090											
	3-2	2	0.5	1	1668	3336	3374	6748	0.1	133375	5.06	6653	0.0104	1	0.014	15	0.0245
			0.5	2	1706	3412											
	3-3	2	0.5	1	1623	3246	3255	6510	0.1	133375	4.88	6415	0.0100	1	0.014	15	0.0236
			0.5	2	1632	3264											
	4-1	2	0.5	1	2160	4320	4368	8736	0.1	133375	6.55	8641	0.0135	1	0.014	15	0.0318
			0.5	2	2208	4416											
	4-2	2	0.5	1	2120	4240	4154	8308	0.1	133375	6.23	8213	0.0128	1	0.014	15	0.0303
			0.5	2	2034	4068											
	4-3	2	0.5	1	2037	4074	4092	8184	0.1	133375	6.14	8089	0.0126	1	0.014	15	0.0298
			0.5	2	2055	4110											
	5-1	2	0.5	1	2347	4694	4696	9392	0.1	133375	7.04	9297	0.0145	1	0.014	15	0.0343
			0.5	2	2349	4698											
	5-2	2	0.5	1	2405	4810	4797	9594	0.1	133375	7.19	9499	0.0148	1	0.014	15	0.0350
			0.5	2	2392	4784											
	5-3	2	0.5	1	2362	4724	4741	9482	0.1	133375	7.11	9387	0.0147	1	0.014	15	0.0346
			0.5	2	2379	4758											
	6-1	2	0.5	1	2637	5274	5280	10560	0.1	133375	7.92	10465	0.0164	1	0.014	15	0.0386
			0.5	2	2643	5286											
	6-2	2	0.5	1	2402	4804	4787	9574	0.1	133375	7.18	9479	0.0148	1	0.014	15	0.0349
			0.5	2	2385	4770											
	6-3	2	0.5	1	2148	4296	4288	8576	0.1	133375	6.43	8481	0.0133	1	0.014	15	0.0313
			0.5	2	2140	4280											

Assay Date	12/15/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	2

Control Type	Portion	Average	SD
Full activity	Beginning	0.0361	0.0008
Full activity	End	0.0369	0.0003
Full activity	Overall	0.0365	0.0007
Background	Beginning	0.0001	2.08486E-05
Background	End	-0.0001	4.69094E-05
Background	Overall	0.0000	7.62175E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4OH ASDN	1	1	1.00E-06	-6.00	0.0031
4OH ASDN	1	2	1.00E-06	-6.00	0.0031
4OH ASDN	1	3	1.00E-06	-6.00	0.0032
4OH ASDN	2	1	1.00E-07	-7.00	0.0172
4OH ASDN	2	2	1.00E-07	-7.00	0.0169
4OH ASDN	2	3	1.00E-07	-7.00	0.0163
4OH ASDN	3	1	5.00E-08	-7.30	0.0226
4OH ASDN	3	2	5.00E-08	-7.30	0.0245
4OH ASDN	3	3	5.00E-08	-7.30	0.0236
4OH ASDN	4	1	2.50E-08	-7.60	0.0318
4OH ASDN	4	2	2.50E-08	-7.60	0.0303
4OH ASDN	4	3	2.50E-08	-7.60	0.0298
4OH ASDN	5	1	1.00E-08	-8.00	0.0343
4OH ASDN	5	2	1.00E-08	-8.00	0.0350
4OH ASDN	5	3	1.00E-08	-8.00	0.0346
4OH ASDN	6	1	1.00E-09	-9.00	0.0386
4OH ASDN	6	2	1.00E-09	-9.00	0.0349
4OH ASDN	6	3	1.00E-09	-9.00	0.0313

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	8.37	8.43	8.78
2	-7.00	47.03	46.39	44.65
3	-7.30	62.03	67.22	64.82
4	-7.60	87.31	82.98	81.73
5	-8.00	93.94	95.98	94.85
6	-9.00	105.74	95.78	85.69

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0194	23687	1220979		
2	0.0196	25605	1306378		
3	0.0195	26370	1352308		
4	0.0193	26381	1366891		
5	0.0196	26145	1333929		
				Average DPM/g soln	1316097
				SD	57773
				CV	4.39
				μCi/g soln	0.593

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	10.3	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	8.0348	g
Mass of dilution B used in substrate prep	4.5214	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.579609	μg/g

Calculation of Substrate Solution Specific Activity

1) Calculate μg [^{3}H]ASDN/g soln. = 0.00671 μg/g soln.
μg/g soln.

a. μCi/g soln 0.593
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.

= 0.579609 + 0.00671
= 0.586320 μg ASDN/g soln.

3) Calculate Solution Specific Activity

= (μCi/g soln.)/(μg ASDN/g soln.)
= 1.011 μCi/μg ASDN

642874 dpm/nmol

Assay Date	12/17/2004	Test Chemical ID	4OH ASDN		# Concentrations tested		6					
Technician ID	BDL/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
	0.285	0.234	0.191	0.161	0.066	0.039	0.000			
	0.298	0.259	0.166	0.132	0.079	0.059	0.000			
	0.282	0.221	0.170	0.124	0.075	0.065	0.000			
Samples:										
	0.078									
	0.076									
	0.077									

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	0.288	0.288	0.0248	m, b	0.093	-0.002
0.8	14.3	25	0.00080	25	0.0200	0.238	0.238	0.0202	se_m, se_b	0.003	0.001
0.6	10.7	25	0.00060	25	0.0150	0.176	0.176	0.0144	r^2, se_y	0.995	0.001
0.4	7.1	25	0.00040	25	0.0099	0.139	0.139	0.0110	F, df	841	4
0.2	3.6	25	0.00020	25	0.0050	0.073	0.073	0.0049	ss_{reg}, ss_{resid}	0.000	0.000
0.14	2.5	25	0.00014	25	0.0035	0.054	0.054	0.0032			

Regression results are calculated using the function LINEST

Blank	0.000	r^2=	0.995	
		m=	0.093	
		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
0.078	0.078	0.005	25	100	5000	0.011	0.011	10.519
0.076	0.076	0.005	25	100	5000	0.010		
0.077	0.077	0.005	25	100	5000	0.011		

Assay Date	12/17/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	3

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	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):	10.519
Protein Concentration (dilution added to assay, mg/mL):	0.021038

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	5.00E-08
4	2.50E-08
5	1.00E-08
6	1.00E-09

Assay Date	12/17/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	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 (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	2811	5622	5310	10620	0.1	131610	8.07	10496	0.0163	1	0.011	15	0.0517
			0.5	2	2499	4998											
	2	2	0.5	1	3064	6128	6168	12336	0.1	131610	9.37	12212	0.0190	1	0.011	15	0.0602
			0.5	2	3104	6208											
	3	2	0.5	1	2839	5678	5659	11318	0.1	131610	8.60	11194	0.0174	1	0.011	15	0.0552
			0.5	2	2820	5640											
	4	2	0.5	1	2893	5786	5734	11468	0.1	131610	8.71	11344	0.0176	1	0.011	15	0.0559
			0.5	2	2841	5682											
Background control	1	2	0.5	1	25	50	59	118	0.1	131610	0.09	-7	0.0000	1	0.011	15	0.0000
			0.5	2	34	68											
	2	2	0.5	1	30	60	58	116	0.1	131610	0.09	-9	0.0000	1	0.011	15	0.0000
			0.5	2	28	56											
	3	2	0.5	1	35	70	68	136	0.1	131610	0.10	12	0.0000	1	0.011	15	0.0001
			0.5	2	33	66											
	4	2	0.5	1	33	66	64	128	0.1	131610	0.10	4	0.0000	1	0.011	15	0.0000
			0.5	2	31	62											
Positive control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
Negative Control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
4OH ASDN	1-1	2	0.5	1	242	484	509	1018	0.1	131610	0.77	894	0.0014	1	0.011	15	0.0044
			0.5	2	267	534											
	1-2	2	0.5	1	270	540	527	1054	0.1	131610	0.80	930	0.0014	1	0.011	15	0.0046
			0.5	2	257	514											
	1-3	2	0.5	1	254	508	526	1052	0.1	131610	0.80	928	0.0014	1	0.011	15	0.0046
			0.5	2	272	544											
	2-1	2	0.5	1	1245	2490	2485	4970	0.1	131610	3.78	4846	0.0075	1	0.011	15	0.0239
			0.5	2	1240	2480											
	2-2	2	0.5	1	1209	2418	2470	4940	0.1	131610	3.75	4816	0.0075	1	0.011	15	0.0237
			0.5	2	1261	2522											
	2-3	2	0.5	1	1234	2468	2508	5016	0.1	131610	3.81	4892	0.0076	1	0.011	15	0.0241
			0.5	2	1274	2548											
	3-1	2	0.5	1	1672	3344	3365	6730	0.1	131610	5.11	6606	0.0103	1	0.011	15	0.0326
			0.5	2	1693	3386											
	3-2	2	0.5	1	1751	3502	3598	7196	0.1	131610	5.47	7072	0.0110	1	0.011	15	0.0349
			0.5	2	1847	3694											
	3-3	2	0.5	1	1851	3702	3709	7418	0.1	131610	5.64	7294	0.0113	1	0.011	15	0.0360
			0.5	2	1858	3716											
	4-1	2	0.5	1	2091	4182	4139	8278	0.1	131610	6.29	8154	0.0127	1	0.011	15	0.0402
			0.5	2	2048	4096											
	4-2	2	0.5	1	2120	4240	4226	8452	0.1	131610	6.42	8328	0.0130	1	0.011	15	0.0410
			0.5	2	2106	4212											
	4-3	2	0.5	1	2211	4422	4423	8846	0.1	131610	6.72	8722	0.0136	1	0.011	15	0.0430
			0.5	2	2212	4424											
	5-1	2	0.5	1	2543	5086	5156	10312	0.1	131610	7.84	10188	0.0158	1	0.011	15	0.0502
			0.5	2	2613	5226											
	5-2	2	0.5	1	2450	4900	4976	9952	0.1	131610	7.56	9828	0.0153	1	0.011	15	0.0484
			0.5	2	2526	5052											
	5-3	2	0.5	1	2479	4958	4994	9988	0.1	131610	7.59	9864	0.0153	1	0.011	15	0.0486
			0.5	2	2515	5030											
	6-1	2	0.5	1	2670	5340	5431	10862	0.1	131610	8.25	10738	0.0167	1	0.011	15	0.0529
			0.5	2	2761	5522											
	6-2	2	0.5	1	2656	5312	5268	10536	0.1	131610	8.01	10412	0.0162	1	0.011	15	0.0513
			0.5	2	2612	5224											
	6-3	2	0.5	1	2663	5326	5340	10680	0.1	131610	8.11	10556	0.0164	1	0.011	15	0.0520
			0.5	2	2677	5354											

Assay Date	12/17/2004	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	3

Control Type	Portion	Average	SD
Full activity	Beginning	0.0560	0.0060
Full activity	End	0.0555	0.0005
Full activity	Overall	0.0558	0.0035
Background	Beginning	0.0000	6.97097E-06
Background	End	0.0000	2.78839E-05
Background	Overall	0.0000	4.58002E-05
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4OH ASDN	1	1	1.00E-06	-6.00	0.0044
4OH ASDN	1	2	1.00E-06	-6.00	0.0046
4OH ASDN	1	3	1.00E-06	-6.00	0.0046
4OH ASDN	2	1	1.00E-07	-7.00	0.0239
4OH ASDN	2	2	1.00E-07	-7.00	0.0237
4OH ASDN	2	3	1.00E-07	-7.00	0.0241
4OH ASDN	3	1	5.00E-08	-7.30	0.0326
4OH ASDN	3	2	5.00E-08	-7.30	0.0349
4OH ASDN	3	3	5.00E-08	-7.30	0.0360
4OH ASDN	4	1	2.50E-08	-7.60	0.0402
4OH ASDN	4	2	2.50E-08	-7.60	0.0410
4OH ASDN	4	3	2.50E-08	-7.60	0.0430
4OH ASDN	5	1	1.00E-08	-8.00	0.0502
4OH ASDN	5	2	1.00E-08	-8.00	0.0484
4OH ASDN	5	3	1.00E-08	-8.00	0.0486
4OH ASDN	6	1	1.00E-09	-9.00	0.0529
4OH ASDN	6	2	1.00E-09	-9.00	0.0513
4OH ASDN	6	3	1.00E-09	-9.00	0.0520

Percent of control values

Level	Log[test substance]	Replicate 1	Replicate 2	Replicate 3
1	-6.00	7.90	8.22	8.20
2	-7.00	42.84	42.57	43.25
3	-7.30	58.40	62.52	64.48
4	-7.60	72.08	73.62	77.11
5	-8.00	90.07	86.88	87.20
6	-9.00	94.93	92.05	93.32

Aliquot #	Weight of aliquot (g)	DPM/Aliq.	DPM/g soln.		
1	0.0195	24387	1250615		
2	0.0194	25716	1325567		
3	0.0195	26703	1369385		
4	0.0195	26668	1367590		
5	0.0194	26261	1353660		
				Average DPM/g soln	1333363
				SD	49470
				CV	3.71
				μCi/g soln	0.601

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	8.0483	g
Mass of dilution B used in substrate prep	4.5293	g
Concentration of nonradiolabeled ASDN in substrate soln.	0.607786	μg/g

Calculation of Substrate Solution Specific Activity

1) Calculate μg [^{3}H]ASDN/g soln. = 0.00680 μg/g soln.
μg/g soln.

a. μCi/g soln 0.601
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.

= 0.607786 + 0.00680
= 0.614585 μg ASDN/g soln.

3) Calculate Solution Specific Activity

= (μCi/g soln.)/(μg ASDN/g soln.)
= 0.977 μCi/μg ASDN

621355 dpm/nmol

Assay Date	2/9/2005	Test Chemical ID	4OH ASDN		# Concentrations tested		6				
Technician ID	BDL/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	0.14	Blank		28	20	210000238
	0.287	0.247	0.203	0.145	0.074	0.059	0.000				
	0.289	0.231	0.191	0.137	0.085	0.053	0.000				
	0.275	0.226	0.208	0.152	0.083	0.057	0.000				
Samples:											
	0.081										
	0.077										
	0.069										

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	0.283	0.283	0.0242	m, b	0.095	-0.003
0.8	14.3	25	0.00080	25	0.0200	0.234	0.234	0.0195	se_m, se_b	0.006	0.001
0.6	10.7	25	0.00060	25	0.0150	0.201	0.201	0.0164	r^2, se_y	0.987	0.001
0.4	7.1	25	0.00040	25	0.0099	0.145	0.145	0.0110	F, df	293	4
0.2	3.6	25	0.00020	25	0.0050	0.081	0.081	0.0049	ss_{reg}, ss_{resid}	0.000	0.000
0.14	2.5	25	0.00014	25	0.0035	0.056	0.056	0.0026			

Regression results are calculated using the function LINEST

Blank	0.000			r^2=	0.987						
				m=	0.095						
				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
0.081	0.081	0.005	25	100	5000	0.010	0.009	8.776
0.077	0.077	0.005	25	100	5000	0.009		
0.069	0.069	0.004	25	100	5000	0.008		

Assay Date	2/9/2005	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	4

Microsome Dilution Details		
Dilution A	0.1	mL microsome Stock used
	5	mL total volume
	50	dilution factor
Dilution B	3	mL microsome Dilution A used
	30	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):	8.776
Protein Concentration (dilution added to assay, mg/mL):	0.017552

Test Chemical Concentrations	
Level	Final Concentration (M)
1	1.00E-06
2	1.00E-07
3	5.00E-08
4	2.50E-08
5	1.00E-08
6	1.00E-09

Assay Date	2/9/2005	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	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
Full activity control	1	2	0.5	1	3195	6390	6349	12698	0.1	133336	9.52	12531	0.0202	1	0.009	15	0.0766
			0.5	2	3154	6308											
	2	2	0.5	1	3075	6150	6199	12398	0.1	133336	9.30	12231	0.0197	1	0.009	15	0.0748
			0.5	2	3124	6248											
	3	2	0.5	1	3034	6068	6098	12196	0.1	133336	9.15	12029	0.0194	1	0.009	15	0.0735
			0.5	2	3064	6128											
	4	2	0.5	1	3087	6174	6147	12294	0.1	133336	9.22	12127	0.0195	1	0.009	15	0.0741
			0.5	2	3060	6120											
Background control	1	2	0.5	1	59	118	107	214	0.1	133336	0.16	47	0.0001	1	0.009	15	0.0003
			0.5	2	48	96											
	2	2	0.5	1	39	78	81	162	0.1	133336	0.12	-6	0.0000	1	0.009	15	0.0000
			0.5	2	42	84											
	3	2	0.5	1	44	88	82	164	0.1	133336	0.12	-4	0.0000	1	0.009	15	0.0000
			0.5	2	38	76											
	4	2	0.5	1	35	70	65	130	0.1	133336	0.10	-38	-0.0001	1	0.009	15	-0.0002
			0.5	2	30	60											
Positive control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
Negative Control	1	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	2	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	3	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
	4	2		1						0		#VALUE!	#VALUE!		0.000		#VALUE!
				2													
4OH ASDN	1-1	2	0.5	1	288	576	595	1190	0.1	133336	0.89	1023	0.0016	1	0.009	15	0.0063
			0.5	2	307	614											
	1-2	2	0.5	1	276	552	556	1112	0.1	133336	0.83	945	0.0015	1	0.009	15	0.0058
			0.5	2	280	560											
	1-3	2	0.5	1	280	560	553	1106	0.1	133336	0.83	939	0.0015	1	0.009	15	0.0057
			0.5	2	273	546											
	2-1	2	0.5	1	1464	2928	2922	5844	0.1	133336	4.38	5677	0.0091	1	0.009	15	0.0347
			0.5	2	1458	2916											
	2-2	2	0.5	1	1467	2934	2944	5888	0.1	133336	4.42	5721	0.0092	1	0.009	15	0.0350
			0.5	2	1477	2954											
	2-3	2	0.5	1	1466	2932	2985	5970	0.1	133336	4.48	5803	0.0093	1	0.009	15	0.0355
			0.5	2	1519	3038											
	3-1	2	0.5	1	2053	4106	4072	8144	0.1	133336	6.11	7977	0.0128	1	0.009	15	0.0488
			0.5	2	2019	4038											
	3-2	2	0.5	1	1955	3910	3898	7796	0.1	133336	5.85	7629	0.0123	1	0.009	15	0.0466
			0.5	2	1943	3886											
	3-3	2	0.5	1	1804	3608	3614	7228	0.1	133336	5.42	7061	0.0114	1	0.009	15	0.0432
			0.5	2	1810	3620											
	4-1	2	0.5	1	2483	4966	5007	10014	0.1	133336	7.51	9847	0.0158	1	0.009	15	0.0602
			0.5	2	2524	5048											
	4-2	2	0.5	1	2400	4800	4749	9498	0.1	133336	7.12	9331	0.0150	1	0.009	15	0.0570
			0.5	2	2349	4698											
	4-3	2	0.5	1	2349	4698	4693	9386	0.1	133336	7.04	9219	0.0148	1	0.009	15	0.0564
			0.5	2	2344	4688											
	5-1	2	0.5	1	2850	5700	5731	11462	0.1	133336	8.60	11295	0.0182	1	0.009	15	0.0690
			0.5	2	2881	5762											
	5-2	2	0.5	1	2736	5472	5439	10878	0.1	133336	8.16	10711	0.0172	1	0.009	15	0.0655
			0.5	2	2703	5406											
	5-3	2	0.5	1	2786	5572	5648	11296	0.1	133336	8.47	11129	0.0179	1	0.009	15	0.0680
			0.5	2	2862	5724											
	6-1	2	0.5	1	3099	6198	6194	12388	0.1	133336	9.29	12221	0.0197	1	0.009	15	0.0747
			0.5	2	3095	6190											
	6-2	2	0.5	1	2858	5716	5721	11442	0.1	133336	8.58	11275	0.0181	1	0.009	15	0.0689
			0.5	2	2863	5726											
	6-3	2	0.5	1	3041	6082	6126	12252	0.1	133336	9.19	12085	0.0194	1	0.009	15	0.0739
			0.5	2	3085	6170											

Assay Date	2/9/2005	Test Chemical ID	4OH ASDN	# Concentrations tested	6	Microsome type	Placental	Microsome ID	11343-7	Technician ID	BDL/TD	Replicate #	4

Control Type	Portion	Average	SD
Full activity	Beginning	0.0757	0.0013
Full activity	End	0.0738	0.0004
Full activity	Overall	0.0748	0.0013
Background	Beginning	0.0001	0.000224766
Background	End	-0.0001	0.000146962
Background	Overall	0.0000	0.000212078
Positive	Beginning	#VALUE!	#VALUE!
Positive	End	#VALUE!	#VALUE!
Positive	Overall	#VALUE!	#VALUE!
Negative	Beginning	#VALUE!	#VALUE!
Negative	End	#VALUE!	#VALUE!
Negative	Overall	#VALUE!	#VALUE!

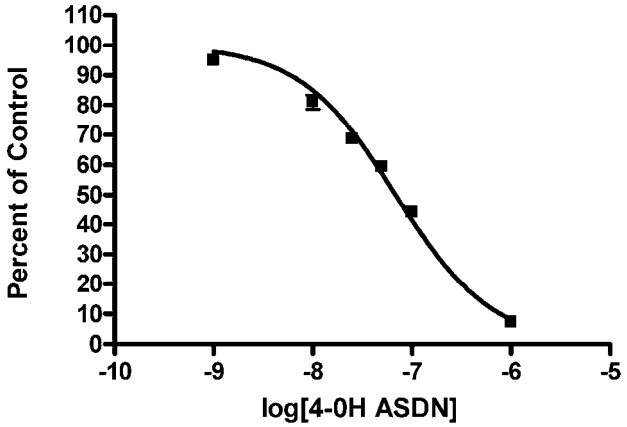
Test Substance	Level	Replicate	[test substance] M	Log[test substance]	Activity
4OH ASDN	1	1	1.00E-06	-6.00	0.0063
4OH ASDN	1	2	1.00E-06	-6.00	0.0058
4OH ASDN	1	3	1.00E-06	-6.00	0.0057
4OH ASDN	2	1	1.00E-07	-7.00	0.0347
4OH ASDN	2	2	1.00E-07	-7.00	0.0350
4OH ASDN	2	3	1.00E-07	-7.00	0.0355
4OH ASDN	3	1	5.00E-08	-7.30	0.0488
4OH ASDN	3	2	5.00E-08	-7.30	0.0466
4OH ASDN	3	3	5.00E-08	-7.30	0.0432
4OH ASDN	4	1	2.50E-08	-7.60	0.0602
4OH ASDN	4	2	2.50E-08	-7.60	0.0570
4OH ASDN	4	3	2.50E-08	-7.60	0.0564
4OH ASDN	5	1	1.00E-08	-8.00	0.0690
4OH ASDN	5	2	1.00E-08	-8.00	0.0655
4OH ASDN	5	3	1.00E-08	-8.00	0.0680
4OH ASDN	6	1	1.00E-09	-9.00	0.0747
4OH ASDN	6	2	1.00E-09	-9.00	0.0689
4OH ASDN	6	3	1.00E-09	-9.00	0.0739

Percent of control values

Level	Log[test substance]	Replicate 1	2	3
1	-6.00	8.36	7.72	7.67
2	-7.00	46.42	46.78	47.45
3	-7.30	65.23	62.38	57.74
4	-7.60	80.52	76.30	75.38
5	-8.00	92.36	87.58	91.00
6	-9.00	99.93	92.19	98.82

Appendix F

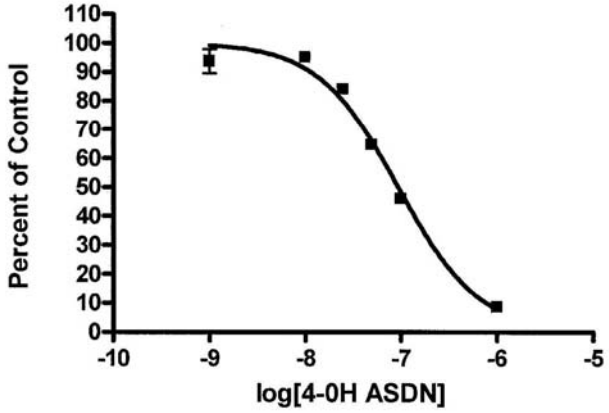
Assay Run 12-13-04



-6.00	7.49	7.05	7.53
-7.00	43.13	46.25	42.97
-7.30	57.99	59.91	60.26
-7.60	70.90	68.41	66.24
-8.00	76.84	80.79	85.02
-9.00	94.49	94.10	96.30

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.166
HILLSLOPE	-0.8969
EC50	6.827e-008
Std. Error	
LOGEC50	0.02004
HILLSLOPE	0.03032
95% Confidence Intervals	
LOGEC50	-7.208 to -7.123
HILLSLOPE	-0.9612 to -0.8327
EC50	6.191e-008 to 7.529e-008
Goodness of Fit	
Degrees of Freedom	16
R^2 (unweighted)	0.9868
Weighted Sum of Squares (1/Y)	3.080
Absolute Sum of Squares	188.5
Sy.x	3.432
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

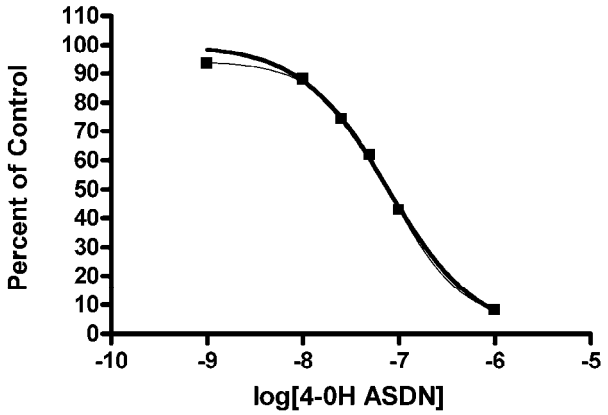
Assay Run 12-15-04



-6.00	8.37	8.43	8.78
-7.00	47.03	46.39	44.65
-7.30	62.03	67.22	64.82
-7.60	87.31	82.98	81.73
-8.00	93.94	95.98	94.85
-9.00	99.50	95.78	85.69

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.028
HILLSLOPE	-1.041
EC50	9.375e-008
Std. Error	
LOGEC50	0.02271
HILLSLOPE	0.04470
95% Confidence Intervals	
LOGEC50	-7.076 to -6.980
HILLSLOPE	-1.136 to -0.9463
EC50	8.391e-008 to 1.047e-007
Goodness of Fit	
Degrees of Freedom	16
R^2 (unweighted)	0.9797
Weighted Sum of Squares (1/Y)	4.386
Absolute Sum of Squares	345.5
Sy.x	4.647
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Assay Run 12-17-04

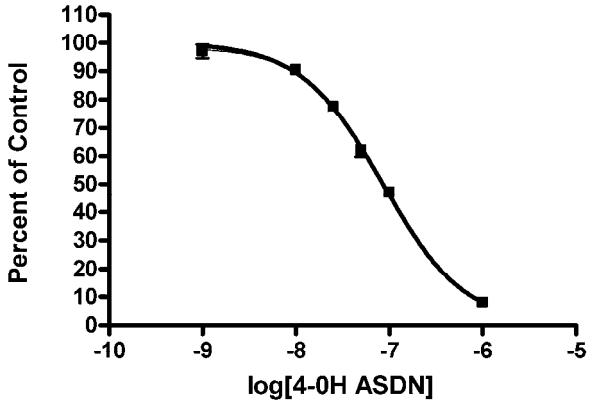


-6.00	7.90	8.22	8.20
-7.00	42.84	42.57	43.25
-7.30	58.40	62.52	64.48
-7.60	72.08	73.62	77.11
-8.00	90.07	86.88	87.20
-9.00	94.93	92.05	93.32

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.112
HILLSLOPE	-0.9511
EC50	7.725e-008
Std. Error	
LOGEC50	0.01416
HILLSLOPE	0.02431
95% Confidence Intervals	
LOGEC50	-7.142 to -7.082
HILLSLOPE	-1.003 to -0.8995
EC50	7.209e-008 to 8.278e-008
Goodness of Fit	
Degrees of Freedom	16
R^2 (unweighted)	0.9915
Weighted Sum of Squares (1/Y)	1.627
Absolute Sum of Squares	130.2
Sy.x	2.853
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Placental microsomes

Assay run 02-09-2005



-6.00	8.36	7.72	7.67
-7.00	46.42	46.78	47.45
-7.30	65.23	62.38	57.74
-7.60	80.52	76.30	75.38
-8.00	92.36	87.58	91.00
-9.00	99.93	92.19	98.82

Sigmoidal dose-response (variable slope)	
Best-fit values	
BOTTOM	0.0
TOP	100.0
LOGEC50	-7.069
HILLSLOPE	-0.9934
EC50	8.531e-008
Std. Error	
LOGEC50	0.01340
HILLSLOPE	0.02403
95% Confidence Intervals	
LOGEC50	-7.097 to -7.041
HILLSLOPE	-1.044 to -0.9425
EC50	7.991e-008 to 9.107e-008
Goodness of Fit	
Degrees of Freedom	16
R^2 (unweighted)	0.9932
Weighted Sum of Squares (1/Y)	1.485
Absolute Sum of Squares	110.4
Sy.x	2.626
Constraints	
BOTTOM	BOTTOM = 0.0
TOP	TOP = 100.0
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Appendix G

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 4

October 14, 2005

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
4-OH ASDN Positive Control Inhibitor Study
Intralaboratory Statistical Analysis of Battelle Data

EPA CONTRACT NUMBER 68-W-01-023
WORK ASSIGNMENT 4-16, TASK 4

Ying-Liang Chou, Author — Date

Paul I. Feder, Reviewer — Date

Offsite 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 Battelle Task Leader/ Battelle Management	Date Reported to Offsite Study Director/ Management
Audit study file	10/12/2005	10/12/2005	10/12/2005
Audit draft report	10/12/2005	10/12/2005	10/12/2005

Quality Assurance Unit	Date
Hillary Flory	10-14-05

This report discusses the methods and results of the intralaboratory statistical analysis on the data collected at Battelle with the placental aromatase assay in the 4-OH ASDN positive control inhibitor study.

Summary and Conclusions

Statistical analyses were carried out on the percent of control responses for aromatase activity in four independent replicates. Within each replicate three repeat tubes were run at each of six graded concentrations of the positive control inhibitor 4-OH ASDN. Additionally two full enzyme activity control tubes and two background activity control tubes were run at the beginning of each replicate and two full enzyme activity and two background activity controls were run at the end.

Concentration response curves were fitted within each replicate to describe the relation between 4-OH ASDN concentration 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. In addition full enzyme activity and background activity control tube responses were compared between beginning and end of each replicate to identify differences within replicates and differences across replicates.

The following results were obtained:

1. The concentration response curves were similar across the four replicates.
2. Replicate 1 had a slightly lower estimated IC_{50} and a less negative slope than replicates 2 to 4. Replicate 2 had a slightly higher estimated IC_{50} and a more negative slope than the other replicates.
3. For the background activity controls, the average percent of control response at the ends of replicates 2 and 4 were lower than at the beginning. For the full enzyme activity controls the average percent of control response at the end of replicate 1 was lower than at the beginning. This provides a suggestion of some reduction in aromatase activity between the beginning and end of a replicate, but it is only tentative since the replicates involved differed between the background activity and the full enzyme activity controls.
4. For both the background activity and the full enzyme activity controls averaged across replicates there were not significant differences between the beginning and the end portions. The variability among repetitions within replicates was large compared to the variation of portion (end vs. beginning) effects among replicates.

Introduction and Background

Task 4 of the Placental Aromatase Validation Study involves the individual laboratories independently carrying out the placental aromatase assay with positive control inhibitor 4-OH ASDN and centrally prepared microsomes, according to a common protocol. This report discusses the methods and results of the intralaboratory statistical analysis performed on the experimental data collected by Battelle. Aromatase activity levels were determined for the full enzyme activity control, the background activity control, and for six graded concentrations of positive control inhibitor 4-OH ASDN.

Four replicates of the positive control inhibitor study were carried out. Within each replicate three repetitions were run at each of the 4-OH ASDN log (base 10) concentrations -6, -7, -7.3, -7.6, -8, and -9. In addition two repeat tubes of the full enzyme activity and background activity controls were run prior to the 4-OH ASDN runs and two repeat tubes of the full enzyme activity and background activity controls were run following the 4-OH ASDN 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 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 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.

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.

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 of test substance 4-OH ASDN.
2. Estimate the IC_{50} concentration, slope, and associated standard errors within each replicate.
3. Combine results across replicates to determine the average IC_{50} concentration, average slope, and associated standard errors.
4. Determine whether there are differences between the full enzyme activity and background activity controls obtained at the beginning and those obtained at the end of each replicate.
5. Assess the consistency of conditions within replicates and across replicates based on the full enzyme activity and background activity control values.

Statistical analyses were carried out based on the results from all four replicates, as well

as the results restricted to replicates 2 to 4.

Statistical Analysis Methods

Concentration Response Trend Curves

Within each replicate a concentration response curve was fitted to the percent of control activity values at the three repetitions at each of the six graded 4-OH ASDN 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 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
β ≡ 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 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). The variance is 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 100 percent of control. Otherwise it is indicated as "II", incomplete but can

extrapolate to $\log_{10}IC_{50}$ or "IX", incomplete but must extrapolate to $\log_{10}IC_{50}$. These are the definitions of response curve "Status" that were used in this analysis. A modified set of descriptors of response curve status, that conform to EPA conventions, will be used in subsequent analyses.

For each replicate the individual percent of control values were plotted versus logarithm of inhibitor compound concentration. The fitted concentration response curve was superimposed on the same plot. These plots display the data, the fitted response curves in relation to these data, and deviations from the fits.

One-way random effects analysis of variance models with heterogeneous variances among the replicates were 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. The within replicate variances were estimated as the squares of the standard errors for each replicate. The analysis of variance fits provide estimated weighted average effects (mean) across the replicates and their associated standard errors. Degrees of freedom associated with the mean effects were calculated based on Satterthwaite's approximation.

The estimated IC_{50} for the test substance was estimated as 10 to the power mean $\log_{10}IC_{50}$. The geometric standard error associated with the estimated IC_{50} was estimated 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 based on this one-way random effects analysis of variance model fit. 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 of the four replicates 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 of the four replicates 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 four replicates, based on the random effects one-way analysis of variance model discussed above. An analogous plot, restricted to replicates 2 to

4, was also prepared.

All concentration response curves were fitted to the data using the non-linear 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 Variance of Full Enzyme Activity Controls and Background Activity Controls Across Replicates

Within each replicate quadruplicate repetitions were made of the full enzyme activity control and the background activity 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 responses were expressed as percent of control. The full enzyme activity and background activity controls percent of control responses were plotted across replicates, with plotting symbol distinguishing between beginning and end, and with reference line 0% (background activity control) or 100% (full enzyme activity control). 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 of 0.

Mixed effects analysis of variance models were fitted to the background activity controls and to the full enzyme activity controls data. The fixed effect factor in the analysis of variance was portion (beginning or end). The random effects were replicate and portion by replicate 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.

This analysis was carried out for replicates 1 to 4 and additionally for replicates 2 to 4, using the SAS statistical analysis system, Version 9.

Statistical Analysis Results

The percent of control responses are displayed in Table A-1 for each replicate and for each 4-OH ASDN ($\log_{10}$) concentration. The full enzyme activity and background activity control percent of control responses are displayed in Table A-2, sorted by replicate and beginning and end within replicate.

Concentration response curves were fitted separately to the repeat tubes data within each replicate and to the averages of the repetitions within each replicate (Table A-1). The parameters of these fitted concentration response curves are displayed in Table 1. The individual repetition data within each replicate are plotted in Figure A-1 through Figure A-4 with the corresponding fitted concentration response curves superimposed in each figure. Figure 1 displays the four concentration response curves fitted to the averages of the three repetitions within each replicate. The concentration response curves for the four replicates are similar. Replicate 1 has a slightly lower estimated IC_{50} as well as a less negative slope β. Replicate 2 has a slightly higher estimated IC_{50} as well as a more negative slope. (Table 1).

The parameters of the average concentration response curve, based on random effects analysis of variance model fits with replicate as a random effect are displayed in Table 1. The average parameters based on replicates 1 to 4 and those based on replicates 2 to 4 are displayed. The average concentration response curve, along with the averages of three repetitions within each replicate are plotted together in Figure 2 (replicates 1 to 4) and in Figure 3 (replicates 2 to 4).

The parameter estimates for each replicate and the average parameter estimates across replicates with their associated 95% confidence intervals are displayed in Table 2 and graphed in Figure 4 for $\log_{10}IC_{50}$ (μ) and Figure 5 for slope (β). Since replicate 1 had a lower IC_{50} and less negative slope (β), the average across replicates 2 to 4 had a higher IC_{50} and a more negative slope (β) than the average across all four replicates. However the differences are slight.

The results of analyses of variance for these estimates are presented in Table 3. 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. The variance components across replicates 1 to 4 are seen to be greater than those across replicates 2 to 4.

For $\log_{10}IC_{50}$ the replicate-to-replicate variation is more than five times the individual replicate within-replicate variances, when all four replicates are considered, and more than two times the individual replicate within-replicates variances when just replicates 2 to 4 are considered.

The background activity control and full enzyme activity control responses for each replicate are displayed in Table A-2. These data are plotted by replicate in Figures 6 and 7, with plotting symbol distinguishing between beginning and end of the replicate. Figures 8 and 9 show the differences between the averages at the beginning and at the end within each replicate (end minus beginning). For background activity controls, the percent of controls measurements on average were lower at the end than at the beginning for replicates 2 and 4 (Figure 8). The average standard error of these differences is about 0.2 percent, so replicates 2 and 4 do appear to be lower at the end. For full enzyme activity control, the percent of controls measurements on averages were lower at the end for replicate 1 (Figure 9). The average standard error of the full

enzyme activity control differences is about 4 percent, so replicate 1 does appear to be lower at the end.

Mixed effects analysis of variance models were fitted to the background activity control and full enzyme activity control data with portion as a fixed effect and with replicate and replicate by portion interaction as random effects. The results are displayed in Table 4. The component of variation due to replicate is constrained to be 0 by the definitions of the background activity and full enzyme activity control responses. 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, averaged across replicates. The right panel displays the estimated variance components. No significant differences between the beginning and the end, averaged across replicates, were observed for either background activity or full enzyme activity controls. The estimated variance for the portion by replicate interaction is considerably smaller than the residual variation, which is based on the variation between the two repetitions carried out within the same portion of the same replicate.

Table 1. 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)[e]	Slope (SE)	Status
Individual Values[a]				
1	-7.166 (0.02004)	$6.827x10^{-8}$ (1.04722)	-0.8969 (0.03032)	C
2	-7.028 (0.02271)	$9.375x10^{-8}$ (1.05368)	-1.041 (0.04470)	C
3	-7.112 (0.01416)	$7.725x10^{-8}$ (1.03314)	-0.9511 (0.02431)	C
4	-7.069 (0.01339)	$8.531x10^{-8}$ (1.03131)	-0.9933 (0.02402)	C
Mean of Replicates 2-4[c]	-7.072 (0.02341)	$8.470x10^{-8}$ (1.05538)	-0.9852 (0.02214)	--
Mean of Replicates 1-4[d]	-7.094 (0.02864)	$8.051x10^{-8}$ (1.06816)	-0.9662 (0.02776)	--
Averages Values[b]				
1	-7.165 (0.03405)	$6.845x10^{-8}$ (1.08156)	-0.8981 (0.05161)	C
2	-7.027 (0.03509)	$9.392x10^{-8}$ (1.08415)	-1.042 (0.06917)	C
3	-7.111 (0.02239)	$7.741x10^{-8}$ (1.05291)	-0.9518 (0.03848)	C
4	-7.068 (0.01079)	$8.557x10^{-8}$ (1.02516)	-0.9943 (0.01938)	C

a. Concentration response curve fitted to the data collected within each replicate, with three repetitions at each 4-OH ASDN concentration level.
b. Concentration response curve fitted to the averages of the three repetitions at each 4-OH ASDN concentration level within each replicate.
c. Weighted averages of the parameter estimates across replicates, estimated based on replicates 2 to 4.
d. Weighted averages of the parameter estimates across replicates, estimated based on replicates 1 to 4.
e. 10 to the power of $log_{10}IC_{50}$ and 10 to the power of its associated standard error.

Table 2. 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 of Replicates 2-4 [b]	Mean of Replicates 1-4 [b]
$Log_{10}IC_{50}$	-7.166 (-7.208, -7.124)	-7.028 (-7.076, -6.980)	-7.112 (-7.142, -7.082)	-7.069 (-7.097, -7.041)	-7.072 (-7.178, -6.966)	-7.094 (-7.189, -7.000)
Slope	-0.897 (-0.961, -0.833)	-1.041 (-1.136, -0.947)	-0.951 (-1.003, -0.900)	-0.993 (-1.044, -0.942)	-0.985 (-1.079, -0.891)	-0.966 (-1.062, -0.871)

a. Parameter estimates and their associated 95% confidence intervals for each replicate, based on the concentration response curves fitted to the individual values within replicates.

b. Mean and its associated 95% confidence interval, based on a heterogeneous variance one-way analysis of variance model with replicate treated as a random effect.

Table 3. 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 for Replicates 2-4		Overall for Replicates 1-4	
	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Random Replicate (p-value)[d]	Variance of Mean	Random Replicate (p-value)[d]	Variance of Mean
$Log_{10}IC_{50}$	0.000402 /df=16	0.000516 /df=16	0.000201 /df=16	0.000179 /df=16	0.001359 /df=2 (p=0.2123)	0.000548 /df=1.894	0.002962 /df=3 (p=0.1421)	0.000820 /df=2.823
Slope	0.000919 /df=16	0.001998 /df=16	0.000591 /df=16	0.000577 /df=16	0.000622 /df=2 (p=0.3526)	0.000490 /df=2.030	0.002153 /df=3 (p=0.2170)	0.000771 /df=2.646

a. The variance estimates for each replicate were based on the concentration response curves fitted to the individual 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 $2*((1/K)* \sum(S_r^2 + S_i^2))^2/(var(S_r^2) +(2/K^2)* \sum(S_i^4 /d\ f_i))$, 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. Variance Components of Full Enzyme Activity Control and Background Activity Control Percent of Control Values. Position Effects and Variation Across Replicates of Portion Effects Within Replicates

Parameter	Difference Between Beginning and End Portions		Variance Components			
	Estimate (%) (Std. Error)	p-Value/ Degree of Freedom	Replicate[a]	Portion* Replicate	Residual (Repetition)	Total Variance
Replicates 1 to 4						
Background Activity	0.1340 (0.08399)	0.1617/df=6	0	0.0010	0.0263	0.0272
Full Enzyme Activity Control	2.5365 (2.0422)	0.2346/df=14	0	<0.000001	16.6823	16.6823
Replicates 2 to 4						
Background Activity	0.1787 (0.1101)	0.1798/df=4	0	0.0055	0.0253	0.0308
Full Enzyme Activity Control	0.3623 (2.1301)	0.8683/df=10	0	0	13.6123	13.6123

a. The replicate component of variation is constrained to be 0, by definition of background activity and full enzyme activity control responses.

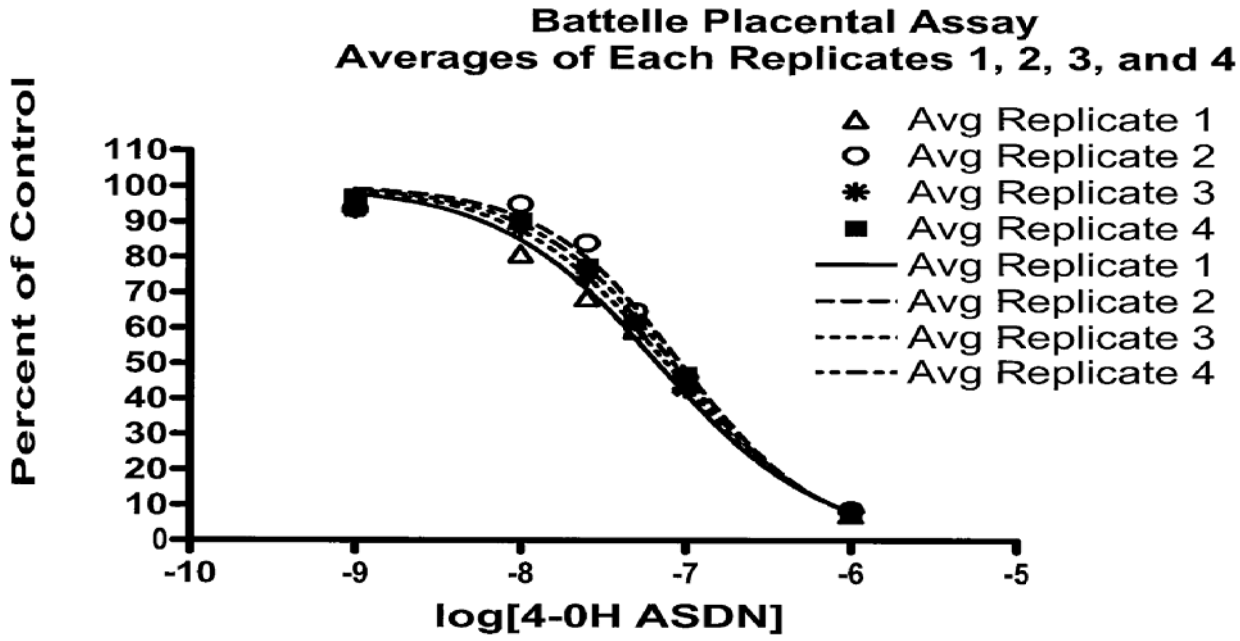


Figure 1. **Concentration Response Curves and Averages of Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. By Replicate**

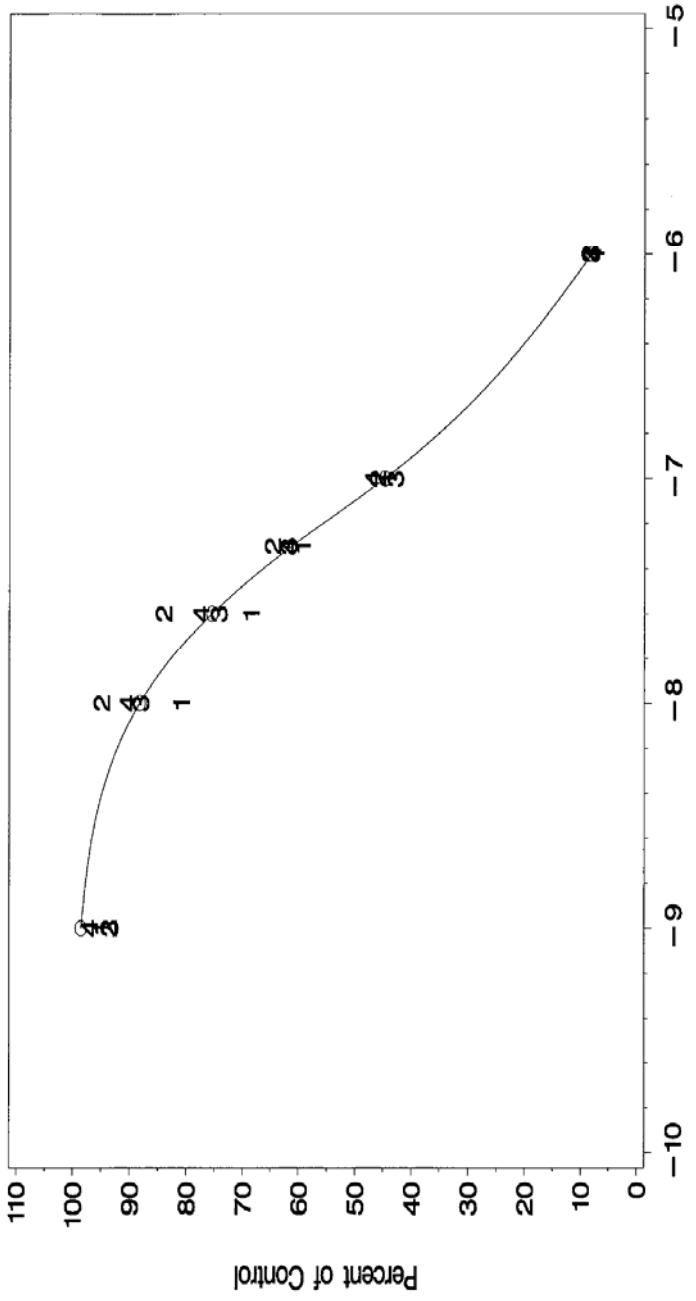


Figure 2. Overall Average Concentration Response Curve Across Replicates 1 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 1 to 4 Parameter Values

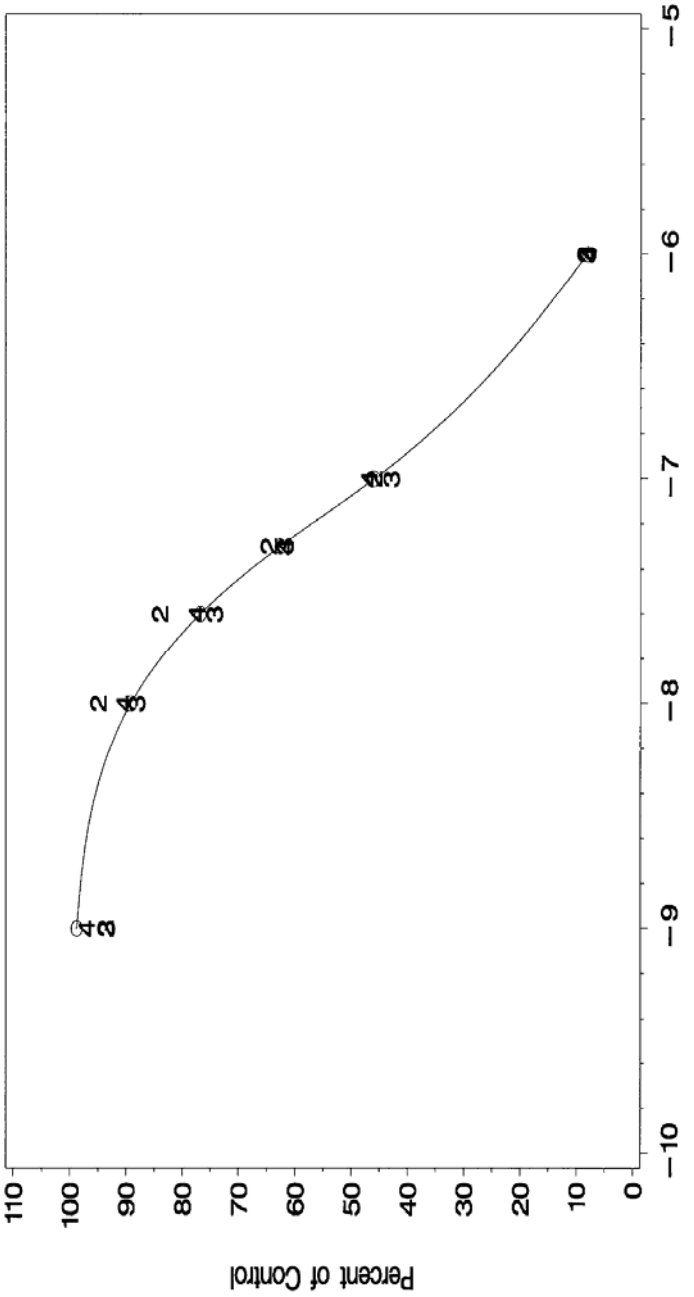


Figure 3. **Overall Average Concentration Response Curve Across Replicates 2 to 4 and Average Responses Across Repetitions Within 4-OH ASDN Concentrations. Placental Aromatase Assay. Parameters of Average Curve Based on One-Way Analysis of Variance Across Replicate 2 to 4 Parameter Values**

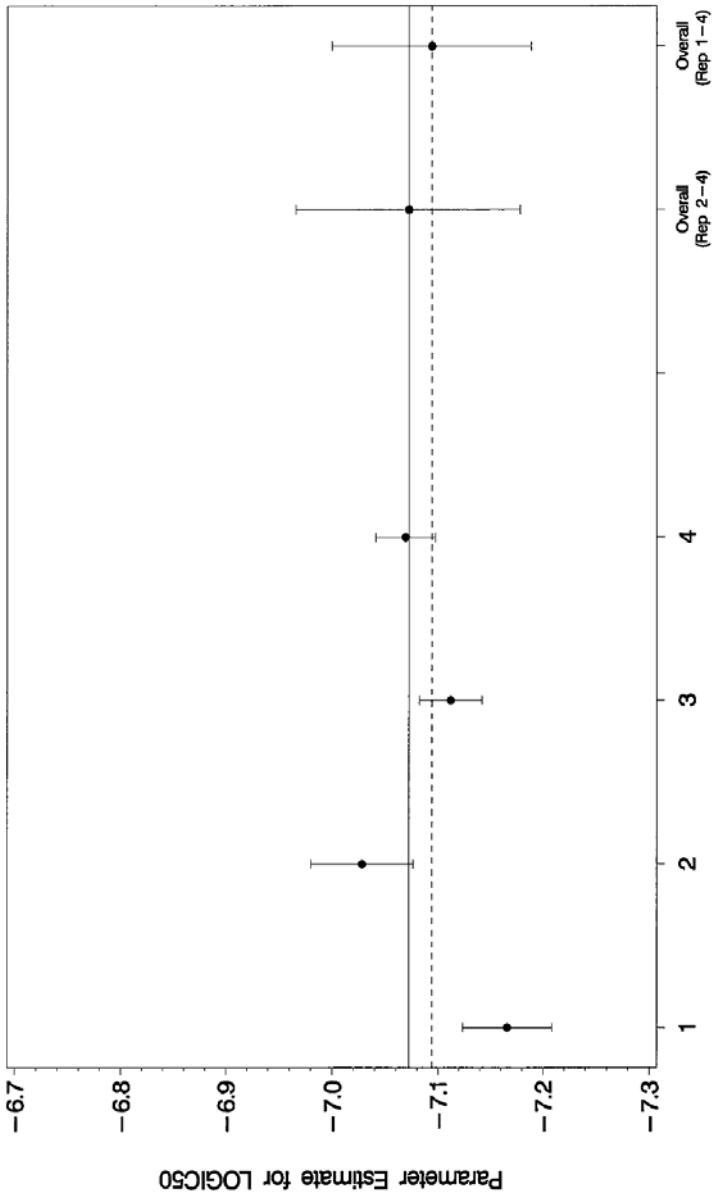


Figure 4. **$Log_{10}IC_{50}$ Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates 1 to 4, 2 to 4. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates 2 to 4. The Dotted Reference Line Corresponds to the Average Across Replicates 1 to 4**

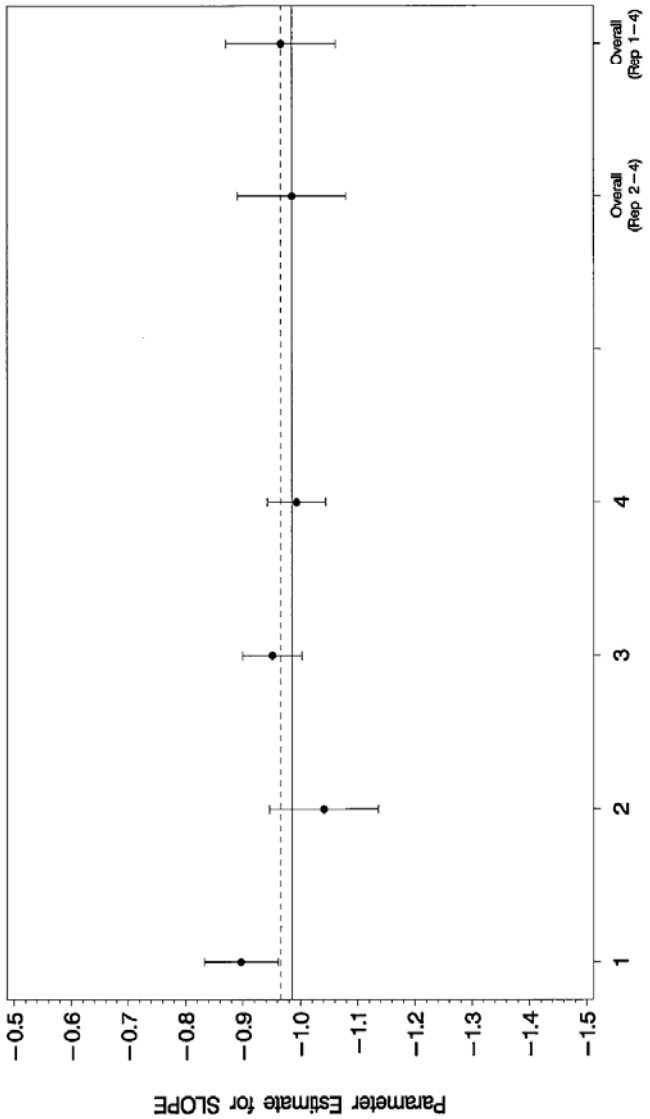


Figure 5. **Slope (β) Parameter Estimates and Their Associated 95% Confidence Intervals for Each Replicate and Across Replicates 1 to 4, 2 to 4. Placental Aromatase Assay. The Solid Reference Line Corresponds to the Average Across Replicates 2 to 4. The Dotted Reference Line Corresponds to the Average across Replicates 1 to 4**

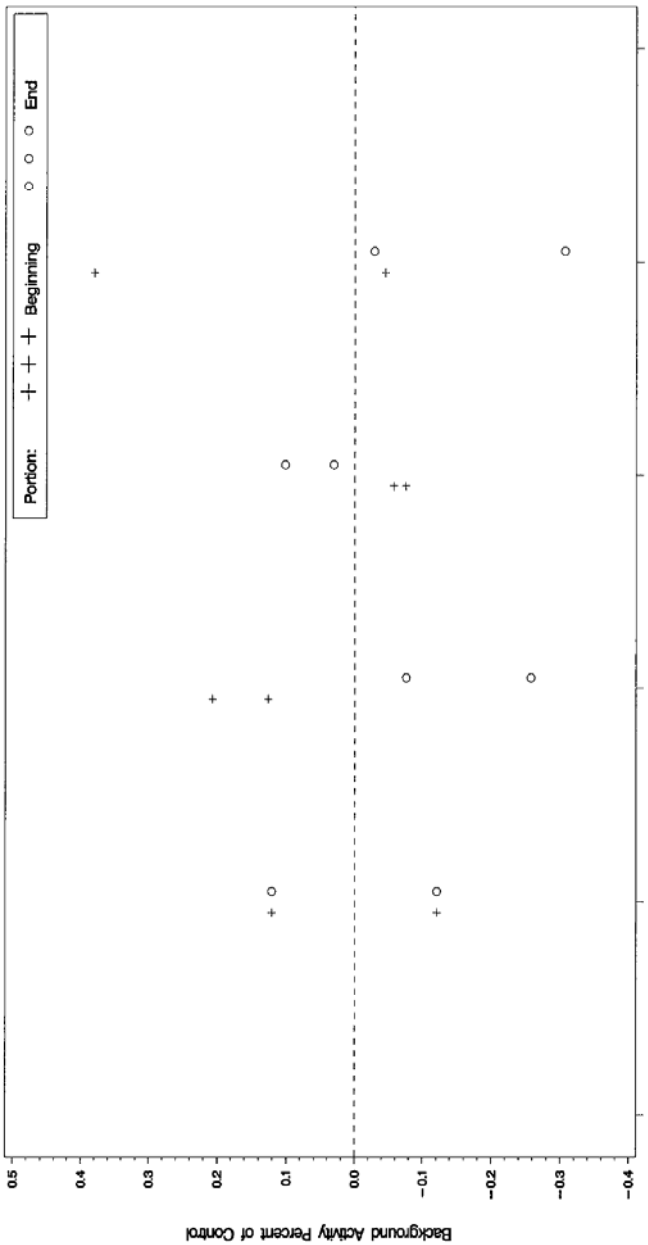


Figure 6. **Background Activity Controls. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 0%**

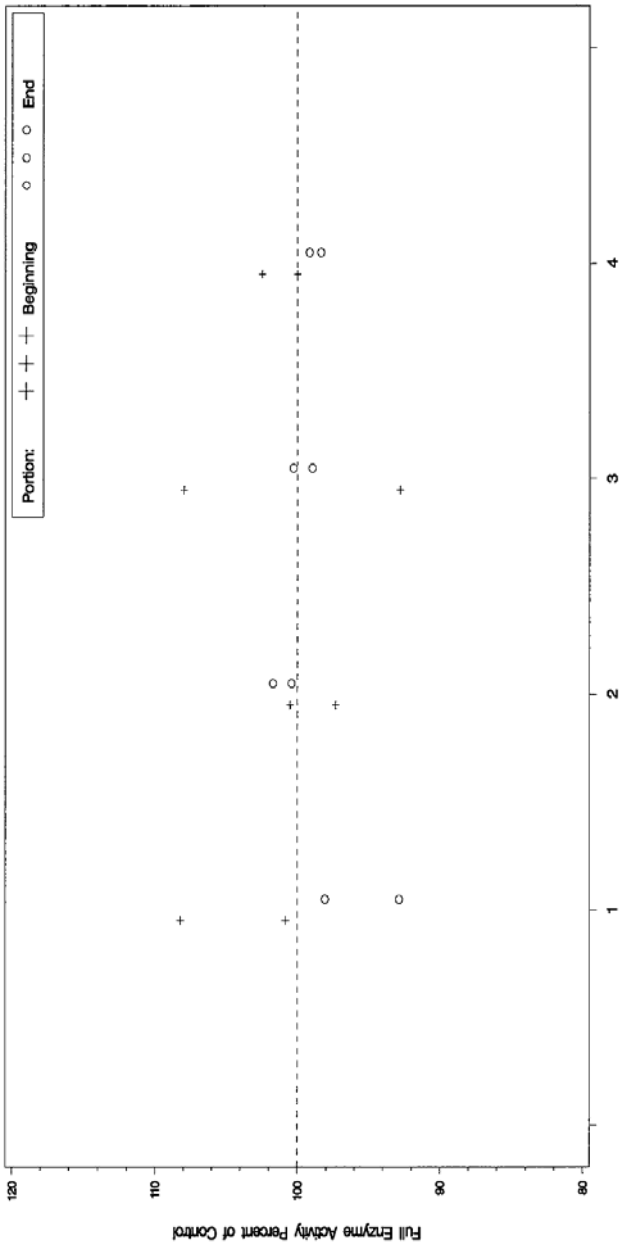


Figure 7. Full Enzyme Activity Controls. Percent of Control by Replicate and Portion of Replicate (Beginning or End). Placental Aromatase Assay. Reference Line at 100%

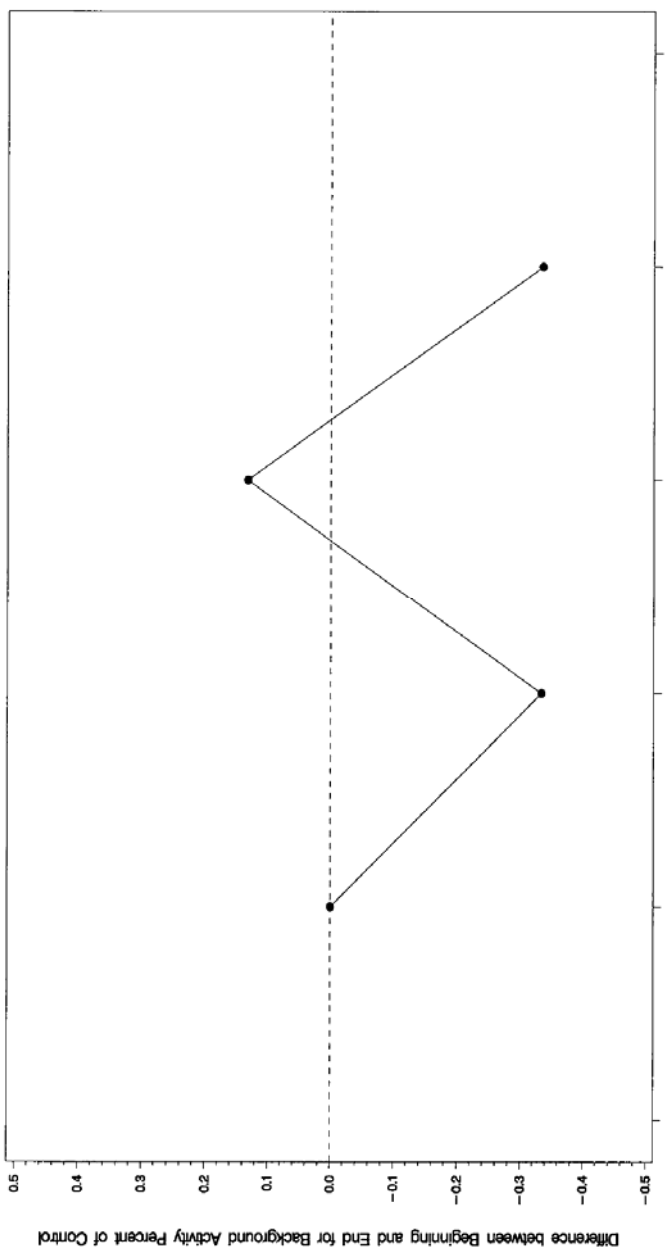


Figure 8. **Background Activity Controls. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Placental Aromatase Assay. Reference Line at 0%**

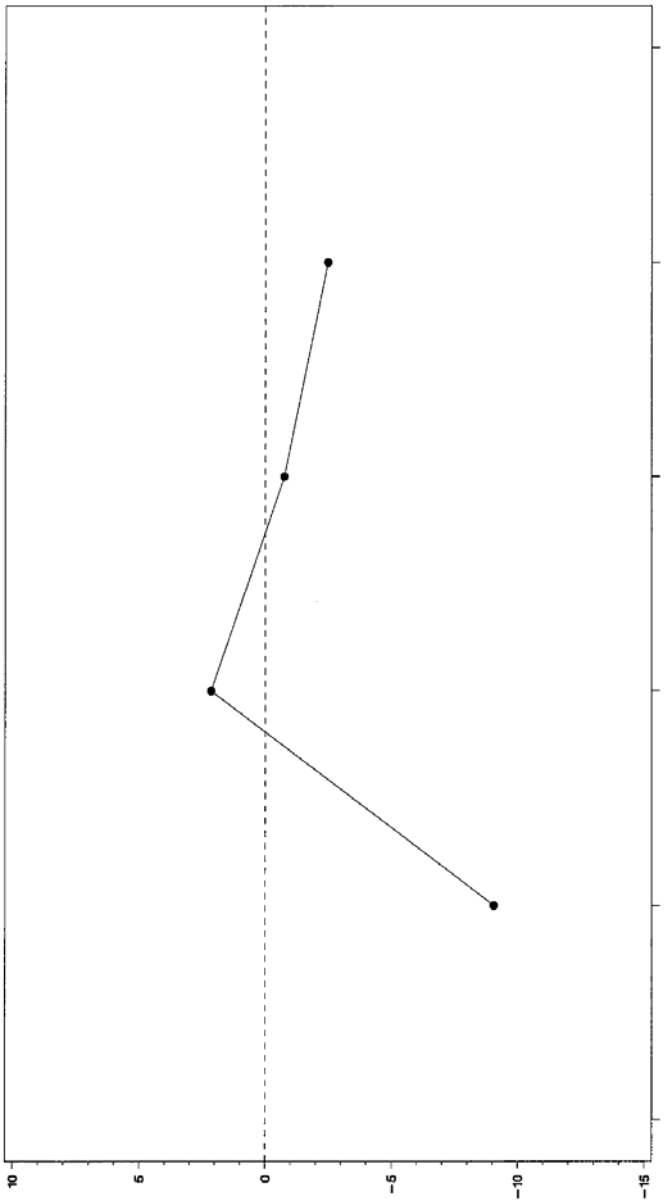


Figure 9. **Full Enzyme Activity Controls. Difference Between the Average of the Two End Percent of Control Responses and the Average of the Two Beginning Responses by Replicate (End Minus Beginning). Placental Aromatase Assay. Reference Line at 0%**

Table A-1. Percent of Control Activity in Placental Assay by Replicate, 4-OH ASDN Concentration Within Replicate, and Repetition Within Concentration

Replicate	Log [4-OH ASDN]	Percent of Control		
		Repetition 1	Repetition 2	Repetition 3
1	-6.00	7.49	7.05	7.53
	-7.00	43.13	46.25	42.97
	-7.30	57.99	59.91	60.26
	-7.60	70.90	68.41	66.24
	-8.00	76.84	80.79	85.02
	-9.00	94.49	94.10	96.30
2	-6.00	8.37	8.43	8.78
	-7.00	47.03	46.39	44.65
	-7.30	62.03	67.22	64.82
	-7.60	87.31	82.98	81.73
	-8.00	93.94	95.98	94.85
	-9.00	105.74	95.78	85.69
3	-6.00	7.90	8.22	8.20
	-7.00	42.84	42.57	43.25
	-7.30	58.40	62.52	64.48
	-7.60	72.08	73.62	77.11
	-8.00	90.07	86.88	87.20
	-9.00	94.93	92.05	93.32
4	-6.00	8.36	7.72	7.67
	-7.00	46.42	46.78	47.45
	-7.30	65.23	62.38	57.74
	-7.60	80.52	76.30	75.38
	-8.00	92.36	87.58	91.00
	-9.00	99.93	92.19	98.82

Table A-2. Background Activity Control and Full Enzyme Activity Control Corrected Aromatase Activity by Replicate and Portion (Beginning or End). Placental Aromatase Assay

Aromatase Activity	Replicate	Portion	Corrected Activity	% of Control[a]
Background Activity Control	1	Beginning	-0.000050	-0.1209
		Beginning	0.000050	0.1209
		End	0.000050	0.1209
		End	-0.000050	-0.1209
	2	Beginning	0.000046	0.1263
		Beginning	0.000076	0.2071
		End	-0.000028	-0.0758
		End	-0.000094	-0.2577
	3	Beginning	-0.000032	-0.0575
		Beginning	-0.000042	-0.0751
		End	0.000057	0.1017
		End	0.000017	0.0309
	4	Beginning	0.000284	0.3802
		Beginning	-0.000034	-0.0450
		End	-0.000021	-0.0286
		End	-0.000229	-0.3066
Full Enzyme Activity Control	1	Beginning	0.044362	108.2124
		Beginning	0.041342	100.8465
		End	0.040206	98.0761
		End	0.038070	92.8650
	2	Beginning	0.036666	100.5254
		Beginning	0.035501	97.3324
		End	0.037101	101.7178
		End	0.036629	100.4244
	3	Beginning	0.051735	92.7902
		Beginning	0.060193	107.9613
		End	0.055175	98.9612
		End	0.055915	100.2873
	4	Beginning	0.076606	102.4655
		Beginning	0.074772	100.0123
		End	0.073537	98.3605
		End	0.074136	99.1618

a. The corrected aromatase activity values were divided by the average of the four full enzyme activity control activity values within the same replicate and multiplied by 100 percent.

Figure A-1 Replicate 1. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay

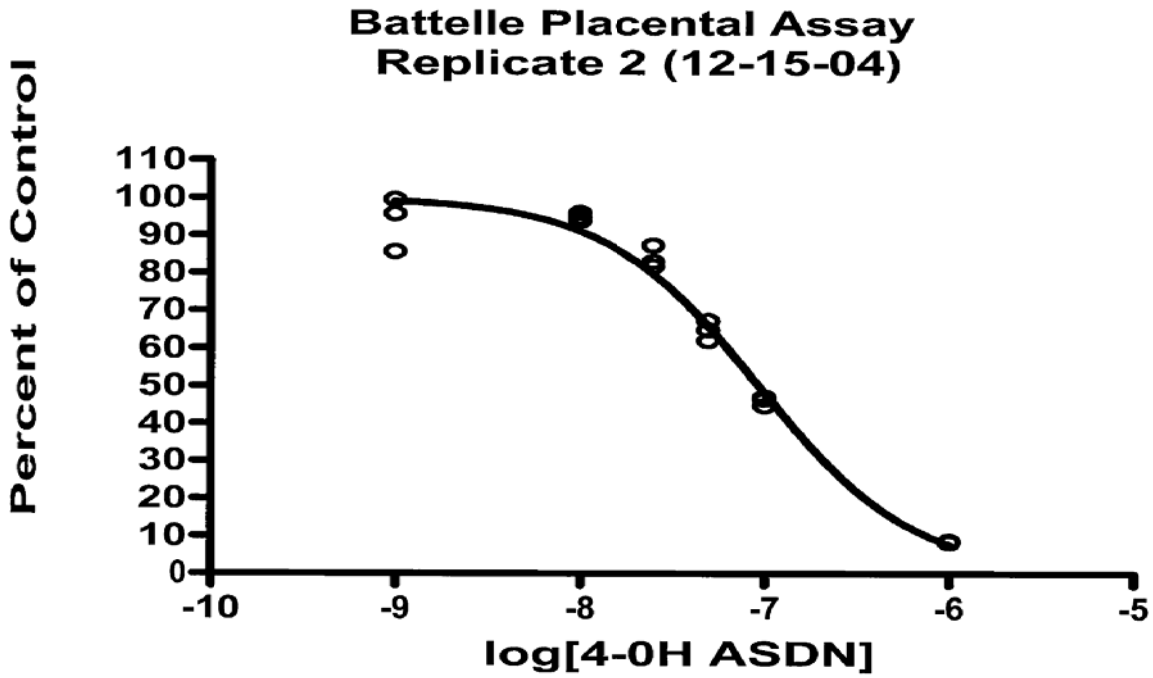


Figure A-2 **Replicate 2. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay**

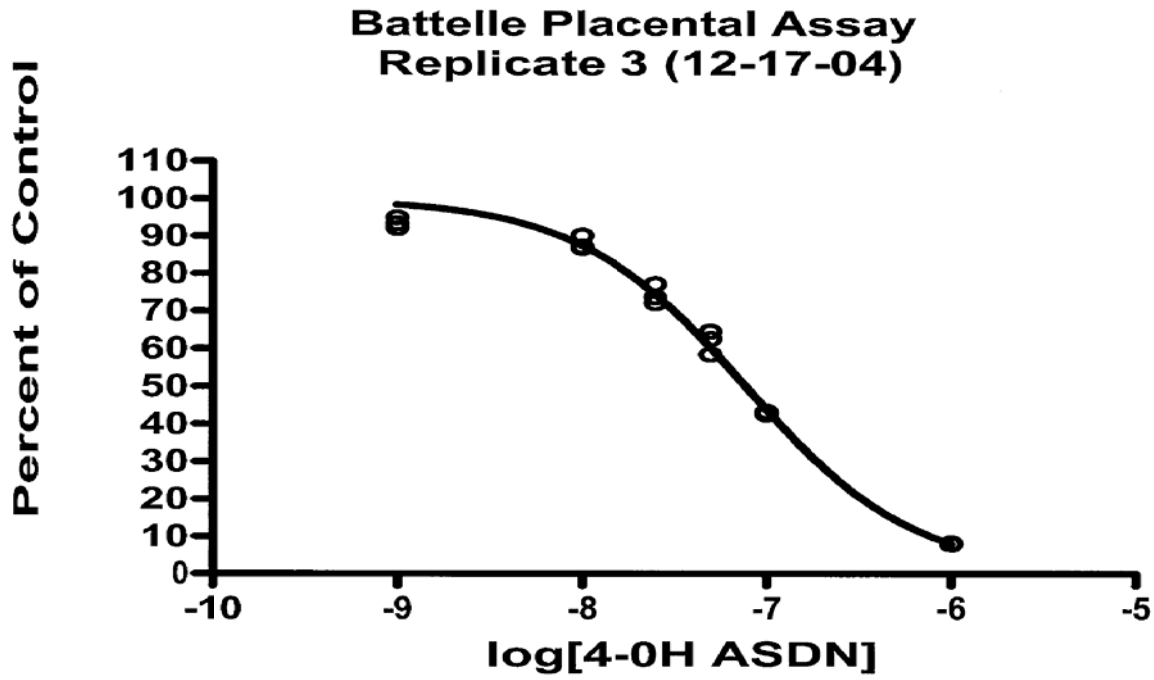


Figure A-3 **Replicate 3. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay**

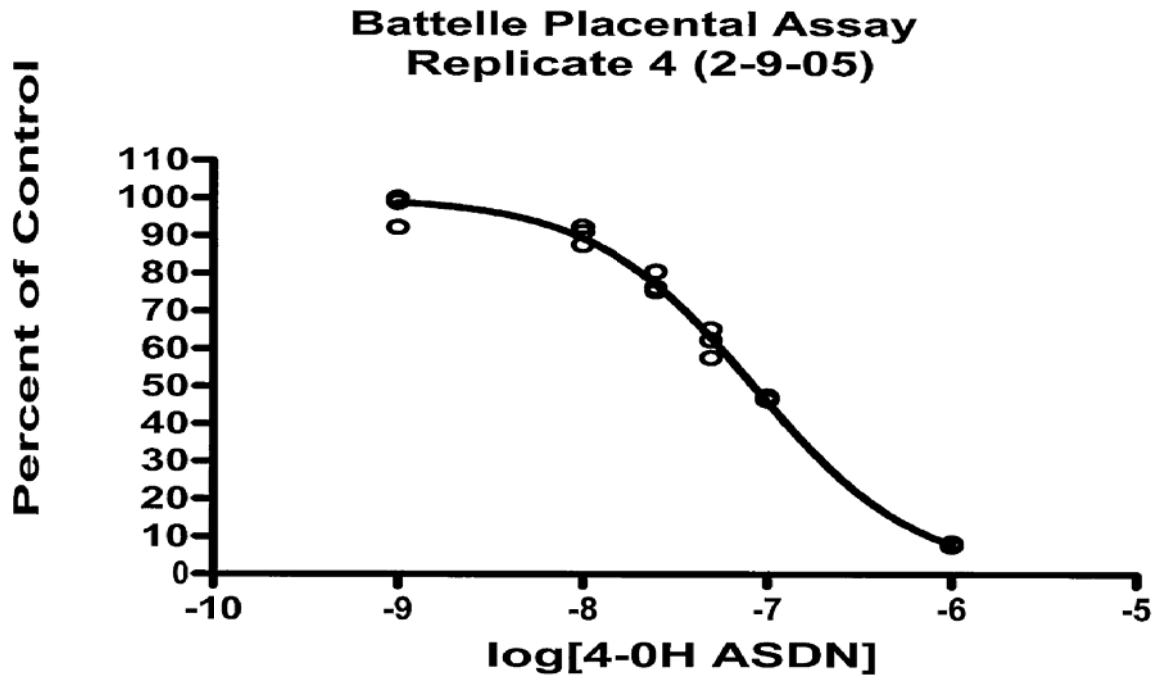


Figure A-4 **Replicate 4. Individual Percent of Control Values Vs. (Base 10) Logarithm of 4-OH ASDN Inhibitor Concentration. Concentration Response Curve Fitted to Average Responses Within Concentrations. Placental Aromatase Assay**