DRAFT FINAL REPORT

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

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

WA 4-16, Task 5

OVERALL TASK DRAFT FINAL REPORT

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This study was inspected by the Quality Assurance Unit and reports were submitted to the Work Assignment Leader (WAL) and Management as follows:

Phase Inspected	**Inspection Date**	**Date Reported to WAL/Management**
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EXECUTIVE SUMMARY

The aromatase assay using human placental microsomes was conducted by staff from a lead laboratory (RTI International) and two participating laboratories (Battelle and WIL Research Laboratories). The identity of the lead laboratory was disclosed as Laboratory A in order to make comparisons to the lead lab, whereas the identities of the participating laboratories were coded as Laboratories B and C for presentation of the results (order of the labs listed above may or may not be different than the aforementioned code). Each laboratory conducted the placental aromatase assay using four reference chemicals: aminoglutethimide, chrysin, econazole, and ketoconazole. The study design involved conducting at least three repetitions at each of eight concentrations of each reference chemical; each of which was tested in at least three independent replicates. In addition, each replicate of the assay included a positive control (4-hydroxy androstenedione) and a negative control (lindane), each tested at a single concentration.

The objectives of this task were to evaluate the responsiveness of the human placental microsomal aromatase assay to the reference chemicals and positive and negative controls when performed by the lead and participating laboratories using microsomes from the lead laboratory, as well as to obtain intralaboratory and interlaboratory values for aromatase full enzyme activity, positive and negative controls on enzyme activity, and aromatase inhibition (IC_{50}) for each of the reference chemicals.

The overall individual laboratory group mean ± SEM protein concentration values were 11.5 ± 0.6, 10.1 ± 0.8, and 11.2 ± 0.4 mg/mL for RTI and Laboratories B and C, respectively. The overall task mean ± SEM protein concentration was 10.9 ± 0.4 mg/mL with a percent CV of 6.8 percent.

Protein QC samples (target concentrations of 0.125, 0.5, and 1.0 mg/mL) had low accuracy (% RE) and precision (% CV) results for the 0.125 mg/mL QC standard but accuracy for the laboratories ranged from -4.8 to 15.9 percent and precision for the laboratories ranged from 1.7 to 4.6 percent for the 0.5 and 1.0 mg/mL QC standards.

The laboratory overall mean (± SEM) full enzyme activity control values were 0.105 ± 0.009, 0.042 ± 0.013 , and 0.049 ± 0.003 nmol/mg protein/min for RTI and Laboratories B and C, respectively. The overall task mean ± SEM full enzyme activity control value was 0.0654 ± 0.020 nmol/mg protein/min with a percent CV of 52.7 percent.

Background enzyme activity controls indicated that there was no background activity that interfered with the interpretation of the results.

For the positive control (4-hydroxyandrostenedione, 4-OH ASDN), the laboratory overall mean (± SEM) enzyme activity values were 0.049 ± 0.002, 0.022 ± 0.006 , and 0.027 ± 0.002 nmol/mg protein/min for RTI and Laboratories B and C, respectively. The overall task mean ± SEM full enzyme activity control value was 0.0328 ± 0.008 nmol/mg protein/min with a percent CV of 43.3 percent. The laboratory overall group mean (± SEM) inhibition values for 4-OH ASDN (as a percent of control) were 47.2 ± 2.3, 54.5 ± 7.1, and 55.9 ± 1.3 percent for RTI and Laboratories B and C, respectively. The overall task mean ± SEM percent of control value was 52.5 ± 2.7 percent with a percent CV of 8.9.

For the negative control (lindane), the laboratory overall mean (± SEM) enzyme activity values were 0.099 ± 0.012, 0.047 ± 0.011, and 0.049 ± 0.003 nmol/mg protein/min for RTI and Laboratories B and C, respectively. The overall task mean ± SEM full enzyme activity control value was 0.0649 ± 0.017 nmol/mg protein/min with a percent CV of 45.9 percent. The laboratory overall group mean (± SEM) percent of control values for lindane were 93.6 ± 4.1, 121.1 ± 24.4, and 99.2 ± 1.0 percent for RTI and Laboratories B and C, respectively. The overall task mean ± SEM percent of control value was 104.6 ± 8.4 percent with a percent CV of 13.9.

For aminoglutethimide in all three laboratories, increasing concentrations of aminoglutethimide decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. At an aminoglutethimide concentration of 10^{-4} M, the laboratory percent of control values ranged from 4 to 5 percent, whereas at a concentration of 10^{-8} M, the laboratory percent of control values ranged from 98 to 100 percent. Overall task mean ± SEM percent of control values at 10^{-4} and 10^{-8} M were 4.50 ± 0.24 and 98.82 (n=2) percent, respectively. The overall task percent CV values ranged from 1 to 2 percent, except at the two lowest concentrations where the percent CV values ranged from 9 to 57 percent. The mean ± SEM IC_{50} values for RTI and Laboratories B and C were 4.3 ± 0.1, 4.3 ± 0.3, and 4.5 ± 0.9 µM; the percent CV values were 4.4, 13.2, and 46.4 percent, respectively. The overall task group mean ± SEM IC_{50} value was 4.4 ± 0.1 µM and the percent CV was 3.6 percent.

For chrysin in all three laboratories, increasing concentrations of chrysin decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. At a chrysin concentration of 10^{-4} M, aromatase inhibition was not complete; the laboratory percent of control values ranged from 9 to 29 percent, whereas at a chrysin concentration of 10^{-7} M, the laboratory percent of control values ranged from 93 to 97 percent. Overall task mean ± SEM percent of control values at 10^{-4} and 10^{-7} M were 20.53 ± 6.16 and 95.15 ± 0.94 percent, respectively. The overall task percent CV values ranged from 2 to 8 percent, except at the lowest concentrations where the percent CV value was 52 percent. The mean ± SEM IC_{50} values for RTI and Laboratories B and C were 4.45 ± 0.54, 3.54 ± 0.33, and 4.17 ± 1.55 µM; the percent CV values were 20.9, 18.7, and 64.3 percent, respectively. The overall task group mean ± SEM IC_{50} value was 4.05 ± 0.27 µM and the percent CV was 11.5 percent.

For econazole in all three laboratories, increasing concentrations of econazole decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. At an econazole concentration of 10^{-7} M, the laboratory percent of control values were approximately 2 percent, whereas at a concentration of 10^{-10} M, the laboratory percent of control values ranged from 93 to 102 percent. Overall task mean ± SEM percent of control values at 10^{-7} and 10^{-10} M were 2.05 ± 0.15 and 96.12 ± 2.8 percent, respectively. The overall task percent CV values ranged from 4 to 12 percent. The mean ± SEM IC_{50} values for RTI and Laboratory C were 2.19 ± 0.22 and 2.12 ± 0.13 nM; the percent CV values were 17.6 and 10.6 percent, respectively. An average IC_{50} value was not calculated for Laboratory B (n=1) because the variability in the full enzyme activity control values precluded estimating a value from the other replicates. The overall task group mean ± SEM IC_{50} value was 2.10 ± 0.06 nM and the percent CV was 4.6 percent.

For ketoconazole in all three laboratories, increasing concentrations of ketoconazole decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. At a ketoconazole concentration of 10^{-4} M, the laboratory percent of control values ranged from 6 to 8 percent, whereas at a concentration of 10^{-7} M, the laboratory percent of control values ranged from 93 to 98 percent. Overall task mean ± SEM percent of control values at 10^{-4} and 10^{-7} M were 6.83 ± 0.78 and 95.77 (n=2) percent, respectively. The overall task percent CV values ranged from less than 5 to 20 percent. The mean ± SEM IC_{50} values for RTI and Laboratories B and C were 7.16 ± 0.17, 8.67 ± 1.08, and 6.53 ± 0.66 µM; the percent CV values were 4.1, 25.0, and 17.5 percent, respectively. The overall task group mean ± SEM IC_{50} value was 7.46 ± 0.64 µM and the percent CV was 14.8 percent.

Inhibition curves were characterized as "complete" by all laboratories for aminoglutethimide, econzole, and ketoconazole. RTI and Laboratory C characterized chrysin as "incomplete-interpolated", whereas Laboratory B characterized it as "complete".

The principal results of the interlaboratory analysis are summarized as follows:

Control activity comparisons among laboratories were made including and excluding the econazole control responses from Laboratory B. When the Laboratory B results were reported excluding econazole the end portion of the replicate was significantly lower than the beginning for each laboratory and averaged across laboratories, for full enzyme activity, negative, and positive controls. When the Laboratory B results were reported including econazole the significant difference between end and beginning no longer held for the positive and negative controls within Laboratory B. The end was significantly greater than the beginning for the full enzyme activity controls within Laboratory B. Averaged across laboratories the end was significantly lower than the beginning for the negative and positive controls but not for the full enzyme activity controls.

There were no significant differences between end and beginning for the background activity controls at RTI and Laboratory B and averaged across laboratories, whether econazole was included in or excluded from the Laboratory B results. In Laboratory C, the end was significantly lower than the beginning.

The $\log_{10}IC_{50}$ estimates were similar among the three laboratories for all four reference chemicals. The among laboratory variance were zero or near zero (p-value=1). Coefficients of variation were calculated for variation in the IC_{50} ($\equiv 10^{\log_{10}IC_{50}}$) estimates across laboratories. The coefficient of variation among laboratories ranged from 2.6% to 8.6%.

The slope estimates for aminoglutethimide, ketoconazole, and econazole were similar among the three laboratories. (The among laboratory variance were zero or near zero and the among laboratories CVs were between 1.4% and 2.3%.

For chrysin the estimated slope for Laboratory B was more than 59% smaller than those for RTI and Laboratory C (-0.94 versus -0.59 and-0.56). The among laboratory variance was 29 times larger than the within laboratory variances and among laboratories coefficient of variation was about 25%.

1.0 INTRODUCTION

1.1 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 $P450_{arom}$ 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 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.

1.2 Task Description and Objectives

In this task, the aromatase assay was conducted by staff from a lead laboratory (RTI International) and two participating laboratories (Battelle and WIL Research Laboratories). A third participating laboratory was planned but scheduling constraints precluded its participation in this task. Each laboratory conducted at least three independent replicates of the placental aromatase assay with aminoglutethimide, chrysin, econazole, and ketoconazole (reference chemicals), 4- hydroxyandrostenedione (positive control) and lindane (negative control). The human placental microsomes were prepared by RTI. The study design involved, for each reference chemical, conducting three repetitions at each of eight concentrations, and for the positive and negative controls, conducting three repetitions at a single concentration, for each of at least three independent replicates. Reagents and assay solutions were made fresh for each replicate so that the replicates were truly independent. The reference chemicals and positive and negative controls were prepared and analyzed at a central laboratory (Chemical Repository at Battelle) before they were distributed to the laboratories.

The objectives of this task were to evaluate the responsiveness of centrally-prepared human placental microsomes (RTI International) that were prepared for conducting the aromatase assay using four reference chemicals and positive and negative controls when the assay was performed by a lead and two participating laboratories, as well as to obtain intralaboratory and interlaboratory values for the aromatase enzyme activity and aromatase inhibition (IC_{50}) for each of the reference chemicals and the positive and negative controls.

1.3 Overall Report Content and Format

The overall report includes salient information about the methods used and results obtained by the individual laboratories, as well as the interlaboratory statistical analysis narrative. Detailed information about the results obtained by the individual laboratories can be found in their reports, which are included in the appendices of the overall report. In addition, there are a few important supplemental documents that were the same for all laboratories, i.e. chemistry reports and quality assurance project plan (QAPP), and others that were laboratory specific, i.e. protocol, spreadsheets, intralaboratory statistical analysis narrative. All of these documents can be found in the appendices of the individual laboratory reports.

The participating laboratories that conducted the experiments of this task are coded in those sections of the overall report where laboratory performance is described or data are presented. Coded presentation is used so that the data could be evaluated in an unbiased manner and, whatever the outcome of the study, there would be no connotation, favorable or otherwise, put on the laboratories. The lead laboratory was identified since some endpoints are expressed in terms relative to the lead laboratory.

2.0 MATERIALS AND METHODS

2.1 Chemistry

2.1.1 Substrate – Androstenedione (ASDN)

The substrate for the assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were obtained by Battelle's Chemical Repository and then distributed to the lead and participating laboratories so that all laboratories used the same lot of the substrate. The non-radiolabeled ASDN had a reported purity of 100%. The radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN 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 and was determined to be 97% (RTI's report for radiochemical purity is included in the individual laboratory report appendices).

ASDN substrate solution preparation is described in detail in the laboratory reports. Briefly, the substrate solution was prepared fresh each day and contained 2 μM ASDN with approximately 1 μCi/mL. The addition of 100 μL of the substrate solution to each 2 mL assay volume yielded a final [^{3}H]ASDN concentration of 100 nM with approximately 0.1 μCi/tube.

2.1.2 Reference Chemicals and Control Substances

The Chemical Repository at Battelle was responsible for the chemistry activities performed on the four reference chemicals and two control substances. The chemistry activities included chemical procurement, solubility, formulation stability assessment, formulation preparation, formulation analysis and shipment of the stock formulations to the lead and participating laboratories. These chemistry activities and results of the analysis and stability determinations are described in the Chemical Repository chemistry reports that are in the appendices of the individual laboratory reports. Table 1 summarizes the salient information for the reference chemicals and control substances.

Table 1. Chemistry Information for the Reference Chemicals and Control Substances

Chemical Name	Mfr. Purity	CAS No.	Molecular Formula	Molecular Weight (g/mol)
Reference Substances				
Aminoglutethimide	>99%	125-84-8	$C_{13}H_{16}N_2O_2$	232.3
Chrysin	98.2%	480-40-0	$C_{15}H_{10}O_4$	254.2
Econazole	98	24169-02-6	$C_{18}H_{15}Cl_3N_2O \cdot HNO_3$	444.7
Ketoconzole	>99	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	531.4
Control Substances				
4-hydroxyandrostenedione (Positive Control)	99%	566-48-3	$C_{19}H_{26}O_3$	302.4
Lindane (Negative Control)	99.6%	58-89-9	$C_6H_6Cl_6$	290.8

The reference chemical and control stock formulations were prepared by the Chemical Repository and shipped to the laboratories. The laboratories instituted appropriate procedures so that the reference chemical stock formulations were tested blind. The reference chemical stock formulation concentrations were 0.01 M for chrysin and ketoconazole and 0.1 M for aminoglutethimide and econazole. The dilution schemes used by the laboratories to prepare the appropriate concentrations of the reference chemicals and control substances for testing are presented in the individual laboratory reports. Fresh dilutions were prepared on the day prior to testing. The reference chemicals were tested at eight different concentrations ranging from 10^{-3} to 10^{-10} M and the control substances were each tested at a single concentration of 5×10^{-8} M for 4-OH ASDN and 10^{-6} M for lindane. The vehicle for the reference chemicals and lindane was dimethylsulfoxide (DMSO), whereas for 4-OH ASDN it was ethanol.

2.2 Human Placental Microsomes

Human placental microsomes were provided to each of the laboratories by RTI (Lot No. 11343-7, 14 mg/mL protein concentration, prepared November, 2004) and were stored at approximately -70°C until the time of the assay. The microsomes received from RTI were thawed, pooled, homogenized, and re-distributed into smaller single-use vials (approximately 100 to 160 µL aliquots) to preclude any further freeze-thaw cycles from occurring when the assay was conducted. On the day of use, the microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter-Elvejhem homogenizer and then kept on ice until used. For use in the assay, the microsomes were diluted in the assay buffer in two serial dilutions. A

50-fold dilution was made to achieve a concentration of approximately 0.28 mg/mL. Another 10-fold dilution was made to achieve the desired final working stock concentration of approximately 0.025 mg/mL. The final target protein concentration in the incubation mixture was approximately 0.0125 mg/mL.

2.3 Other Assay Components

Information about the other assay components is provided in Table 2. The Chemical Repository obtained the NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, reduced form), DMSO and ethanol and distributed it to the participating laboratories so that it would be from the same supplier and lot.

Table 2. Other Assay Components

Component	Supplier		
	Battelle	RTI	WIL
NADPH (co-factor)[a]	Sigma	Sigma	Sigma-Aldrich
Propylene glycol	Spectrum Chemical	J. T. Baker	J. T. Baker
Sodium phosphate dibasic (buffer)	Sigma	J. T. Baker	J. T. Baker
Sodium phosphate monobasic (buffer)	Sigma	J. T. Baker	J. T. Baker

NADPH was prepared fresh each day (6 mM, 5 mg/mL) in 0.1 M sodium phosphate buffer (pH of 7.4). Further details about preparation, storage, and expiration are provided in the laboratory reports (see appendices).

2.4 Protein Determination

The microsomal protein concentration was determined using a DC Protein Assay kit from Bio-Rad (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 0.13 to 1.5 mg protein/mL (varied slightly for each laboratory). The absorbance at a wavelength of 750 nm was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined from the absorbance value using linear regression to the absorbance of the protein standards.

During the conduct of the work assignment, quality control (QC) samples were included in the assay. QC samples were selected from a set of pre-diluted protein standards at concentrations of 0.125, 0.500, and 1.000 mg/mL. The QC samples were analyzed in duplicate using the protein determination method described in the preceding paragraph. Additional information about the unknowns and QC sample protein determinations can be found in the laboratory reports (see appendices).

2.5 Aromatase Assay Procedure

Details of how the assay was actually performed by each participating laboratory are presented in the individual laboratory reports. The general procedure is presented as follows. The assays were performed in 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 a given reference chemical or control substance to the total volume of 1.0 mL. The final concentrations for the assay major components are presented in Table 3. 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.

Table 3. Aromatase Assay Conditions

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)
Reference Chemical, Control Substance, or Vehicle	20 µL	Varied
Incubation Time	15 min	Not Applicable

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

- Full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of reference chemical solutions] and microsomes).

- Background activity controls (all components found in the full aromatase activity control, except NADPH).

- Positive controls (all components found in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a concentration of 5 x 10^{-8} M).

- Negative controls (all components found in full aromatase activity controls, except vehicle, and with the addition of lindane at a concentration of 1 x 10^{-6} M).

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

The total assay volume was 2.0 mL and the tubes were incubated for 15 minutes at 37 ± 1°C. The incubations were stopped by the addition of methylene chloride (2 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 to extract unreacted ASDN, then centrifuged for 10 minutes to facilitate separation of the organic and aqueous layers. The methylene chloride layer was removed and discarded; the aqueous layers were extracted two more times, each time with 2 mL of methylene chloride. 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 was added to each counting vial and the vials shaken to mix.

Analysis of the samples was performed using liquid scintillation spectrometry (LSS). Radioactivity found in the aqueous fractions represented ^3H_2O formed from the hydrolysis of [^{3}H]-ASDN. One H_2O molecule was released per molecule of ASDN converted to estrogen in a stereospecific reaction. Thus, the amount of 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). Results are presented as the activity (velocity) of the enzyme reaction and expressed in nmol (mg protein)$^{-1}$min^{-1}.

Each laboratory performed at least three independent replicates and, for a given replicate, each reference chemical or control substance concentration was performed in triplicate. The study design is summarized in Table 4.

Table 4. Reference Chemical Study Design

Sample Type	Repetitions (Test Tubes)	Description	Final Reference Chemical or Control Substance Concentration (M)
Full Enzyme 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
Positive Control	4	Complete assay with positive control chemical (4-OH ASDN) added	5×10^{-8}
Negative Control	4	Complete assay with negative control chemical (lindane) added	1×10^{-6}
Reference Chemical Concentration 1	3	Complete assay with Reference Chemical added	1×10^{-3}
Reference Chemical Concentration 2	3	Complete assay with Reference Chemical added	1×10^{-4}
Reference Chemical Concentration 3	3	Complete assay with Reference Chemical added	1×10^{-5}
Reference Chemical Concentration 4	3	Complete assay with Reference Chemical added	1×10^{-6}
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1×10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1×10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1×10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1×10^{-10}

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

2.6 Data Analysis - Aromatase Activity and Percent of Control Calculations

Each laboratory entered data into an Excel spreadsheet (provided by RTI and Battelle) for calculation of aromatase activity and percent of control. For each repeat tube (full enzyme activity control, background activity control, positive and negative controls and each reference chemical concentration), the Excel spreadsheet included total observed (uncorrected) disintegration per minute (dpm) per tube and total aromatase activity per tube. The dpm and aromatase activity values were corrected for the background dpm's, as measured by the average of the background activity control tubes. The aromatase activity was calculated as the corrected dpm, normalized by the specific activity of the [^{3}H]ASDN, the mg of protein of the aromatase, and the incubation time. The average (corrected) dpm and aromatase activity across the four background activity control repeat tubes were necessarily equal to 0 (zero) within each replicate.

For each tube, percent of control was determined by dividing the background corrected aromatase activity for that tube by the average background corrected aromatase activity for the four full enzyme activity control tubes and multiplying by 100. It was expected that for an inhibitor the percent of control activity values could vary between approximately 0% near the high inhibition concentrations and approximately 100% near the low inhibitions concentrations. However, due to experimental variation individual observed percent of control values sometimes extended below 0% or above 100%.

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

2.7 Statistical Analysis

2.7.1 Intralaboratory Statistical Analysis

2.7.1.1 Concentration Response Fits for the Reference Chemicals

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

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

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

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

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

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits were carried out using Prism software (Version 3.02 or higher). Observed individual percent activity values above 100% were set to 99.5%. Observed individual percent activity values below 0% were set to 0.5%.

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

2.7.1.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fit

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

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

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

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

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

2.7.1.4 Statistical Software

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

2.7.2 Interlaboratory Statistical Analysis

The interlaboratory analysis was based on the $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined in the intralaboratory analyses for each reference chemical. The interlaboratory analysis also compared across the three laboratories the full enzyme activity, background activity, negative, and positive control responses at the beginnings and the ends of the replicates. It determined whether the control activity differed between the beginning and the end of each replicate.

In some instances the entries in the tables in the interlaboratory analysis report tables differ from corresponding entries in the intralaboratory analysis reports tables by one or a small number of trailing digits in the last decimal place. This is due to differences in rounding in intermediate calculations between the intralaboratory analyses and the interlaboratory analysis.

2.7.2.1 Objectives of the Interlaboratory Analysis

The objectives of the interlaboratory statistical analysis were to:

- Determine the average values and the variabilities among laboratories for the above parameters.

- Determine the coefficients of variation among laboratories for the $\log_{10}IC_{50}$ and the slope parameters[1].
- Estimate the ratio of the among laboratory variation to the average within laboratory variation for the parameters mentioned above.

The interlaboratory analyses for $\log_{10}IC_{50}$ and the slope parameters were carried out separately for each reference chemical, including the data from each laboratory for each chemical. The interlaboratory analyses for the control activity responses were carried out on the results combined across reference chemicals, based on two versions of the data:

- Including the data from each laboratory for each reference chemical.
- Excluding the econazole results from Laboratory B.

2.7.2.2 Test Organization

Placental aromatase assay activity levels were determined for graded concentrations of each of the four reference chemicals: aminoglutethimide, chrysin, ketoconazole, and econazole. One to five replicates of the positive control study were carried out at each laboratory for each reference chemical (Table 5). Within each replicate three repetitions were run at each of the chemical concentrations. In addition, for each reference chemical, two repeat tubes of the full enzyme activity, background activity, negative, and positive controls were run prior to the concentration response runs and two repeat tubes of the full enzyme activity, background activity, negative, and positive controls were run following the concentration response runs.

Table 5. Number of Replicates at Each Laboratory for Each Reference Chemical

Chemical	RTI	Laboratory B	Laboratory C
Aminoglutethimide	3	3	5
Chrysin	3	3	4
Ketoconazole	4[2]	3	3
Econazole	4[2]	1	3

In order to fully explain the interlaboratory statistical analysis, it is necessary to describe some aspects of the intralaboratory statistical analysis.

Intralaboratory statistical analyses were carried out on the percent of control activity responses. Percent of control activity is defined as the ratio of the background adjusted aromatase activity in the tube under consideration to the average background adjusted aromatase activity among the four full enzyme activity control tubes within the replicate, times 100. The average percent of control among the four background adjusted full enzyme activity control tubes is necessarily 100 percent within each replicate. The average percent of control among the

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[1] Coefficient of variation was not calculated for the control results because differences between the beginning and the end portions of replicates were reported and the distributions of these differences were anticipated to straddle 0.

[2] Three replicates were used in the analyses

four background adjusted background activity control tubes is necessarily 0 percent within each replicate.

Nominally for an inhibitor the percent of control activity values vary between approximately 0% near the high chemical concentrations and approximately 100% near the low chemical concentrations, but this may vary with the inhibitor.

Intralaboratory statistical analyses were performed based on a common statistical analysis plan. The following results were reported in each intralaboratory analysis.

- Concentration curve fits within each replicate of each reference chemical to describe the trend in the percent of control activity across varying concentrations of each reference chemical.
- Estimates of the $\log_{10}IC_{50}$ concentration, slope, and associated standard errors within each replicate for each reference chemical.
- Average $\log_{10}IC_{50}$ concentration, average slope, and associated standard errors across replicates for each chemical.
- Results of analysis of variance applied to the data for the full enzyme activity, background activity, negative, and positive activity controls tested at the beginning and those tested at the end of each replicate. RTI reported least squares means results at the end and at the beginning, separately for each chemical, Laboratory B reported least squares means results at the end and at the beginning combined across chemicals, and Laboratory C reported the differences between the beginning and the end combined across chemicals. In addition, laboratory B reported results both including and excluding econazole (i.e., results across all four chemicals and results across three chemicals only).

The interlaboratory statistical analysis combines summary results from each of the intralaboratory analyses to assess relationships among the results at each laboratory, the extent of laboratory-to-laboratory variation, and overall consensus estimates among the laboratories with associated variability estimates (incorporating laboratory-to-laboratory variability). The interlaboratory analysis is based on the average $\log_{10}IC_{50}$ and slope parameters of the concentration response curve fits determined by each of the test laboratories, as reported in the intralaboratory analyses. It also compares among laboratories the differences between results obtained at the end of each replicate and those obtained at the beginning for full enzyme activity, background activity, negative, and positive controls.

2.7.2.3 Statistical Analysis Methods

Statistical analyses were carried out for each of the six endpoints discussed above: $\log_{10}IC_{50}$, slope, portion effects (beginning minus end) for background activity, full enzyme activity, negative, and positive controls. The analyses for $\log_{10}IC_{50}$ and slope were carried out separately for each reference chemical, while the analyses for the control activity responses were carried out combining all chemicals.

For each endpoint a one-way random effects analysis of variance model with heterogeneous variances among the participating laboratories was fitted to the summary responses within laboratories. Laboratory was treated as a random effect. The within laboratory variances were based on the squares of the standard errors associated with the endpoint estimates in each of the intralaboratory analyses. The analysis of variance resulted in a weighted average across all the laboratories and its associated standard error as well as an estimate of the laboratory-to-laboratory component of variation. The weights included in the weighted averages incorporated both laboratory-to-laboratory variation and within laboratory variation. The degrees of freedom associated with the overall weighted averages were calculated based on Satterthwaite's approximation as

$$2*[((1/K)* \Sigma(S_L^2 + S_i^2))^2]/[(\mathrm{var}(S_L^2)+(2/K^2)* \Sigma(S_i^4/df_i))]$$

where S_L^2 is the random laboratory to laboratory variance, S_i^2 and df_i are the reported within laboratory variance and degrees of freedom for the i^{th} laboratory, $\mathrm{var}(S_L^2)$ is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).

For each endpoint, the estimated overall average and its associated standard error (incorporating both within laboratory and among laboratory components of variation) and associated degrees of freedom were used to construct a 95% confidence interval based on the t-distribution. For each laboratory the individual effect and associated 95% confidence interval (based on the within laboratory standard error) were also determined. These were plotted side-by-side to provide a graphical comparison among the laboratories.

When calculating the within laboratory mean $\log_{10}IC_{50}$ and slope across replicates, all three laboratories incorporated the replicate-to-replicate component of variation into the standard errors of the averages.

The three laboratories reported different summary statistics for the control activity responses. The differences between the beginning and the end results (i.e., end minus beginning) were therefore determined differently for each laboratory:

- RTI reported least squares means and associated standard errors for the beginning and the end portions, separately for each reference chemical. For RTI, the least squares means were first calculated for each position, beginning or end, by averaging the values for the position across the reference chemicals. The differences were calculated as end minus beginning. The associated standard errors for the differences between beginning and end (beginning minus end) were calculated as (LS Mean)/ (F-value)$^{1/2}$, where the F-values and associated p-values were reported for the portion effects in the intralaboratory analysis report.

- Laboratory B reported least squares means and associated standard errors for the beginning and the end portions, combined across reference chemicals. The difference was calculated as end minus beginning. The associated standard errors for the differences between beginning and end (beginning minus end) were calculated as (LS

Mean)/(F-value)$^{1/2}$, where the F-values and associated p-values were reported for the portion effects in the intralaboratory analysis report.

- Results for Laboratory C are as reported in the intralaboratory analysis report.

To describe the variability among the individual laboratory values relative to the overall average value, coefficients of variation (CV) and their associated 95% confidence intervals (95% CIs) were calculated for the $\log_{10}IC_{50}$ and the slope parameters. The coefficient of variation is defined as the standard deviation of the effect response divided by its mean. The methods for calculating the CV and the associated 95% CI were different depending on the underlying assumption about the distribution of the endpoint parameter.

For $\log_{10}IC_{50}$, the CV was determined for the variation of the IC_{50} among laboratories because this is the physically meaningful parameter. $IC_{50} \equiv 10^{\log_{10}IC_{50}}$ and the distribution of $\log_{10}IC_{50}$ was assumed to be approximately normally distributed, so the individual IC_{50} values were assumed to be approximately log normally distributed. The CV therefore is expressed as

$$CV = [10^{(S2)}-1]^{½} \times 100\%$$

where S^2 is the total variance (of $\log_{10}IC_{50}$) among the three laboratories. S^2 is approximated by $3(se)^2$ where se is the standard error of the pooled mean estimate of $\log_{10}IC_{50}$. This would be exact if the within laboratory variances were equal across laboratories.

The 95% confidence interval is based on the chi square distribution and is calculated as

$$[(10^{(df*S2/(\chi2_{df,\,0.975}))} - 1)^{½} \times 100\%, (10^{(df*S2/(\chi2_{df,\,0.025}))} - 1)^{½} \times 100\%]$$

where df is the estimated degree of freedom among the three laboratories.

For slope (β), the measurements are assumed to be approximately normal. The CV therefore is expressed as

$$CV = S/\beta_{avg} \text{ x } 100\ \%$$

where S^2 is the total variance among the three laboratories, defined as above and $S = \sqrt{S^2}$. The endpoints of the confidence interval for CV are based on the noncentral t distribution (Lehmann, 1986).

To describe the variability among laboratories relative to variability within laboratories the ratio of the variance among laboratories to the average variance within laboratories was calculated as

$$R = S^2_{lab}\ [⅓(s_1^2 + s_2^2 + s_3^2)]$$

where S^2_{lab} is the component of variance among the three laboratories and (s_1^2, s_2^2, s_3^2) are the squares of the within laboratory standard errors at the three laboratories. A confidence interval for this ratio

$$[R/F^{-1}(0.975), R/F^{-1}(0.025)]$$

was based on the F-distribution with (ν_{lab}, ν_{wi}) degree of freedom, where ν_{lab}=2 and ν_{wi} is based on Satterthwaite's approximation

$$\nu_{wi} \approx [(s_1^2 + s_2^2 + s_3^2)^2]/[s_1^4/\nu_1 + s_2^4/\nu_2 + s_3^4/\nu_3].$$

This ratio was calculated for each of the six endpoint parameters.

In several places entries in the tables in the interlaboratory analysis report tables may differ from corresponding entries in the intralaboratory analysis report tables by one or a small number of trailing digits in the last decimal place. This is often due to differences between the intralaboratory analyses and the interlaboratory analysis in rounding in intermediate calculations.

2.8 Good Laboratory Practices

The toxicology laboratories at RTI, Battelle (and Battelle's chemistry laboratories), and WIL Research Laboratories are operated in compliance with the U.S. EPA FIFRA Good Laboratory Practices (GLP) Standards. Thus, these studies were conducted in compliance with EPA FIFRA Regulations for GLPs.

2.9 Personnel

The personnel involved in the conduct of this task are listed in their respective laboratory reports that are included in the appendix. The study directors and the lead technician for the study at each of the laboratories were:

- James M. Mathews, Ph.D., D.A.B.T. and Sherry Black, B.S. – RTI International
- Bozena D. Lusiak, Ph.D. and Thomas Deck, B.S. – Battelle
- Jennifer A. Thomas-Wohlever, Ph.D. and Justin Godsey, B.S. – WIL Research Laboratories

3.0 RESULTS

3.1 [^{3}H]-ASDN Radiochemical Purity

The radiochemical purity for the substrate was 97 percent. The radiochemical purity report is included as an appendix of the individual laboratory reports.

3.2 Reference Chemical and Control Substances Analyses

The actual reference chemical and control substance stock formulation concentrations were within less than approximately 4 percent of their respective target concentrations (Table 6). The formulations were determined to be stable when stored refrigerated for at least the period of time reported in the table. The chemistry reports are included in the appendices of the individual laboratory reports.

Table 6. Analysis Results and Stability of Reference Chemical and Control Substances Stock Formulations

Reference Chemical or Control Substance	Target Conc (mg/mL)	Measured Conc (mg/mL)	% of Target	Stability[a] (Days)
Aminoglutethimide	23.2	22.9	-1.3	59
Chrysin	2.54	2.47	-2.8	100
Econazole	44.4	46.1	3.8	56
Ketoconazole	5.31	5.13	-3.4	60
4-OH ASDN	3.02	3.07	1.7	173
Lindane	29.1	29.9	2.7	168

a. Based on information at the conclusion of Work Assignment 4-16.

3.3 Microsomal Protein Analysis

The microsomal protein concentration was determined on the day that the microsomes were used in the assay. Since the laboratories conducted 3 -5 replicates for a given reference chemical, the number of protein analyses performed varied for the labs, e.g. RTI, n=14; Laboratory B, n=13; Laboratory C, n=15 (one of the determinations for Laboratory B was not included since the microsomes used were from a different source). The overall individual laboratory group mean (± Standard Error of the Mean, SEM) protein concentration values were 11.5 ± 0.6, 10.1 ± 0.8, and 11.2 ± 0.4 mg/mL for RTI and Laboratories B and C, respectively (Table 7). Comparison of the lead laboratory to the individual laboratories resulted in a percent relative error (%RE) of -12.1 percent for Laboratory B and -2.1 percent for Laboratory C. The overall task mean ± SEM protein concentration was 10.9 ± 0.4 mg/mL with a percent CV of 6.8 percent.

Table 7. Human Placental Microsomal Protein Concentration[a]

Parameter	RTI	Laboratory B	Laboratory C
Overall Average (mg/mL)	11.5	10.1	11.2
Overall sd	2.1	2.9	1.4
Overall SEM	0.6	0.8	0.4
Minimum (mg/mL)	8.95	4.35	8.84
Maximum (mg/mL)	17.0	13.2	14.2
Overall % CV	18.1	28.8	12.8

a. Table values were based on all protein concentrations reported by labs for all replicates, regardless of whether the replicates were used or not for reporting enzyme activity and IC_{50} value.

Protein QCs were analyzed with the unknowns during a portion of the study. The target QC concentrations were 0.125, 0.5, and 1.0 mg/mL. In general, accuracy (% RE) and precision (% CV) were low for the 0.125 mg/mL QC standard. In contrast, accuracy for the laboratories ranged from -4.8 to 15.9 percent and precision for the laboratories ranged from 1.7 to 4.6 percent for the 0.5 and 1.0 mg/mL QC standards (Table 8).

Table 8. Protein QC Standards

Parameter	RTI	Lab B	Lab C
0.125 mg/mL			
% RE	-29.2	-10.7	-32.5
% CV	11.3	29.5	29.0
0.5 mg/mL			
% RE	14.8	15.9	0.3
% CV	3.1	2.7	1.7
1.0 mg/mL			
% RE	7.2	11.8	-4.8
% CV	4.6	2.8	3.6

3.4 Full Enzyme Aromatase Activity

Full enzyme activity controls were conducted in duplicate repetitions at the beginning and end of each replicate of the assay (a total of four tubes/replicate and at least three replicates). Full enzyme control activities were calculated from three replicates/reference chemical for 4 of 4, 3 of 4, and 2 of 4 reference chemicals for RTI, Laboratories B and C, respectively; five replicates for aminoglutethimide and four replicates for chrysin were used by Laboratory C and five replicates for econazole were used by Laboratory B. These additional replicates were performed because the laboratories believed there was an error that occurred during the conduct of the assay but the absence of knowing for certain precluded exclusion of the data from consideration.

The laboratory overall average ± SEM full enzyme activity control values were 0.105 ± 0.009, 0.042 ± 0.013, 0.049 ± 0.003 nmol/mg protein/min for RTI, Battelle and Laboratories B and C, respectively (Table 9). Comparison of the lead laboratory to the individual laboratories resulted in a %RE of -59.8 percent for Laboratory B and -53.3 percent for Laboratory C. The overall task mean ± SEM full enzyme activity control value was 0.0654 ± 0.020 nmol/mg protein/min with a percent CV of 52.7 percent.

Table 9. Human Placental Full Enzyme Activity Control Determinations[a]

	Aromatase Activity (nmol/mg protein/min)		
Parameter	RTI	Lab B	Lab C
Overall Average	0.105	0.042	0.049
Overall sd	0.019	0.026	0.006
Overall SEM	0.009	0.013	0.003
% CV	17.7	60.4	11.7

a. The overall average value for the laboratory was calculated using the mean values determined for the four reference chemicals.

A reason for the high % CV value reported for Laboratory B was not found. However, the variability may have been related to a change in the microsomes during the latter period of the study. The intrareplicate % CV values were less than 10 percent for all three replicates conducted for aminoglutethimide, chrysin, and ketoconazole, whereas for econazole, the fourth chemical tested, the intra-replicate % CV values ranged from 8 to 113 percent. In addition, the overall % CV values, in the order that the reference chemicals were tested, was 7, 28, 52, and 81 percent. These results suggested that the microsomes or some component of the assay was degrading, which introduced an increasing degree of variability into the outcome of the assay. It was for this reason that a separate source of microsomes was sent to the laboratory to use in the assay but there was no improvement relative to the results obtained earlier.

3.5 Background Activity

Background enzyme activity controls were conducted in duplicate repetitions at the beginning and end of each replicate of the assay (a total of four tubes/replicate). For the most part, the aromatase activity in these samples for all laboratories and reference chemicals was negligible, indicating that there was no background activity that interfered with the interpretation of the results.

3.6 Positive Control Activity

4-OH ASDN, at a final concentration of 5×10^{-8} M, was included as the positive control with each replicate of the assay because it is a known aromatase inhibitor and this concentration would result in an approximately 50 percent inhibition of the enzyme.

The laboratories overall average ± SEM enzyme activity values in the presence of 4-OH ASDN were 0.049 ± 0.002, 0.022 ± 0.006, and 0.027 ± 0.002 nmol/mg protein/min for RTI and Laboratories B and C, respectively (Table 10). Comparison of the lead laboratory to the individual laboratories resulted in a %RE of -54.9 percent for Laboratory B and -43.9 percent for Laboratory C. The overall task mean ± SEM full enzyme activity control value was 0.0328 ± 0.008 nmol/mg protein/min with a percent CV of 43.3 percent.

Table 10. Human Placental Enzyme Activity in the Presence of 4-OH ASDN (Positive Control)[a]

	Aromatase Activity (nmol/mg protein/min)		
Parameter	**RTI**	**Lab B**	**Lab C**
Overall Average	0.049	0.022	0.027
Overall sd	0.005	0.012	0.004
Overall SEM	0.002	0.006	0.002
% CV	9.4	52.5	15.0

a. The overall average value for the laboratory was calculated using the mean values determined for the four reference chemicals.

The laboratory overall group mean ± SEM inhibition values for 4-OH ASDN (as a percent of control) were 47.2 ± 2.3, 54.5 ± 7.1, and 55.9 ± 1.3 percent for RTI and Laboratories B and C, respectively (Table 11). Comparison of the lead laboratory to the individual laboratories resulted in a %RE of 15.3 percent for Laboratory B and 18.4 percent for Laboratory C. The overall task mean ± SEM percent of control value was 52.5 ± 2.7 percent with a percent CV of 8.9.

Table 11. Human Placental Enzyme Activity Inhibition by 4-OH ASDN (Positive Control)[a]

Parameter	Percent of Control		
	RTI	Lab B	Lab C
Overall Average	47.2	54.5	55.9
Overall sd	4.6	14.3	2.5
Overall SEM	2.3	7.1	1.3
% CV	9.8	26.2	4.6

a. The overall average value for the laboratory was calculated using the mean values determined for the four reference chemicals.

3.7 Negative Control Activity

Lindane, at a final concentration of 10^{-6} M, was included as the negative control with each replicate of the assay because it is known to not inhibit aromatase at this concentration.

The laboratories overall average ± SEM enzyme activity values in the presence of lindane were 0.099 ± 0.012, 0.047 ± 0.011, and 0.049 ± 0.003 nmol/mg protein/min for RTI and Laboratory B and C, respectively (Table 12). Comparison of the lead laboratory to the individual laboratories resulted in a %RE of -52.9 percent for Laboratory B and -51.0 percent for Laboratory C. The overall task mean ± SEM full enzyme activity control value was 0.0649 ± 0.017 nmol/mg protein/min with a percent CV of 45.9 percent.

Table 12. Human Placental Enzyme Activity in the Presence of Lindane (Negative Control)[a]

Parameter	Aromatase Activity (nmol/mg protein/min)		
	RTI	Lab B	Lab C
Overall Average	0.099	0.047	0.049
Overall sd	0.024	0.022	0.006
Overall SEM	0.012	0.011	0.003
% CV	24.4	46.2	13.1

a. The overall average value for the laboratory was calculated using the mean values determined for the four reference chemicals.

The laboratory overall group mean ± SEM percent of control values for lindane were 93.6 ± 4.1, 121.1 ± 24.4, and 99.2 ± 1.0 percent for RTI and Laboratories B and C, respectively (Table 13). Comparison of the lead laboratory to the individual laboratories resulted in a %RE of 29.4 percent for Laboratory B and 6.0 percent for Laboratory C. The overall task mean ± SEM percent of control value was 104.6 ± 8.4 percent with a percent CV of 13.9.

Table 13. Human Placental Enzyme Activity Percent of Control Values for Lindane (Negative Control)[a]

Parameter	Percent of Control		
	RTI	Lab B	Lab C
Overall Average	93.6	121.1	99.2
Overall sd	8.2	48.8	2.0
Overall SEM	4.1	24.4	1.0
% CV	8.8	40.3[b]	2.0

a. The overall average value for the laboratory was calculated using the mean values determined for the four reference chemicals.

b. High value reflects the variability in the full enzyme activity control values.

3.8 Aminoglutethimide Inhibition of Aromatase Activity

The effect of increasing the concentrations of aminoglutethimide on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual replicate percent of control results for each laboratory can be found in the appendices. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 14.

Table 14. Effect of Aminoglutethimide on Aromatase Activity (Percent of Control)

Laboratory	Log Aminoglutethimide Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
RTI	-3.00	0.35	0.01	0.01	2.51
	-4.00	4.38	0.14	0.08	3.14
	-5.00	30.92	0.93	0.54	3.00
	-5.30	47.73[b]	--	--	--
	-5.60	63.18[b]	--	--	--
	-6.00	78.09	2.53	1.46	3.24
	-7.00	95.17	0.83	0.48	0.87
	-8.00	97.93	1.38	0.80	1.41
	-9.00	NC[a]	NC	NC	NC
	-10.0	NC	NC	NC	NC
Lab B	-3.00	0.91	1.73	1.00	190.45
	-4.00	4.16	0.89	0.51	21.38
	-5.00	30.61	2.15	1.24	7.04
	-5.12	38.01[b]	--	--	--
	-5.60	63.70[b]	--	--	--
	-6.00	79.25	2.87	1.66	3.62
	-7.00	95.40	2.76	1.59	2.89
	-8.00	NC	NC	NC	NC
	-9.00	95.84	3.36	1.94	3.50
	-10.0	NC	NC	NC	NC

Laboratory	Log Aminoglutethimide Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
Lab C	-3.00	0.39	0.13	0.06	34.35
	-4.00	4.95	2.47	1.10	49.78
	-5.00	30.46	10.11	4.52	33.20
	-5.30	46.66	12.23	6.12	26.22
	-5.60	63.03	12.91	6.45	20.48
	-6.00	79.15	7.77	3.48	9.82
	-7.00	92.20	8.79	3.93	9.53
	-8.00	99.71	4.15	1.85	4.16
	-9.00	NC	NC	NC	NC
	-10.0	NC	NC	NC	NC
Overall Task	Log Aminoglutethimide Conc (M)	Overall Mean	Overall sd[b]	Overall SEM[b]	Overall %CV[b]
	-3.00	0.55	0.31	0.18	56.8
	-4.00	4.50	0.41	0.24	9.1
	-5.00	30.66	0.23	0.14	0.8
	-5.12	(38.01)[c]	--	--	--
	-5.30	47.20	--	--	--
	-5.60	63.30	0.35	0.20	0.6
	-6.00	78.83	0.64	0.37	0.8
	-7.00	94.26	1.78	1.03	1.9
	-8.00	98.82	--	--	--
	-9.00	(95.84)[c]	--	--	--
	-10.00	--	--	--	--

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.

The individual aminoglutethimide inhibition response curves by replicate for each laboratory are reported in the appendices. The overall inhibition response curves by laboratory are shown in Figure 1 and the overall task curve is shown in Figure 2. The curves in these figures are not fitted by the model but are representative of the curve as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of aminoglutethimide decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs aminoglutethimide curve was sigmoidal. At an aminoglutethimide concentration of 10^{-4} M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 4 to 5 percent. In contrast, at an aminoglutethimide concentration of 10^{-8} M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 98 to 100 percent. Overall task mean ± SEM percent of control values at 10^{-4} and 10^{-8} M were 4.50 ± 0.24 and 98.82 (n=2) percent, respectively.

The CV percent of control for each replicate and laboratory are reported in the appendix. The overall percent CV values by laboratory (Table 14) were equal to or less than 3, 21, and 34 percent for RTI and Laboratories B and C, respectively, except at an aminoglutethimide concentration of 10^{-3} M for Laboratory B and 10^{-4} M for Laboratory C, which had overall percent CV values of 50 and 190 percent. The overall task percent CV values ranged from 1 to 2 percent, except at the two lowest concentrations where the percent CV values ranged from 9 to 57 percent.

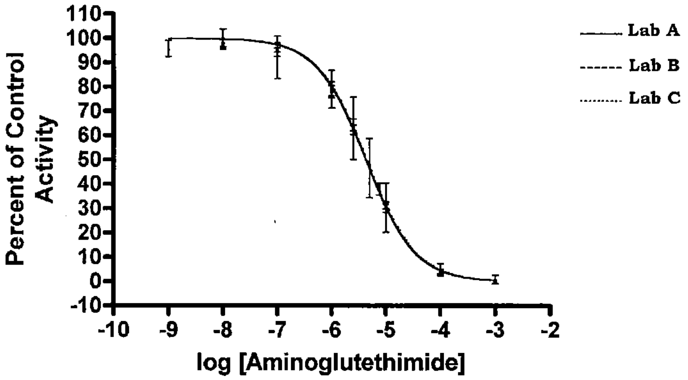

Figure 1. Overall Aminoglutethimide Inhibition Response Curve by Laboratory

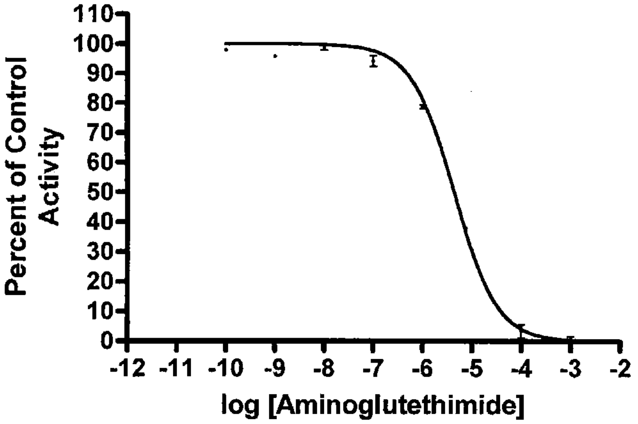

Figure 2. Overall Task Aminoglutethimide Inhibition Response Curve

3.9 Chrysin Inhibition of Aromatase Activity

The effect of increasing the concentrations of chrysin on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual replicate percent of control results for each laboratory can be found in the appendices. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 15.

Table 15. Effect of Chrysin on Aromatase Activity (Percent of Control)

Laboratory	Log Chrysin Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
RTI	-4.00	23.65	2.25	1.30	9.53
	-5.00	28.20	2.50	1.44	8.86
	-5.30	45.20	1.74	1.23	3.85
	-5.60	62.29[b]	--	--	--
	-6.00	78.20[b]	--	--	--
	-6.30	85.90	2.48	1.43	2.88
	-7.00	95.35	2.03	1.17	2.13
	-8.00	96.66	0.58	0.34	0.60
	-9.00	NC[a]	NC	NC	NC
	-10.0	NC	NC	NC	NC
Laboratory B	-4.00	8.65	6.11	3.53	70.59
	-5.00	25.44	3.03	1.75	11.92
	-5.30	38.30[b]	--	--	--
	-5.60	56.08[b]	--	--	--
	-6.00	77.95	6.04	3.49	7.75
	-6.30	99.22[b]	--	--	--
	-7.00	96.67	8.99	5.19	9.30
	-7.30	NC	NC	NC	NC
	-8.00	99.95	10.98	6.34	10.98
	-9.00	NC	NC	NC	NC
	-10.0	NC	NC	NC	NC
Laboratory C	-4.00	29.29	6.28	3.14	21.46
	-5.00	29.50	11.73	5.86	39.76
	-5.60	64.90	8.13	4.69	12.53
	-6.00	76.06	11.62	5.81	15.28
	-6.30	88.23	5.05	2.92	5.73
	-6.60	89.67	3.85	1.92	4.29
	-7.00	93.42	3.63	1.82	3.89
	-8.00	99.04	3.09	1.55	3.12
	-9.00	NC	NC	NC	NC
	-10.0	NC	NC	NC	NC
Overall Task	**Log Chrysin Conc (M)**	**Overall Mean**	**Overall sd[b]**	**Overall SEM[b]**	**Overall %CV[b]**
	-4.00	20.53	10.67	6.16	52.0
	-5.00	27.71	2.07	1.20	7.5
	-5.30	41.75	--	--	--
	-5.60	61.09	4.53	2.62	7.4
	-6.00	77.40	1.17	0.68	1.5
	-6.30	91.12	7.11	4.11	7.8
	-6.60	(89.67)[c]	--	--	--
	-7.00	95.15	1.63	0.94	1.7
	-7.30	NC	--	--	--
	-8.00	98.55	1.70	0.98	1.7
	-9.00	NC	--	--	--
	-10.00	NC	--	--	--

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.

The individual chrysin inhibition response curves by replicate for each laboratory are reported in the appendices. The overall inhibition response curves by laboratory are shown in Figure 3 and the overall task curve is shown in Figure 4. The curves in these figures are not fitted by the model but are representative of the curve as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of chrysin decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs chrysin curve was sigmoidal. At a chrysin concentration of 10^{-4} M, aromatase inhibition was <u>not</u> complete; the laboratory percent of control values ranged from 9 to 29 percent. In contrast, at a chrysin concentration of 10^{-7} M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 97 percent. Overall task mean ± SEM percent of control values at 10^{-4} and 10^{-7} M were 20.53 ± 6.16 and 95.15 ± 0.94 percent, respectively.

The CV percent of control for each replicate and laboratory are reported in the appendix. The overall percent CV values by laboratory (Table 15) were equal to or less than 10, 21, and 21 percent for RTI and Laboratories B and C, respectively, except at a chrysin concentration of 10^{-4} M for Laboratory B and 10^{-5} M for Laboratory C, which had overall percent CV values of 40 and 71 percent. The overall task percent CV values ranged from 2 to 8 percent, except at the lowest concentration where the percent CV value was 52 percent.

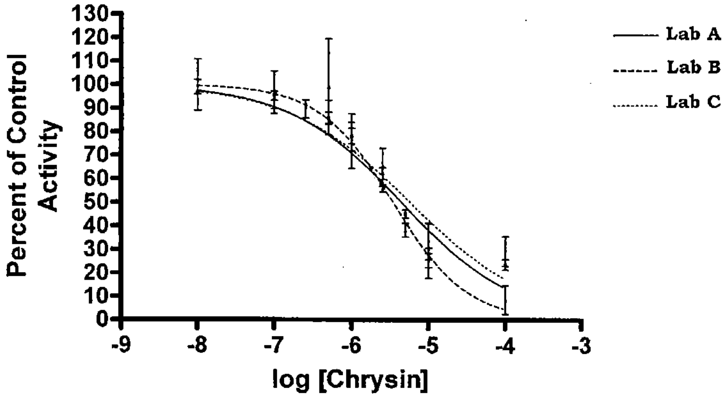

Figure 3. Overall Chrysin Inhibition Response Curve by Laboratory

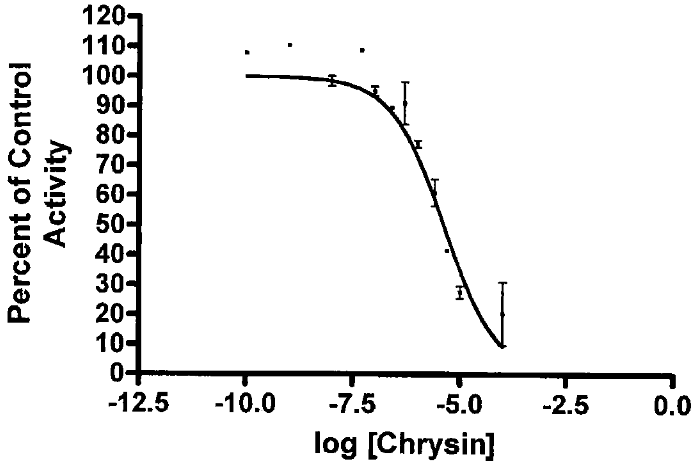

Figure 4. Overall Task Chrysin Inhibition Response Curve

3.10 Econazole Inhibition of Aromatase Activity

The effect of increasing the concentrations of econazole on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual replicate percent of control results for each laboratory can be found in the appendices. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 16.

Table 16. Effect of Econazole on Aromatase Activity (Percent of Control)

Laboratory	Log Econazole Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
RTI	-3.00	NC[a]	NC	NC	NC
	-4.00	NC	NC	NC	NC
	-5.00	NC	NC	NC	NC
	-6.00	NC	NC	NC	NC
	-7.00	1.97	0.29	0.17	14.69
	-8.00	18.24	4.45	2.57	24.40
	-8.30	28.54	2.09	1.20	7.31
	-8.60	46.78	5.82	3.36	12.45
	-9.00	71.59	6.14	3.54	8.58
	-9.30	89.00	13.98	8.07	15.71
	-9.60	91.01	4.60	2.66	5.05
	-10.0	101.63	14.08	8.13	13.85

Laboratory	Log Econazole Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
Laboratory B[d]	-3.00	0.58	--	--	--
	-4.00	0.54	--	--	--
	-5.00	-0.03	--	--	--
	-6.00	0.17	--	--	--
	-7.00	2.34	--	--	--
	-8.00	16.56	--	--	--
	-9.00	66.46	--	--	--
	-10.0	92.99	--	--	--
Laboratory C	-3.00	NC	NC	NC	NC
	-4.00	NC	NC	NC	NC
	-5.00	NC	NC	NC	NC
	-6.00	0.18	0.12	0.07	69.86
	-7.00	1.85	0.18	0.10	9.69
	-7.30	NC	NC	NC	NC
	-7.60	NC	NC	NC	NC
	-8.00	16.04	1.77	1.02	11.03
	-8.30	NC	NC	NC	NC
	-8.60	44.49	NC	NC	NC
	-9.00	69.76	1.74	1.01	2.50
	-9.60	NC	NC	NC	NC
	-10.0	93.73	NC	NC	NC
Overall Task	Log Econazole Conc (M)	Overall Mean	Overall sd[b]	Overall SEM[b]	Overall %CV[b]
	-3.00	(0.58)[c]	--	--	--
	-4.00	(0.54)[c]	--	--	--
	-5.00	(-0.03)[c]	--	--	--
	-6.00	0.17	--	--	--
	-7.00	2.05	0.26	0.15	12.4
	-8.00	16.95	1.15	0.66	6.8
	-8.30	(28.54)[c]	--	--	--
	-8.60	45.64	--	--	--
	-9.00	69.27	2.60	1.50	3.8
	-9.30	(89.00)[c]	--	--	--
	-9.60	(91.01)[c]	--	--	--
	-10.0	96.12	4.79	2.77	5.0

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.
d. Variability in full enzyme activity control values precluded averaging replicates. Tabled values are the results of the first replicate only.

The individual econazole inhibition response curves by replicate for each laboratory are reported in the appendices. For Laboratory B, only replicate 1 was used to characterize the percent of control curve due to the large variability in the full enzyme control activity. The overall inhibition response curves by laboratory are shown in Figure 5 and the overall task curve is shown in Figure 6. The curves in these figures are not fitted by the model but are representative of the curve as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of econazole decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs econazole curve was sigmoidal. At an econazole concentration of 10^{-7} M, aromatase inhibition was almost complete; the laboratory percent of control was approximately 2 percent. In contrast, at an econazole concentration of 10^{-10} M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 102 percent. Overall task mean ± SEM percent of control values at 10^{-7} and 10^{-10} M were 2.05 ± 0.15 and 96.12 ± 2.8 percent, respectively.

The CV percent of control for each replicate and laboratory are reported in the appendix. The overall percent CV values for RTI and Laboratory C (Table 16) were equal to or less than 24 and 11 percent (no values could be estimated for Laboratory C, except at an econazole concentration of 10^{-6} M for Laboratory C, which had overall percent CV value of 70 percent. The overall task percent CV values ranged from 4 to 12 percent.

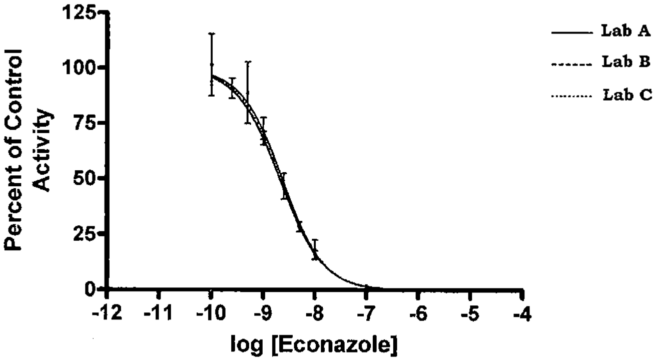

Figure 5. Overall Econazole Inhibition Response Curve by Laboratory

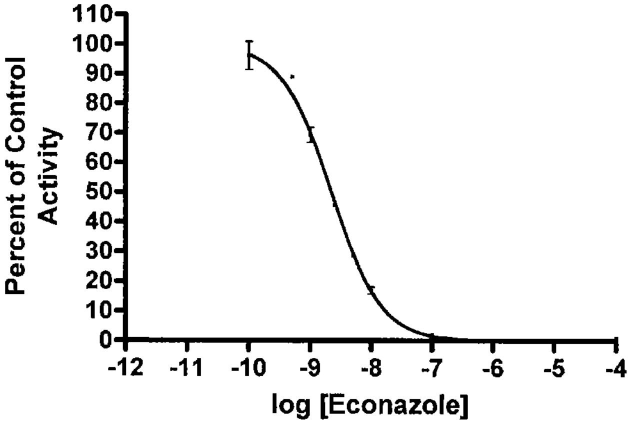

Figure 6. Overall Task Econazole Inhibition Response Curve

3.11 Ketoconazole Inhibition of Aromatase Activity

The effect of increasing the concentrations of ketoconazole on aromatase activity was determined and the results were expressed as a percent of the control aromatase activity. The individual replicate percent of control results for each laboratory can be found in the appendices. The overall percent of control results by laboratory and the overall percent of control results for the task are summarized in Table 17.

Table 17. Effect of Ketoconazole on Aromatase Activity (Percent of Control)

Laboratory	Log Ketoconazole Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
RTI	-4.00	6.05	0.08	0.05	1.40
	-4.30	12.90[b]	--	--	--
	-4.60	22.79[b]	--	--	--
	-5.00	42.15	0.64	0.37	1.53
	-5.30	57.92[b]	--	--	--
	-6.00	86.57	1.37	0.79	1.59
	-7.00	98.32	1.84	1.06	1.88
	-8.00	98.80	1.62	0.94	1.64
	-9.00	NC[a]	NC	NC	NC
	-10.0	NC	NC	NC	NC
	-11.0	NC	NC	NC	NC
Laboratory B	-4.00	8.39	0.51	0.29	6.06
	-4.40	18.20[b]	--	--	--
	-4.80	35.79[b]	--	--	--
	-5.00	NC	NC	NC	NC
	-5.19	56.63[b]	--	--	--
	-5.60	75.91[b]	--	--	--
	-6.00	86.76	2.73	1.57	3.14
	-7.00	NC	NC	NC	NC
	-7.30	99.74	4.97	2.87	4.99
	-8.00	NC	NC	NC	NC
	-9.00	97.22	2.35	1.36	2.42
	-10.0	NC	NC	NC	NC
Laboratory C	-4.00	6.04	0.35	0.20	5.79
	-4.60	21.93	NC	NC	NC
	-5.00	42.20	2.19	1.26	5.18
	-5.30	51.43	NC	NC	NC
	-5.60	70.07	NC	NC	NC
	-6.00	79.95	6.15	3.55	7.69
	-6.60	92.28	6.57	3.79	7.12
	-7.00	93.22	4.34	2.51	4.66
	-8.00	NC	NC	NC	NC
	-9.00	NC	NC	NC	NC
	-10.0	NC	NC	NC	NC

Laboratory	Log Ketoconazole Conc (M)	Overall Percent of Control by Laboratory			
		Mean	sd	SEM	%CV
Overall Task	-4.00	6.83	1.35	0.78	19.8
	-4.30	(12.90)[c]	--	--	--
	-4.40	(18.20)[c]	--	--	--
	-4.60	22.36	--	--	--
	-4.80	(35.79)[c]	--	--	--
	-5.00	42.18	--	--	--
	-5.20	(56.63)[c]	--	--	--
	-5.30	54.68	--	--	--
	-5.60	72.99	--	--	--
	-6.00	84.43	3.88	2.24	4.6
	-6.60	(92.28)[c]	--	--	--
	-7.00	95.77	--	--	--
	-7.30	(99.74)[c]	--	--	--
	-8.00	(98.80)[c]	--	--	--
	-9.00	(97.22)[c]	--	--	--
	-10.00	--	--	--	--

a. NC – Not Calculated. Concentration only tested in one replicate so the mean, sd, SEM, and %CV were not calculated.
b. In those instances when n = 2 only the mean was reported.
c. Value is not an average of multiple laboratories as only one laboratory tested this concentration.

The individual ketoconazole inhibition response curves by replicate for each laboratory are reported in the appendices. The overall inhibition response curves by laboratory are shown in Figure 7 and the overall task curve is shown in Figure 8. The curves in these figures are not fitted by the model but are representative of the curve as denoted by the symbols (mean data). For all three laboratories, increasing concentrations of ketoconazole decreased the activity of the placental microsomal aromatase activity and the decrease was concentration-dependent. The shape of the enzyme activity vs ketoconazole curve was sigmoidal. At a ketoconazole concentration of 10^{-4} M, aromatase inhibition was almost complete; the laboratory percent of control values ranged from 6 to 8 percent. In contrast, at a ketoconazole concentration of 10^{-7} M, there was little to no aromatase inhibition; the laboratory percent of control values ranged from 93 to 98 percent. Overall task mean ± SEM percent of control values at 10^{-4} and 10^{-7} M were 6.83 ± 0.78 and 95.77 (n=2) percent, respectively.

The CV percent of control for each replicate and laboratory are reported in the appendix. The overall percent CV values by laboratory (Table 17) were equal to or less than 8, 20, and 8 percent for RTI and Laboratories B and C, respectively, except for Laboratory B at a ketoconazole concentration of 4 x 10^{-5} M, which had an overall percent CV value of 37 percent. The overall task percent CV values ranged from less than 5 to 20 percent.

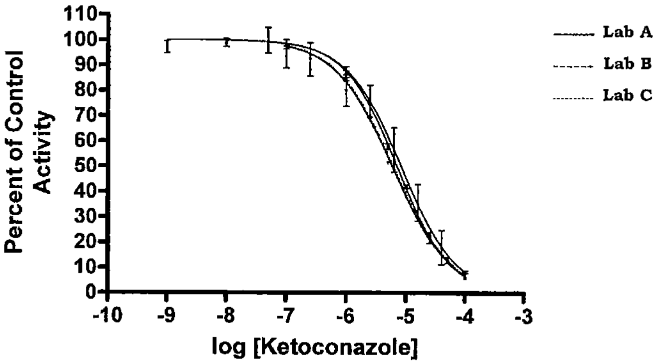

Figure 7. Overall Ketoconazole Inhibition Response Curve by Laboratory

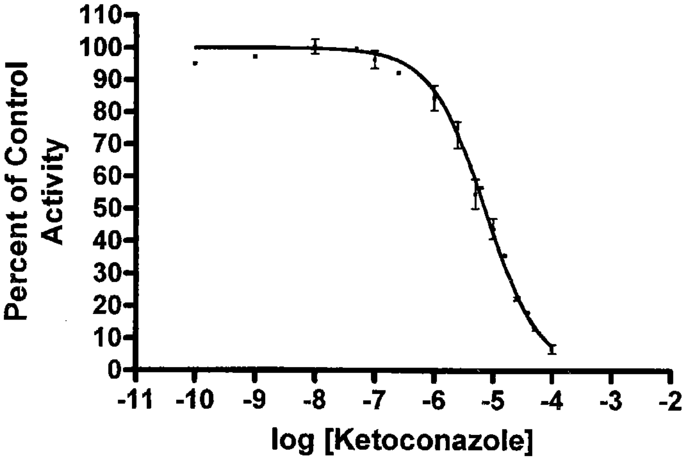

Figure 8. Overall Task Ketoconazole Inhibition Response Curve

3.12 IC_{50} and Slope Determination and Curve Classification

3.12.1 Laboratory IC_{50} Values

Based on the curve-fit of the percent of control aromatase activity values across the various number of concentrations for each reference chemical, the calculated IC_{50} values by replicate and laboratory are summarized in Table 18.

For aminoglutethimide, the average ± SEM IC_{50} values for RTI and Laboratories B and C were 4.26 ± 0.11, 4.28 ± 0.33, and 4.54 ± 0.94 μM; the percent CV values were 4.4, 13.2, and 46.4 percent, respectively. The overall task group mean ± SEM IC_{50} value was 4.36 ± 0.09 μM and the percent CV was 3.6 percent.

For chrysin, the average ± SEM IC_{50} values for RTI and Laboratories B and C were 4.45 ± 0.54, 3.54 ± 0.33, and 4.17 ± 1.55 μM; the percent CV values were 20.9, 18.7, and 64.3

percent, respectively. Laboratory C reported an IC_{50} value for a fourth replicate but it was considered an outlier and, for this reason, it was not included in calculating the laboratory mean value and the overall task value. The overall task group mean ± SEM IC_{50} value was 4.05 ± 0.27 µM and the percent CV was 11.5 percent.

For econazole, the average ± SEM IC_{50} values for RTI and Laboratory C were 2.19 ± 0.22 and 2.12 ± 0.13 nM; the percent CV values were 17.6 and 10.6 percent, respectively. An average IC_{50} value was not calculated for Laboratory B (n=1) because the variability in the full enzyme activity control values precluded estimating a value from the other replicates. The overall task group mean ± SEM IC_{50} value was 2.10 ± 0.06 nM and the percent CV was 4.6 percent.

For ketoconazole, the average ± SEM IC_{50} values for RTI and Laboratories B and C were 7.16 ± 0.17, 8.67 ± 1.08, and 6.53 ± 0.66 µM; the percent CV values were 4.1, 25.0, and 17.5 percent, respectively. The overall task group mean ± SEM IC_{50} value was 7.46 ± 0.64 µM and the percent CV was 14.8 percent.

Table 18. Reference Chemical IC_{50} Values

	IC_{50} Values		
Replicate	**RTI**	**Laboratory B**	**Laboratory C**
	Aminoglutethimide (µM)		
1	4.09	3.98	3.39
2	4.23	3.92	2.14
3	4.46	4.93	3.66
4	--	--	6.91
5	--	--	6.60
Average	4.26	4.28	4.54
sd	0.19	0.57	2.10
SEM	0.11	0.33	0.94
% CV	4.4	13.2	46.4
	Chrysin (µM)		
1	3.75	4.28	1.56
2	5.50	3.32	4.04
3	4.09	3.01	6.92
Average	4.45	3.54	4.17
sd	0.93	0.66	2.68
SEM	0.54	0.33	1.55
% CV	20.9	18.7	64.3
	Econazole (nM)		
1	2.11	2.00	2.36
2	1.85	--[a]	2.10
3	2.61	--	1.91
Average	2.19	(2.00)[b]	2.12
sd	0.39	--	0.23
SEM	0.22	--	0.13
% CV	17.6	--	10.6

Ketoconazole (µM)			
1	7.44	8.81	7.83
2	6.85	10.77	6.08
3	7.20	6.44	5.68
Average	7.16	8.67	6.53
sd	0.30	2.17	1.14
SEM	0.17	1.08	0.66
% CV	4.1	25.0	17.5

a. Variability of the full enzyme activity control value precluded calculating IC_{50} values from the other replicates.
b. Not an average value (n=1).

3.12.2 Laboratory Slope Values

The slope values by replicate and laboratory are summarized in Table 19 for each reference chemical.

For aminoglutethimide, the average ± SEM slope values for RTI and Laboratories B and C were -0.9728 ± 0.0095, -0.9765 ± 0.0284, and -0.9814 ± 0.0119; the percent CV values were 1.7, 5.0, and 2.7 percent, respectively. The overall task group mean ± SEM slope value was -0.9769 ± 0.0025 and the percent CV was 0.4 percent.

For chrysin, the average ± SEM slope values for RTI and Laboratories B and C were -0.5881 ± 0.0059, -0.9393 ± 0.0132, and -0.6188 ± 0.0302; the percent CV values were 1.7, 2.4, and 9.8 percent, respectively. Battelle reported a slope value for a fourth replicate but it was considered an outlier and, for this reason, it was not included in calculating the laboratory mean value and the overall task value. The overall task group mean ± SEM slope value was -0.7154 ± 0.1123 and the percent CV was 27.2 percent.

For econazole, the average ± SEM slope values for RTI and Laboratory C were -1.047 ± 0.017 and -1.054 ± 0.015; the percent CV values were 2.9 and 2.4 percent, respectively. An average IC_{50} value was not calculated for Laboratory B (n=1) because the variability in the full enzyme activity control values precluded estimating a value from the other replicates. The overall task group mean ± SEM slope value was -1.025 ± 0.025 and the percent CV was 4.2 percent.

For ketoconazole, the average ± SEM slope values for RTI and Laboratories B and C were -1.009 ± 0.019, -1.002 ± 0.004, and -0.9439 ± 0.0518; the percent CV values were 3.3, 0.7, and 9.5 percent, respectively. The overall task group mean ± SEM IC_{50} value was -0.9847 ± 0.0205 and the percent CV was 3.6 percent.

Table 19. Reference Chemical Slope Values

Replicate	Slope Values		
	RTI	Lab B	Lab C
Aminoglutethimide			
1	-0.9702	-1.011	-1.018
2	-0.9578	-0.9202	-0.9532
3	-0.9904	-0.9984	-0.9816
4	--	--	-0.9950
5	--	--	-0.9592
Average	-0.9728	-0.9765	-0.9814
sd	0.0165	0.0492	0.0265
SEM	0.0095	0.0284	0.0119
% CV	1.7	5.0	2.7
Chrysin			
1	-0.5892	-0.9651	-0.6870
2	-0.5774	-0.9219	-0.5970
3	-0.5976	-0.9308	-0.5723
Average	-0.5881	-0.9393	-0.6188
sd	0.0101	0.0228	0.0604
SEM	0.0059	0.0132	0.0302
% CV	1.7	2.4	9.8
Econazole			
1	-1.037	-0.9756	-1.043
2	-1.023	--[a]	-1.083
3	-1.081	--	-1.035
Average	-1.047	(-0.9756)[b]	-1.054
sd	0.030	--	0.026
SEM	0.017	--	0.015
% CV	2.9	--	2.4
Ketoconazole			
1	-1.047	-1.001	-1.043
2	-0.9929	-0.9947	-0.9201
3	-0.9865	-1.009	-0.8685
Average	-1.009	-1.002	-0.9439
sd	0.033	0.007	0.0896
SEM	0.019	0.004	0.0518
% CV	3.3	0.7	9.5

a. Variability of the full enzyme activity control value precluded calculating slope values from the other replicates.
b. Not an average value (n=1).

3.12.3 Laboratory Curve Classifications

All three laboratories characterized the concentration response curve as “Complete (C)” for aminoglutethimide and ketoconazole since the percent of control data essentially spanned the 0-100 percent range. RTI and Laboratory B also characterized econazole as “C” for the same reason, although Laboratory C had one replicate that was not fully characterized due to a technical error. RTI and Laboratory C characterized chrysin as “Incomplete-Interpolated (II)” since the percent of control data ranged from approximately 15 to 22 percent at the low concentrations tested and 100 percent at the high concentrations tested. Laboratory B characterized the concentration response curves for econazole and chrysin as “C”.

3.13 Intralaboratory Statistical Analysis

The intralaboratory analyses from each laboratory for background activity, full enzyme activity, and negative and positive controls, IC_{50}s, and slopes are provided in the appendices of the individual laboratory reports, which can be found in their entirety in the appendices of this overall report. Due to the integrated manner in which the intralaboratory statistical analysis reports were presented, as well as the interrelationships among the endpoints, it was decided to present the salient information from the intralaboratory statistical analysis reports in a single section rather than attempt to extract information about individual endpoints and incorporate them into their previously presented respective sections.

3.13.1 RTI Intralaboratory Statistical Analysis

The full statistical analysis report and corresponding tables and graphs can be found in the appendices of the RTI report.

The percent of control values for the background control were fairly consistent and close to 0% of control across the replicates and chemicals, except for ketoconazole replicate 2 and econazole replicates 1 and 2. The percent of control values for the full enzyme activity controls were fairly consistent and close to 100% of control across the replicates and chemicals, except for econazole replicate 1. The percent of control values of the negative control were fairly consistent and close to 100% of control, except for econazole replicate 1. The percent of control values of the positive control were fairly consistent and close to 45% of control, except for ketoconazole replicate 2 and econazole replicate 1.

Differences of the means of the beginning repetitions and the means of the end repetitions within each replicate and chemical were determined. The differences appearing above the zero reference line indicate that the beginning percent of control values were consistently larger than the end percent of control values for the full enzyme activity control. The same pattern of consistently larger beginning values compared to the end values of percent of control were also observed for the differences of portion means for negative and positive controls.

A mixed effects model was fit to the percent of control values for each control. The fixed effects were assigned as the chemical type, portion, and portion by chemical interaction. The random effects were assigned as replicates within reference chemical and portion by replicate interaction within chemical. For background activity control, none of the fixed effects had a significant effect on the variation in the percent of control values. For the full enzyme activity control and negative and positive controls, portion had a significant effect on the variation in the percent of control values. For background activity and full enzyme activity controls, both the replicate within chemical and portion by replicate within chemical covariance parameters were estimated to be zero, leaving the remainder of the variation in the residual term. There was a significant amount of variation in percent of control attributed to variation between the replicates within the chemicals for negative control and an indication of some variation in percent of control, but not quite statistically significant, attributed to variation between the replicates within chemicals for positive control.

The standard errors of the parameter estimates of $\log_{10}IC_{50}$ for aminoglutethimide and ketoconazole were fairly consistent across the replicates. The standard errors of estimates of $\log_{10}IC_{50}$ for chrysin showed a decreasing value across the replicates.

Aminoglutethimide, chrysin and ketoconazole had similar values for the overall mean of $\log_{10}IC_{50}$ but the standard error for chrysin was about 4 times larger than the standard errors for aminoglutethimide and ketoconazole. The slope estimates for aminoglutethimide, ketoconazole and econazole were similar in value but the standard errors of aminoglutethimide and econazole were about half the size of the standard error for ketoconazole. The slope estimate for chrysin was about half the value of the other slope estimates and had a standard error more than 3 times the size of the standard errors for the slope estimates of the other chemicals.

3.13.2 Laboratory B Intralaboratory Statistical Analysis

The intralaboratory statistical analysis did not include an interpretation of the results. Tables and graphs of the statistical analysis results were included in the main body of the laboratory report, which supplemented the statistical analysis report found in the laboratory report appendices. There was no discussion or summarization of the results.

3.13.3 Laboratory C Intralaboratory Statistical Analysis

For all four control types (full enzyme activity control, background activity control, and positive and negative controls) the differences between the beginning and the end portions, when averaged across replicates, were significant. The end portion was significantly lower than the beginning portion. This implied a reduction in aromatase activity between the beginning and the end of a replicate.

For all the control types chemical by portion interaction was not significant and was not included in the tables.

For aminoglutethimide, the variation in Replicate 1 for the full enzyme activity controls and for the negative controls was substantially larger and out of line with than that for any replicate for all the chemicals.

For positive controls, the majority of variation was from replicate-to-replicate.

The estimated $\log_{10}IC_{50}$ ranged from -5.671 to -5.159 for the five replicates of aminoglutethimide, ranged from -5.808 to -4.752 for the four replicates of chrysin, ranged from -8.719 to -8.628 for the three replicates of econazole, and ranged from -5.350 to -5.105 for the three replicates of ketoconazole. Econazole had orders of magnitude lower IC_{50} than the other three reference chemicals.

For aminoglutethimide, econazole, and ketoconazole, the estimated slopes were close to -1. The estimated slopes ranged from -1.018 to -0.916 for aminoglutethimide, ranged from -1.083 to -1.035 for econazole, and ranged from -1.045 to -0.803 for ketoconazole. Chrysin had a flatter slope, averaging -0.56 across the four replicates.

For chrysin, the bottom threshold was between 20% and 40%, depending on the replicate. The two-parameter concentration response model used in this report assumed a bottom threshold of 0% and so it did not fit the chrysin response data. This implied that a four parameter, variable top and bottom threshold models will be needed in future response curve fits for this reference chemical and, possible, the assay.

The majority of variation for $\log_{10}IC_{50}$ was from replicate-to-replicate variation. It ranged from 0.0127 to 0.1832 for the four reference chemicals. The within-replicate variations were of lower order of magnitude for all four reference chemicals. The replicate-to-replicate and within-replicate variations for slope were all small and or the most part of the same order of magnitude for all four reference chemicals.

3.14 Interlaboratory Statistical Analysis

The interlaboratory analyses for background activity, full enzyme activity, negative, and positive activity controls were carried out combined across chemicals, with or without the results for the econazole controls in Laboratory B. The interlaboratory analyses for $\log_{10}IC_{50}$ and the slope parameters were carried out separately for each reference chemical. The complete interlaboratory report can be found in the appendix of this overall report.

3.14.1 Control Activity

Table 20 displays the estimated parameter values and the associated within laboratory 95% confidence intervals about these values[3]. It also displays the overall mean values across laboratories and their associated 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. These mean values and confidence intervals are graphically displayed in Figures 17 to 24. Each figure includes reference lines corresponding to the overall average. Figures 17 to 20 display results corresponding to when the Laboratory B results excluded econazole. Figure 21 to 24 display results corresponding to when the Laboratory B results included econazole.

Table 21 displays the within laboratory variances and their associated degrees of freedom for each laboratory. These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 21 also displays the laboratory to laboratory random variation and the p-values, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95% confidence intervals.

When the Laboratory B results excluded econazole, the end portion was statistically significantly lower than the beginning portion for the full enzyme activity, negative, and positive controls, for each individual laboratory and for the average across laboratories. When the Laboratory B results included econazole, statistical significances between the beginning and the end portions no longer existed for the full enzyme activity controls for the average across

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[3] The confidence intervals are based on the least squares means, standard errors, and degrees of freedom shown in Table A-4 in Appendix D. The degrees of freedom in Table A-4 are based on those in Tables A-1 to A-3, which in turn are based on those reported in the intralaboratory analyses.

laboratories. The estimated variance among the laboratories was more than 7.4 times higher than the average within-laboratory variance. Statistical significance between beginning and end no longer existed for Laboratory B for the negative and positive controls. For the full enzyme activity controls for Laboratory B the end portion was significantly greater than the beginning.

3.14.2 Concentration Response Relations for $Log_{10}IC_{50}$ and Slope

Table 22 displays the estimated parameter values and the associated within laboratory 95% confidence intervals about these values.[4]

Table 22 also displays the overall mean values across laboratories and their associated 95% confidence intervals, incorporating among laboratory variation based on the random effects analysis of variance. These means and confidence intervals are graphically displayed in Figures 9 through 16. Each figure includes reference lines corresponding to the overall average. The estimated among laboratories CVs and their associated 95% confidence intervals for the overall means for the IC_{50} and slope parameters are also presented in Table 22.

Table 23 displays the within laboratory variances and their associated degrees of freedom for each laboratory.[5] These are the squares of the within laboratory standard errors associated with the estimated parameter values. Table 23 also displays the laboratory to laboratory random variation and the p-values, and the squares of the standard errors of the overall mean values, as well as their associated degrees of freedom. The ratios of the among laboratory variances to the unweighted average within laboratory variances are also displayed, with their associated 95% confidence intervals.

The estimates for $\log_{10}IC_{50}$ were similar among the three laboratories for all four chemicals (Table 22). The among laboratory variances were zero or near zero. The p-values were 1.00 for each of the four reference chemicals (Table 23). The CVs for the IC_{50} ranged from 2.6% to 8.6%.

For chrysin the estimated slope for Laboratory B was more than 59% smaller than those for RTI and Laboratory C (-0.94 versus -0.59 or -0.56). For chrysin, the among laboratory variance (p-value=0.12) was about 29 times the average within laboratory variance. The coefficient of variation among laboratories was about 25%. For the other chemicals, the slope estimates were similar among the three laboratories (Table 22). The among laboratory variances were zero or near zero (Table 23). The p-values for ketoconazole and econazole were 1.00. The p-value for aminoglutethimide was 0.37. The CV results ranged from 1.4% to 2.3%.

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[4] The confidence intervals in Table 22 were calculated for the interlaboratory analysis based on the least squares means, standard errors, and degrees of freedom reported in the intralaboratory analyses within each laboratory. The confidence intervals in Table 22 thus may differ in the low significant digits from those displayed in the intralaboratory analysis reports due to round off error in intermediate calculations.

[5] Degrees of freedom (Table 23) for Laboratory C were based on those specified in the Laboratory C inter-laboratory analysis report. Degrees of freedom for Laboratory C were based on the number of replicates (3) minus 1, except for econazole for which there was just one replicate. One degree of freedom was assigned for this situation. The degrees of freedom for Laboratory A were based on an analysis of variance model for all four chemicals combined. There were 12 observations (4 chemicals × 3 replicates per chemical) and four effects estimated, leaving 8 degrees of freedom for residual.

Table 20. Parameter Estimates and 95% Confidence Intervals for Differences Between Beginning and End for the Percent of Control Responses for the Placental Aromatase Assay.

Parameter (End – Beginning)	Estimate and 95% Confidence Interval			
	Lab A[1,2]	Lab B[1,2]	Lab C[1,2]	Average[3]
Three Chemicals in Lab B[2]				
Background Activity Control	0.023 (-0.157, 0.204)	-0.454 (-1.121, 0.213)	-0.165 (-0.247, -0.083)	-0.113 (-0.252, 0.025)
Full Enzyme Activity Control	-9.253 (-17.787, -0.718)	-4.382 (-8.751, -0.013)	-12.955 (-17.211, -8.699)	-8.683 (-15.116, -2.250)
Negative Control	-5.983 (-7.534, -4.431)	-12.695 (-17.568, -7.822)	-6.391 (-11.130, -1.652)	-8.033 (-13.405, -2.660)
Positive Control	-4.115 (-7.345, -0.885)	-8.665 (-17.144, -0.186)	-2.299 (-4.507, -0.091)	-3.200 (-4.978, -1.423)
Four Chemicals in Lab B[2]				
Background Activity Control	0.023 (-0.157, 0.204)	-0.238 (-0.830, 0.354)	-0.165 (-0.247, -0.083)	-0.105 (-0.232, 0.023)
Full Enzyme Activity Control	-9.253 (-17.787, -0.718)	24.148 (5.968, 42.328)	-12.955 (-17.211, -8.699)	-1.145 (-32.436, 30.146)
Negative Control	-5.983 (-7.534, -4.431)	-12.394 (-28.049, 3.261)	-6.391 (-11.130, -1.652)	-6.070 (-7.465, -4.675)
Positive Control	-4.115 (-7.345, -0.885)	-4.765 (-13.522, 3.992)	-2.299 (-4.507, -0.091)	-2.979 (-4.678, -1.279)

1. The estimates and 95% confidence intervals are based on the intralaboratory analyses for the three participating laboratories.
2. The results from the three laboratories are listed in Table A-4 (Appendix D). The results from laboratories A and C are based on data from all four chemicals (aminoglutethimide, chrysin, econazole, and ketoconazole), while results for laboratory B are based on data with and without econazole (i.e., four chemicals and three chemicals respectively).
3. The overall effects and confidence intervals in this table were estimated using a one-way random effects analysis of variance, with heterogeneous variances among the three laboratories. The variances for each laboratory were specified as the squares of the within laboratory standard errors.

Table 21. Variance Components and Ratios of Between to Within Laboratories Variances. Placental Aromatase Assay. Percent of Control.

Parameter (End – Beginning)	Within Laboratory Variance[1]				Among Laboratory Variance[4] and (p-value) (df=2)	Mean Variance[5,6]	Ratio and 95% CI[7]
	Lab A	Lab B	Lab C	Pooled Average[3]			
Three Chemicals in Lab B[2]							
Background Activity Control	0.006/df=8.0	0.074/df=6.0	0.002/df=51.0	0.027/df=7.3	0.004 (0.3302)	0.003/df=6.9	0.142 (0.022, 5.584)
Full Enzyme Activity Control	13.698/df=8.0	3.188/df=6.0	4.207/df=21.8	7.031/df=17.1	10.591 (0.1889)	5.515/df=4.1	1.506 (0.327, 59.412)
Negative Control	0.453/df=8.0	3.965/df=6.0	5.058/df=17.3	3.159/df=21.8	5.896 (0.2104)	2.855/df=3.0	1.867 (0.425, 73.638)
Positive Control	1.962/df=8.0	12.009/df=6.0	0.978/df=9.8	4.983/df=9.1	0.000 (1.0)	0.619/df=9.1	0.000 (0.000, 0.000)
Four Chemicals in Lab B[2]							
Background Activity Control	0.006/df=8.0	0.071/df=10.0	0.002/df=51.0	0.026/df=12.2	0.004 (0.3189)	0.003/df=10.3	0.140 (0.028, 5.508)
Full Enzyme Activity Control	13.698/df=8.0	66.577/df=10.0	4.207/df=21.8	28.160/df=15.3	209.17 (0.1603)	78.125/df=2.5	7.428 (1.566, 292.897)
Negative Control	0.453/df=8.0	49.366/df=10.0	5.058/df=17.3	18.292/df=12.3	0.000 (1.0)	0.412/df=12.3	0.000 (0.000, 0.000)
Positive Control	1.962/df=8.0	15.445/df=10.0	0.978/df=9.8	6.128/df=13.8	0.000 (1.0)	0.626/df=13.8	0.000 (0.000, 0.000)

1. The within laboratory variance for each laboratory is the square of the standard error associated with parameter estimate, which was reported in the intralaboratory analyses for each of the three participating laboratories (see Appendix D, Table A-4).
2. The results from laboratories A and C are based on data from all four chemicals (aminoglutethimide, chrysin, econazole, and ketoconazole), while results for laboratory B are based on data with and without econazole (i.e., four chemicals and three chemicals respectively).
3. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the three laboratories. Associated degrees of freedom were based on Satterthwaite's approximation.
4. Variance among laboratories is based on a one-way random effects analysis of variance model with heterogeneous within laboratory variances among the three laboratories, equal to the squares of the within laboratory standard errors.
5. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
6. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)*\sum(S_L^2+S_i^2))^2/(var(S_L^2)+(2/K^2)*\sum(S_i^4/df_i))$, where S_L^2 is the among laboratory variance, S_i^2 and df_i are the reported variance and degrees of freedom for laboratory i, var(S_L^2) is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).
7. Ratio of the among-laboratory variance and the pooled average within laboratory variance.

Table 22. Parameter Estimates and 95% Confidence Intervals for $Log_{10}IC_{50}$ and Slope Parameter of Concentration Response Curves for the Placental Aromatase Assay. By Chemical.

Chemical	Parameter	Estimate and 95% Confidence Interval				CV(%) and 95% CI[3]
		Lab A[1]	Lab B[1]	Lab C[1]	Average[2]	
Aminoglutethimide	$Log_{10}IC_{50}$	-5.370(-5.397,-5.342)	-5.368(-5.514,-5.222)	-5.387(-5.656,-5.118)	-5.370(-5.398,-5.341)	2.966(1.867,7.063)
	Slope	-0.975(-1.002,-0.948)	-0.997(-1.049,-0.945)	-0.963(-0.987,-0.939)	-0.978(-1.006,-0.951)	1.416(0.780,6.158)
Chrysin	$Log_{10}IC_{50}$	-5.354(-5.467,-5.242)	-5.448(-5.642,-5.254)	-5.276(-5.980,-4.572)	-5.402(-5.498,-5.306)	8.612(5.025,28.130)
	Slope	-0.589(-0.673,-0.504)	-0.936(-1.018,-0.854)	-0.558(-0.687,-0.429)	-0.698(-1.013,-0.384)	24.899(13.486,119.132)
Ketoconazole	$Log_{10}IC_{50}$	-5.143(-5.167,-5.120)	-5.069(-5.344,-4.794)	-5.218(-5.531,-4.905)	-5.143(-5.171,-5.116)	2.581(1.542,7.503)
	Slope	-1.010(-1.056,-0.964)	-0.998(-1.080,-0.916)	-0.927(-1.238,-0.616)	-1.001(-1.048,-0.953)	2.330(1.274,10.580)
Econazole	$Log_{10}IC_{50}$	-8.679(-8.762,-8.595)	-8.711(-9.181,-8.241)	-8.681(-8.794,-8.568)	-8.687(-8.733,-8.641)	4.631(2.856,11.889)
	Slope	-1.029(-1.052,-1.006)	-0.976(-1.535,-0.417)	-1.058(-1.110,-1.006)	-1.034(-1.087,-0.981)	1.444(0.703,16.025)

1. The estimates and 95% confidence intervals are based on the intralaboratory analyses for the three participating laboratories. The intralaboratory analyses were carried out separately for each reference chemical. The confidence intervals were calculated based on the least squares means, standard errors, and degrees of freedom from the intralaboratory analysis reports. They may thus differ from those in the intralaboratory analysis reports in the low significant digits due to round off error.
2. The overall estimates and confidence intervals were estimated based on one-way random effects analysis of variance with heterogeneous within laboratory variances among the three laboratories. The within laboratory variances are the squares of the within laboratory standard errors.
3. CV was calculated for the overall average results for the IC_{50} and slope parameters.

Table 23. Variance Components and Ratio of Between and Within Laboratories Variances for $Log_{10}IC_{50}$ and Slope Parameters of Concentration Response Curves for the Placental Aromatase Assay. By Chemical.

		Within Laboratory Variance[1]						
Chemical	Parameter	RTI[7]	Lab B[8]	Lab C	Pooled Average[2]	Among Laboratory Variance[3] and (p-value) (df=2)	Mean Variance[4, 5]	Ratio and 95% CI[6]
Aminoglutethimide	Log_IC50	0.000/df=8.0	0.001/df=2.0	0.010/df=4.2	0.004/df=5.3	0.000 (1.00)	0.000/df=5.3	0.000 (0.000, 0.000)
Aminoglutethimide	Slope	0.000/df=8.0	0.000/df=2.0	0.000/df=53.2	0.000/df=13.7	0.000 (0.37)	0.000/df=2.6	0.360 (0.074, 14.206)
Chrysin	Log_IC50	0.002/df=8.0	0.002/df=2.0	0.048/df=3.0	0.017/df=3.5	0.000 (1.00)	0.001/df=3.5	0.000 (0.000, 0.000)
Chrysin	Slope	0.001/df=8.0	0.000/df=2.0	0.002/df=4.1	0.001/df=10.3	0.029 (0.12)	0.010/df=3.1	22.163 (4.113, 873.261)
Econazole	Log_IC50	0.001/df=8.0	0.001/df=1.0	0.001/df=1.8	0.001/df=4.7	0.000 (1.00)	0.000/df=4.7	0.000 (0.000, 0.000)
Econazole	Slope	0.000/df=8.0	0.002/df=1.0	0.000/df=3.7	0.001/df=1.5	0.000 (1.00)	0.000/df=1.5	0.000 (0.000, 0.000)
Ketoconazole	Log_IC50	0.000/df=8.0	0.004/df=2.0	0.005/df=1.9	0.003/df=3.9	0.000 (1.00)	0.000/df=3.9	0.000 (0.000, 0.000)
Ketoconazole	Slope	0.000/df=8.0	0.000/df=2.0	0.005/df=1.9	0.002/df=2.5	0.000 (1.00)	0.000/df=2.5	0.000 (0.000, 0.000)

1. The within laboratory variance for each laboratory is the square of the standard error associated with the parameter estimate, as reported in the intralaboratory analyses for the three participating laboratories.
2. Pooled average for the within laboratory variances is the unweighted average of the within laboratory variances among the three laboratories. Associated degrees of freedom were based on Satterthwaite's approximation
3. Among laboratories variance is based on a one-way random effects analysis of variance model with heterogeneous within laboratory variances, equal to the squares of the within laboratory standard errors.
4. Mean variance is the square of the standard error of the pooled weighted mean value. It includes both within and among laboratory variation.
5. Degrees of freedom for the (mean) overall effect variance were estimated as $2*((1/K)* \sum(S_L^2 + S_i^2))^2/(var(S_L^2)+(2/K^2)* \sum(S_i^4/df_i))$, where S_L^2 is the among laboratory variance, S_i^2 and df_i are the reported variance and degrees of freedom for laboratory i, $var(S_L^2)$ is the variance of S_L^2, and K is the number of laboratories (Hartung and Makambi, 2001).
6. Ratio of the among-laboratory variance and the pooled average within laboratory variance.
7. Degrees of freedom for RTI were based on a mixed effects analysis of variance for the four chemicals combined.
8. 1 degree of freedom for econazole for Laboratory B was specified because the results were based on one replicate.

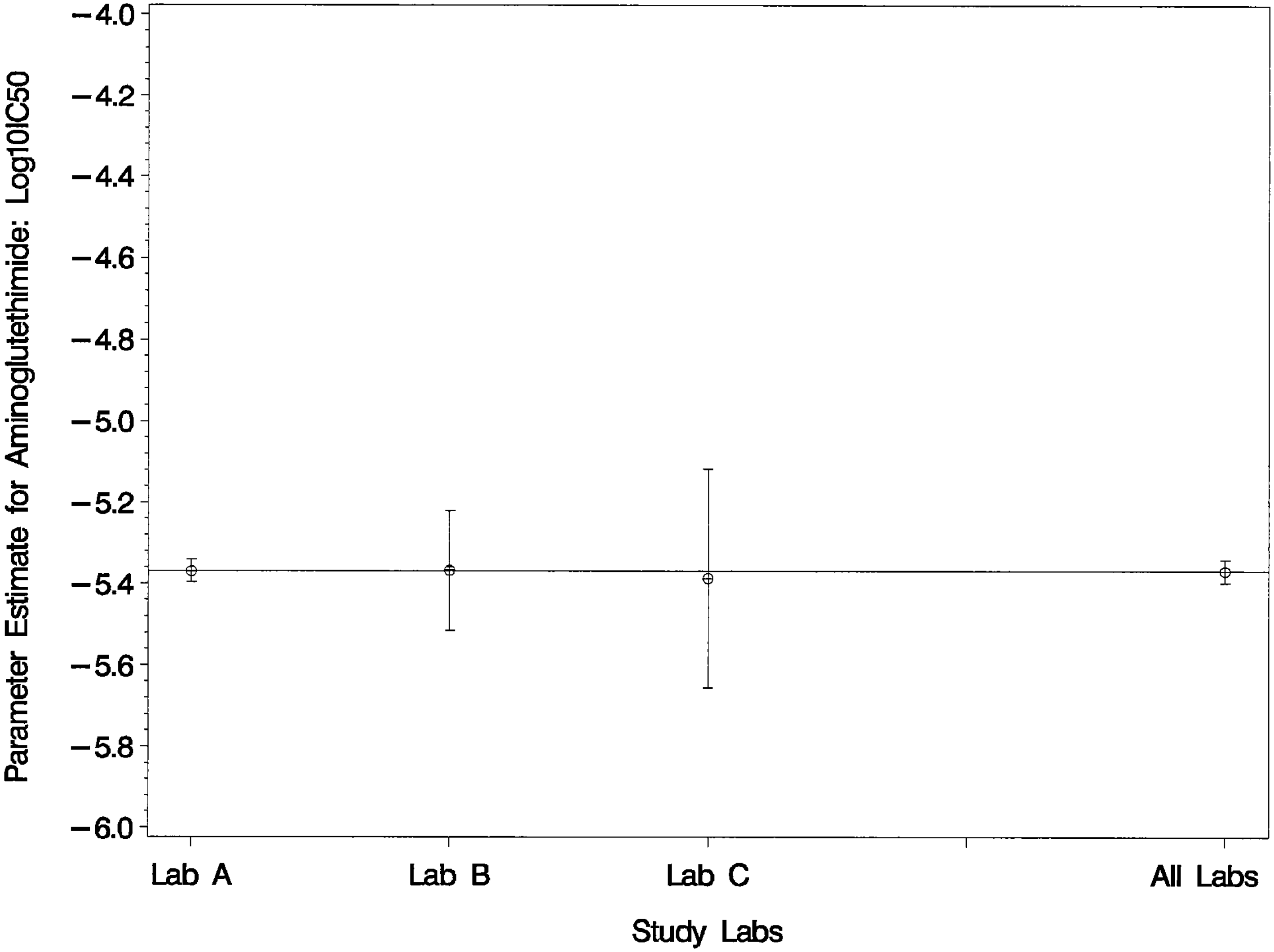

Figure 9. Aminoglutethimide: Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)

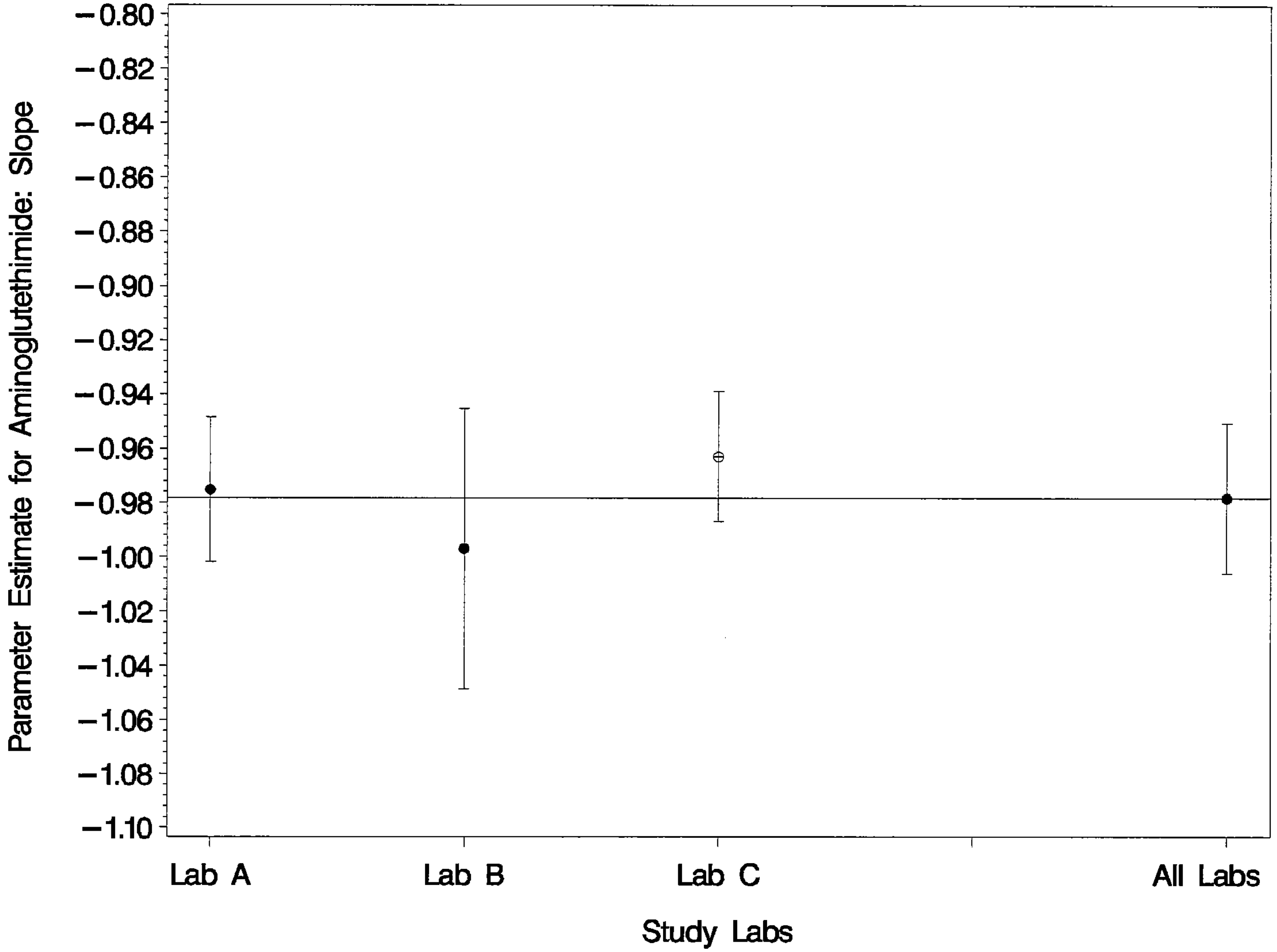

Figure 10. **<u>Aminoglutethimide</u>: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)**

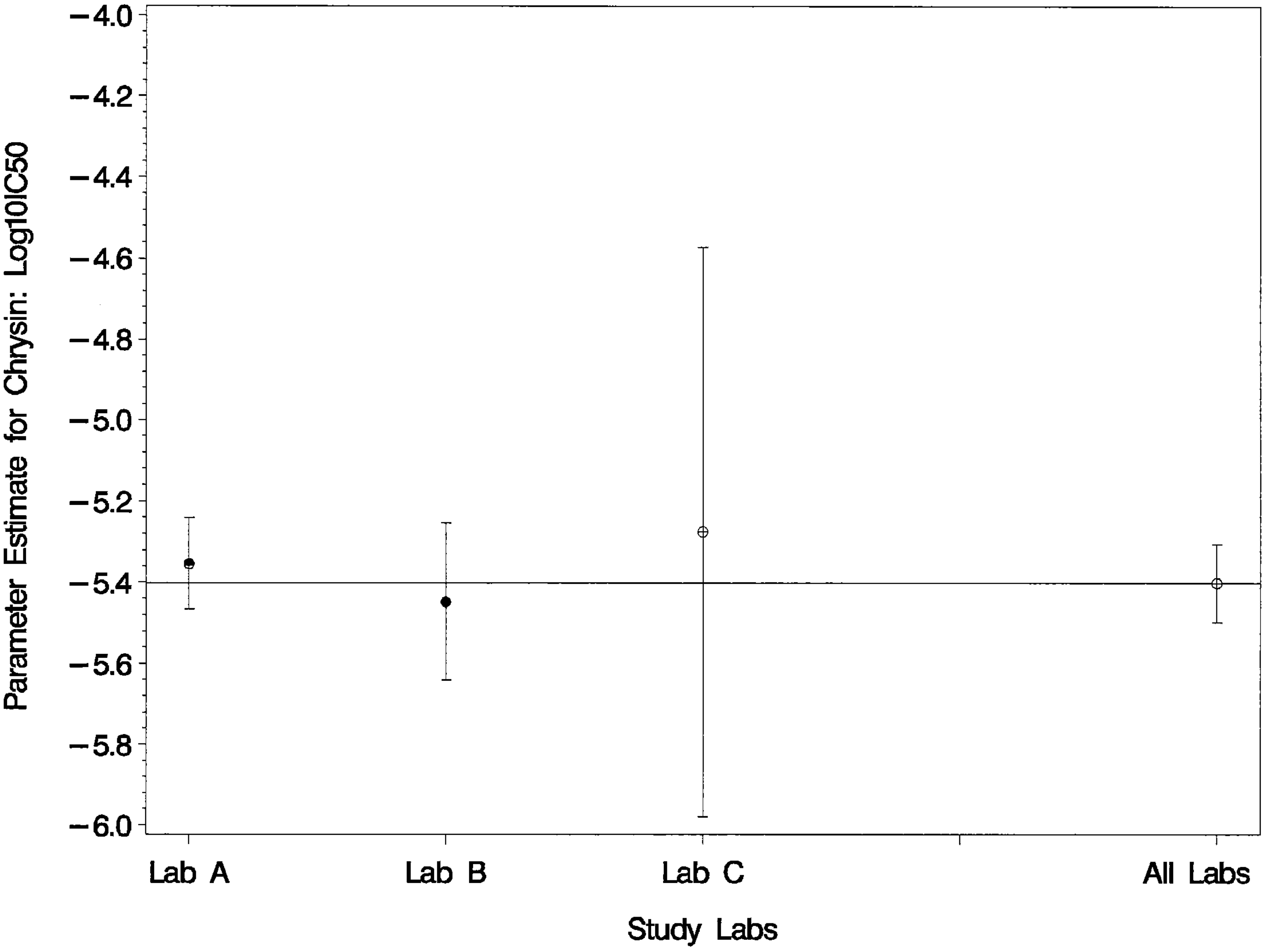

Figure 11. **Chrysin: Parameter Estimates and Their Associated 95% Confidence Intervals for $\text{Log}_{10}\text{IC}_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)**

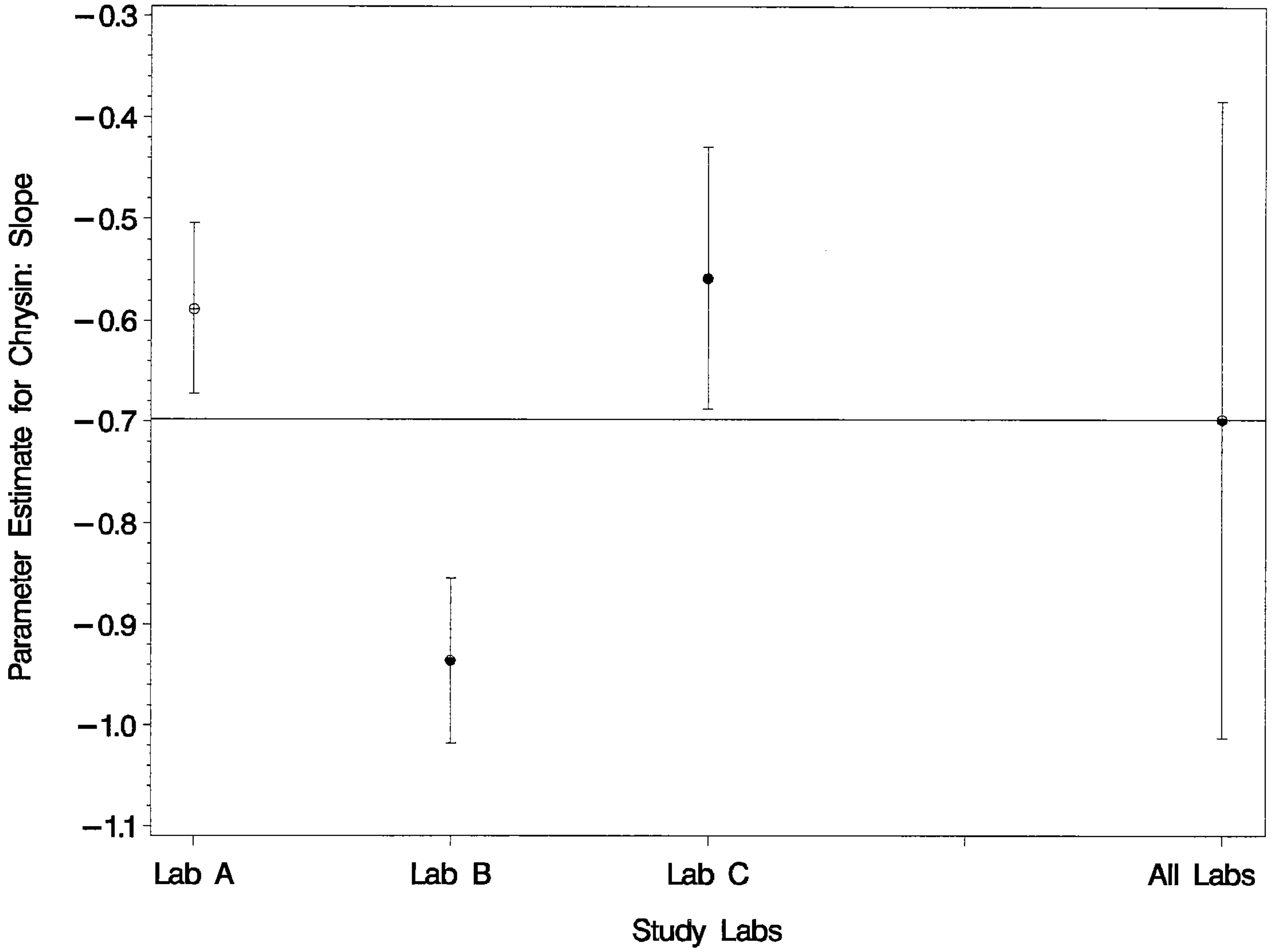

Figure 12. **<u>Chrysin</u>: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)**

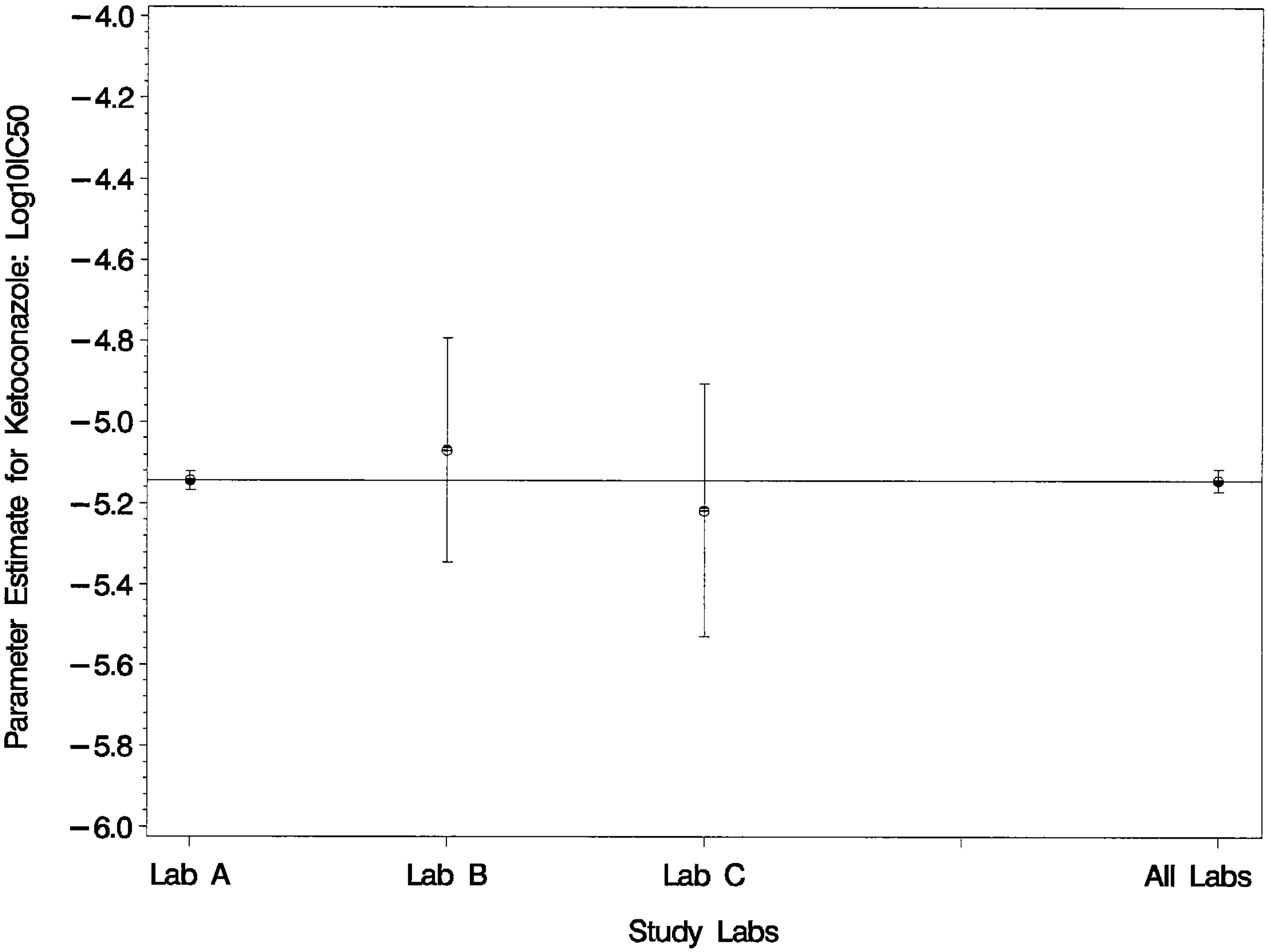

Figure 13. Ketoconazole: Parameter Estimates and Their Associated 95% Confidence Intervals for $Log_{10}IC_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)

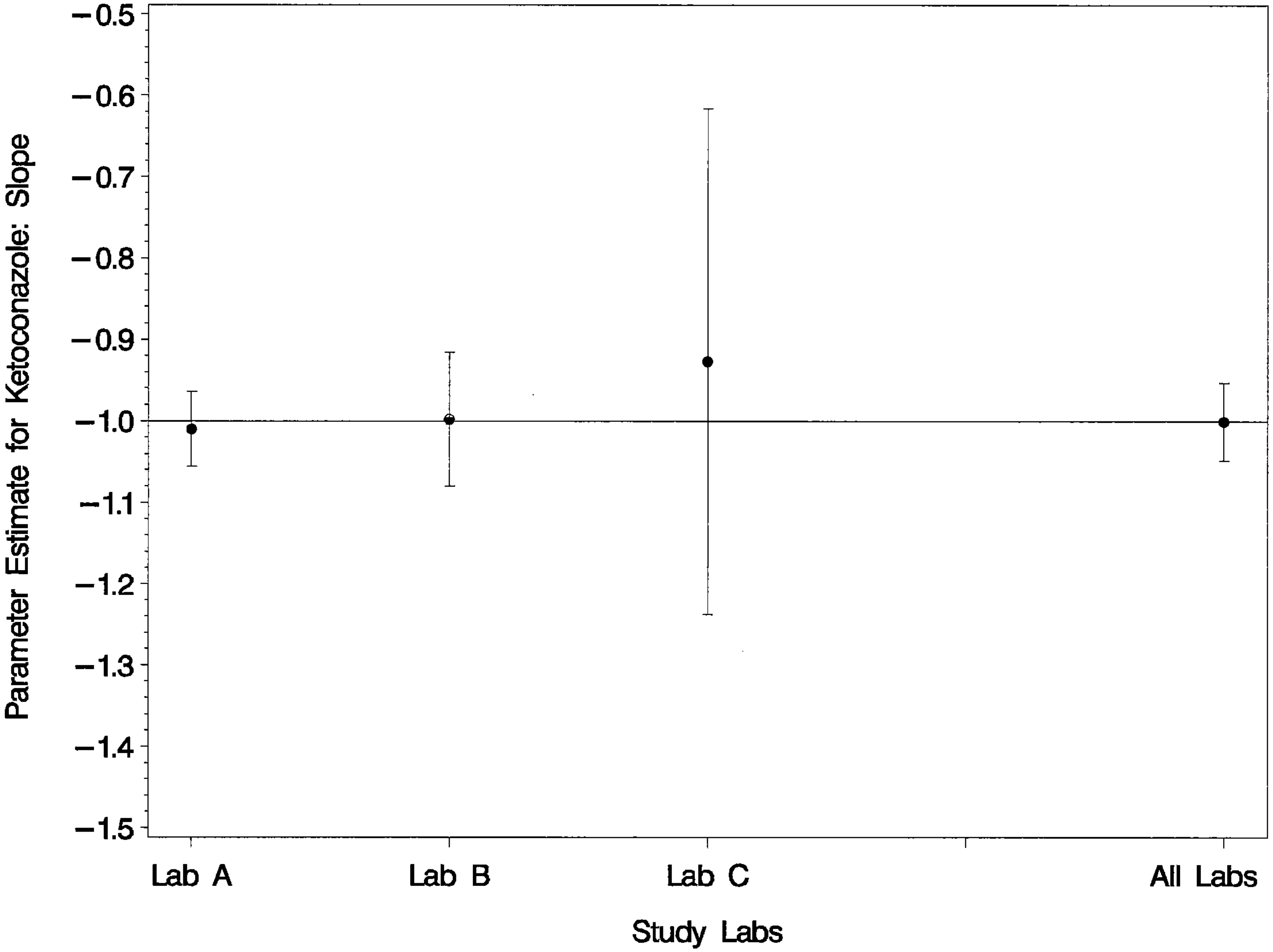

Figure 14. **Ketoconazole: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)**

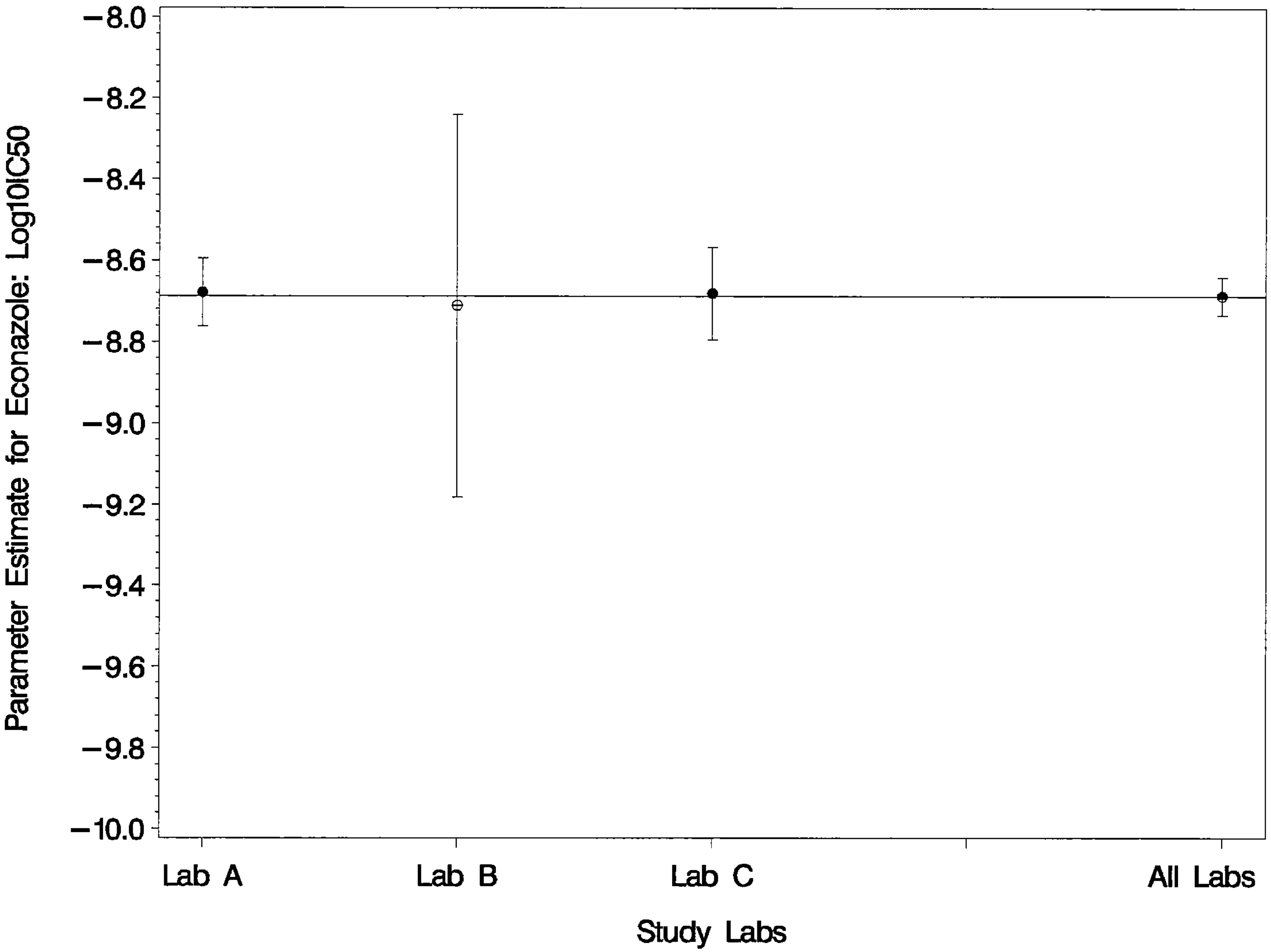

Figure 15. Econazole: Parameter Estimates and Their Associated 95% Confidence Intervals for $\text{Log}_{10}\text{IC}_{50}$ in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)

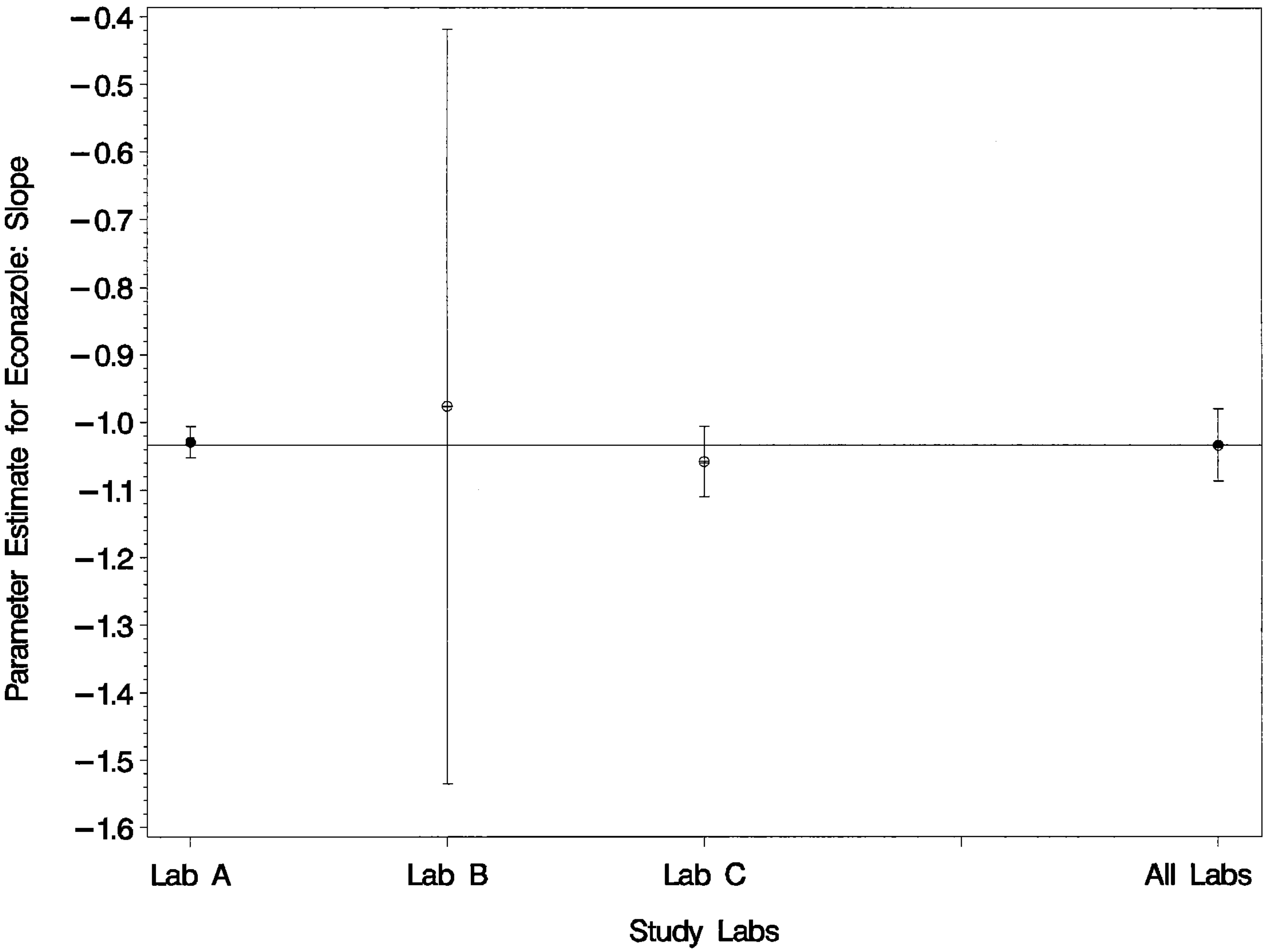

Figure 16. Econazole: Parameter Estimates and Their Associated 95% Confidence Intervals for Slope in the Placental Aromatase Assay. Within and Among Laboratories. The Reference Line Corresponds to the Average Across Laboratories. (Lab A is RTI)

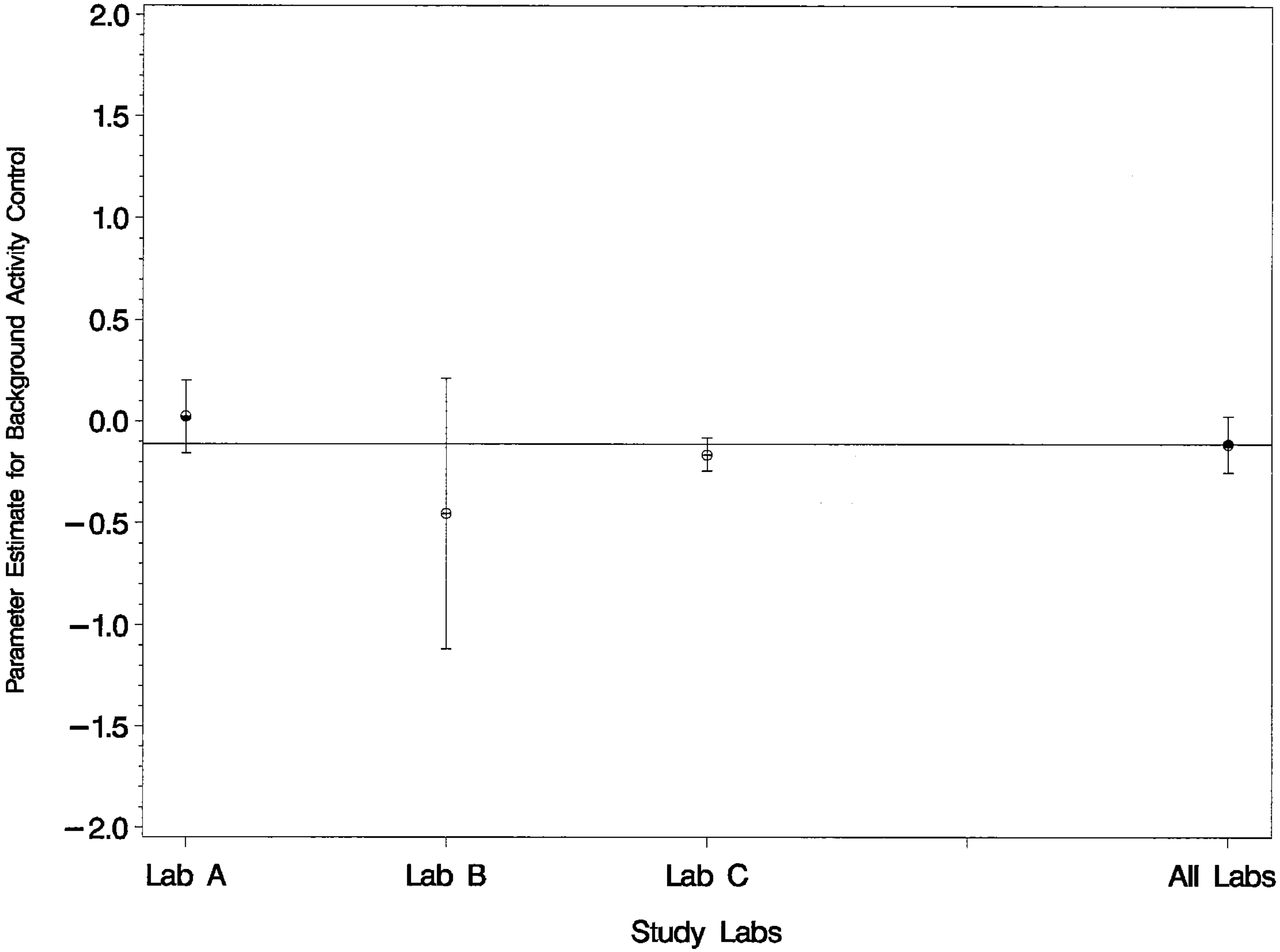

Figure 17. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Background Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B does not Include Econazole. (Lab A is RTI)**

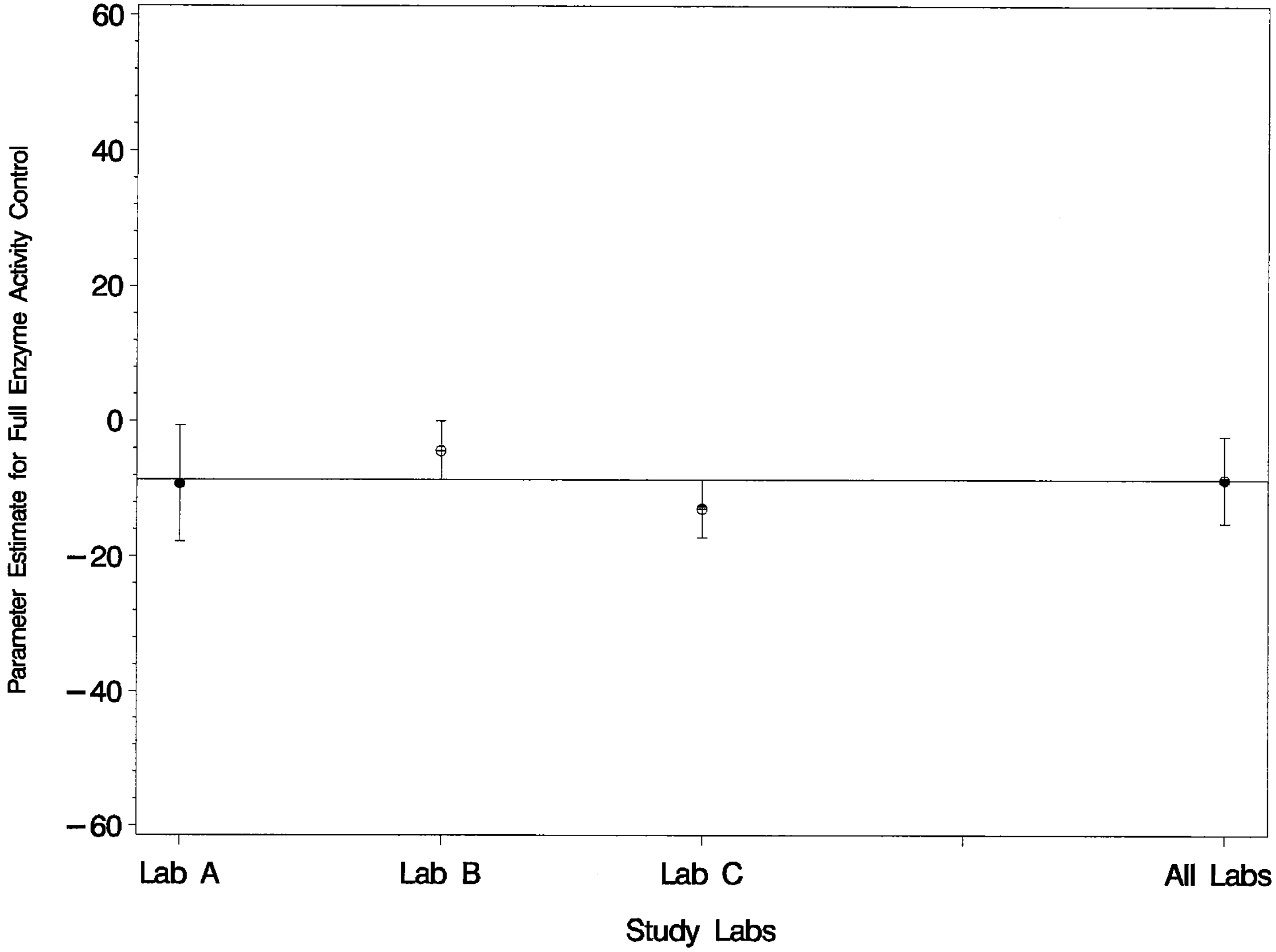

Figure 18. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Full Enzyme Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B does not Include Econazole. (Lab A is RTI)

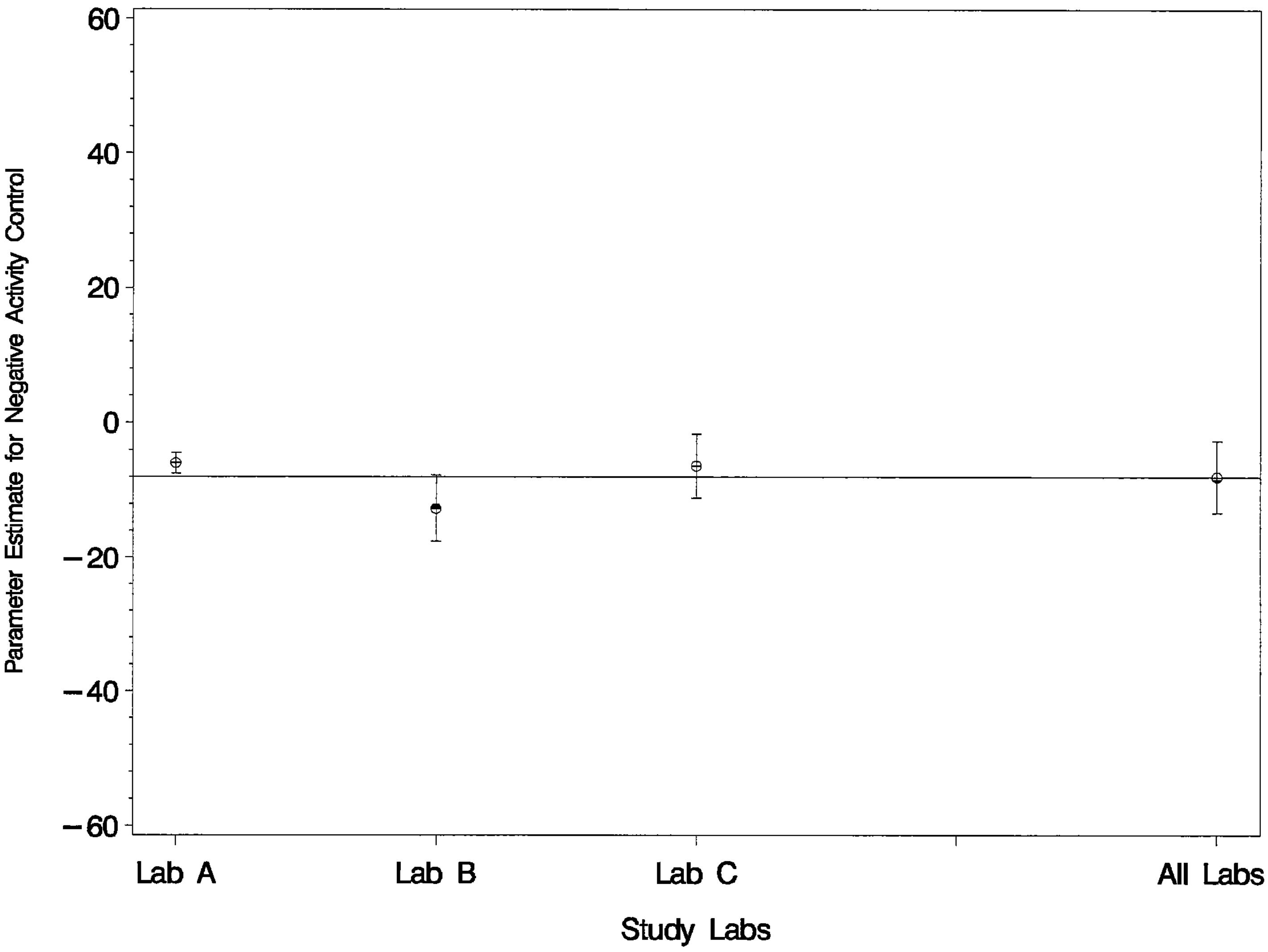

Figure 19. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Negative Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B does not Include Econazole. (Lab A isRTI)**

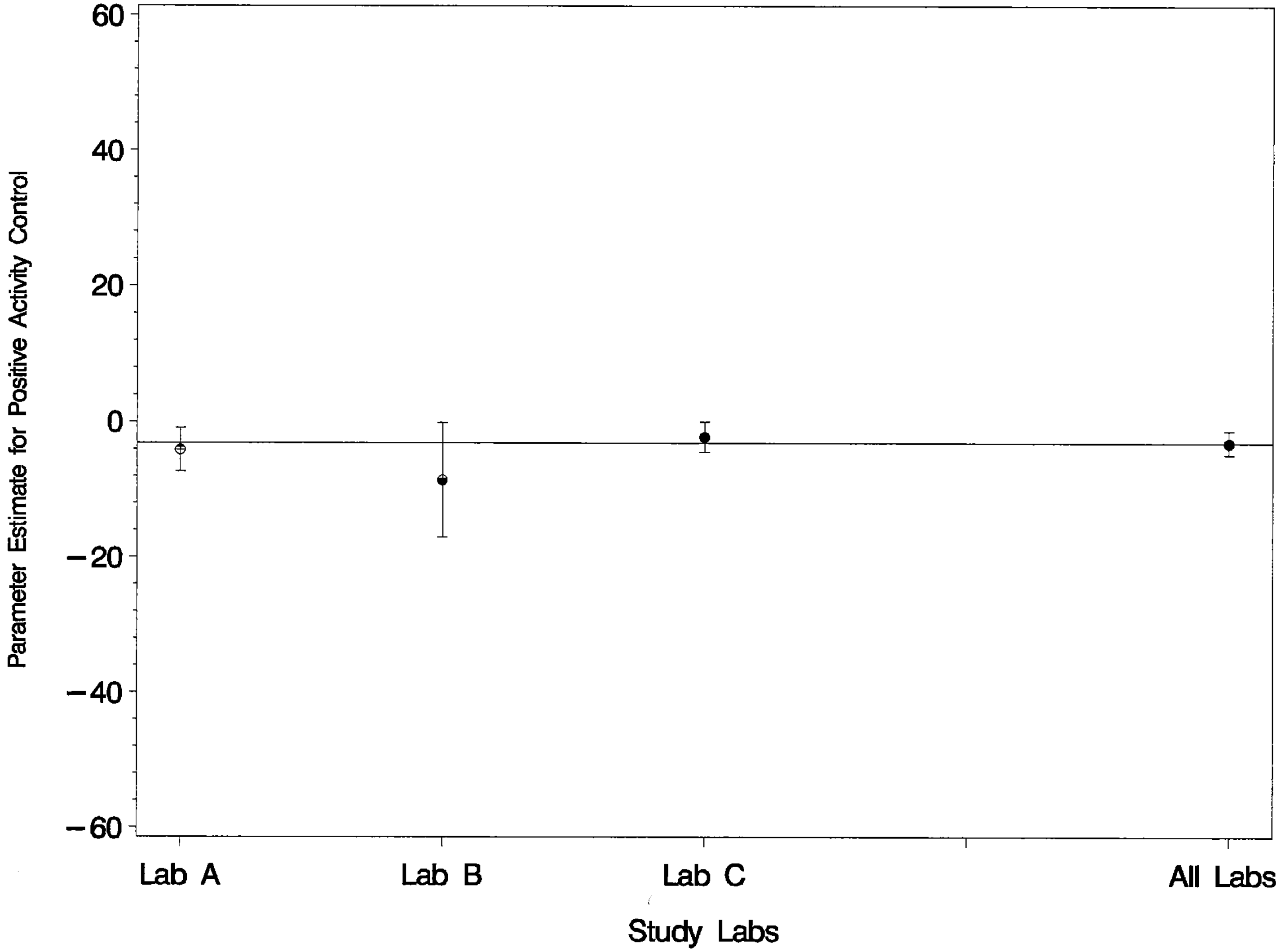

Figure 20. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Positive Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B does not Include Econazole. (Lab A is RTI)

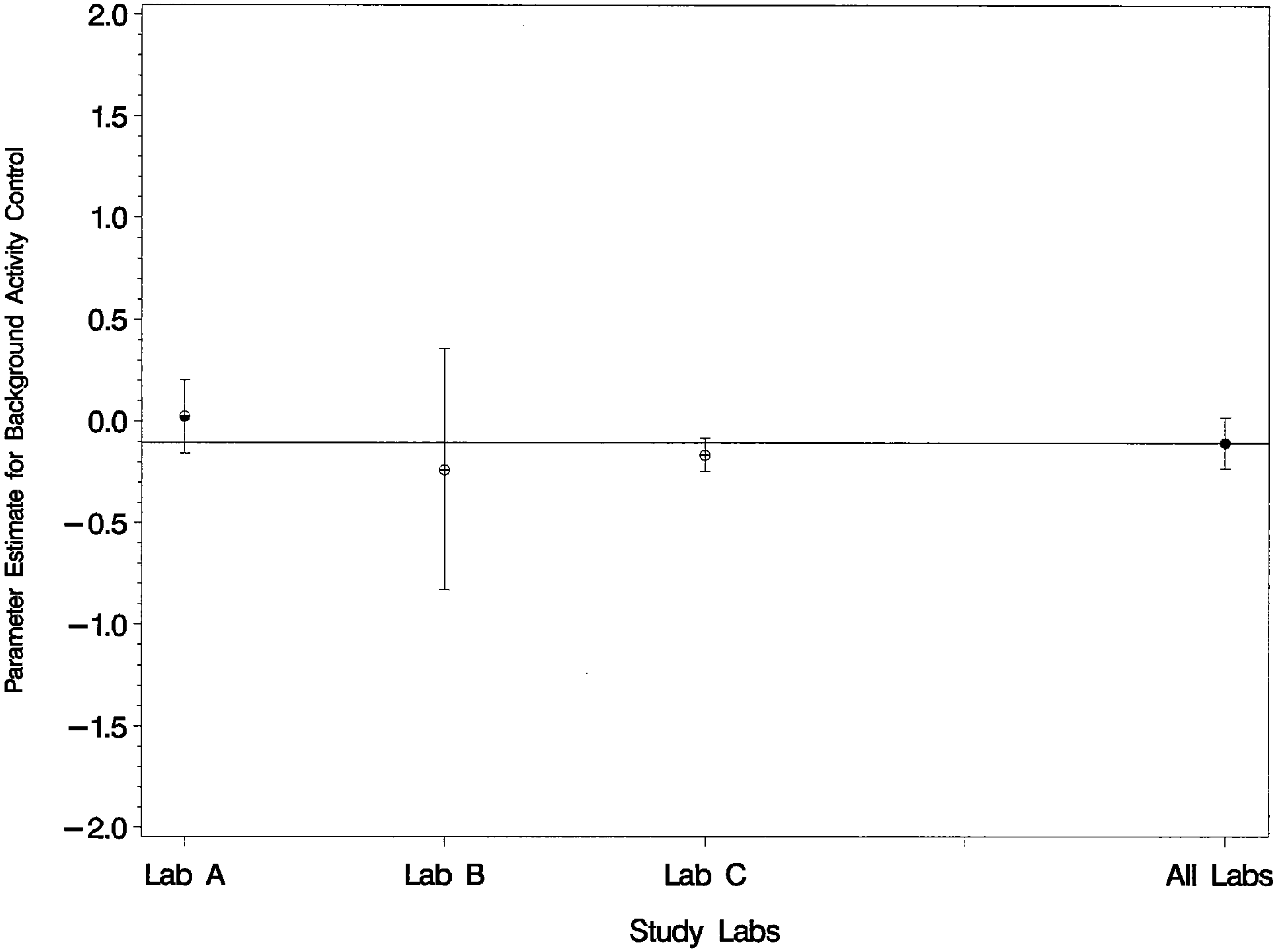

Figure 21. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Background Activity Controls in the Placental Aromatase Assay, by Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole. (Lab A is RTI)

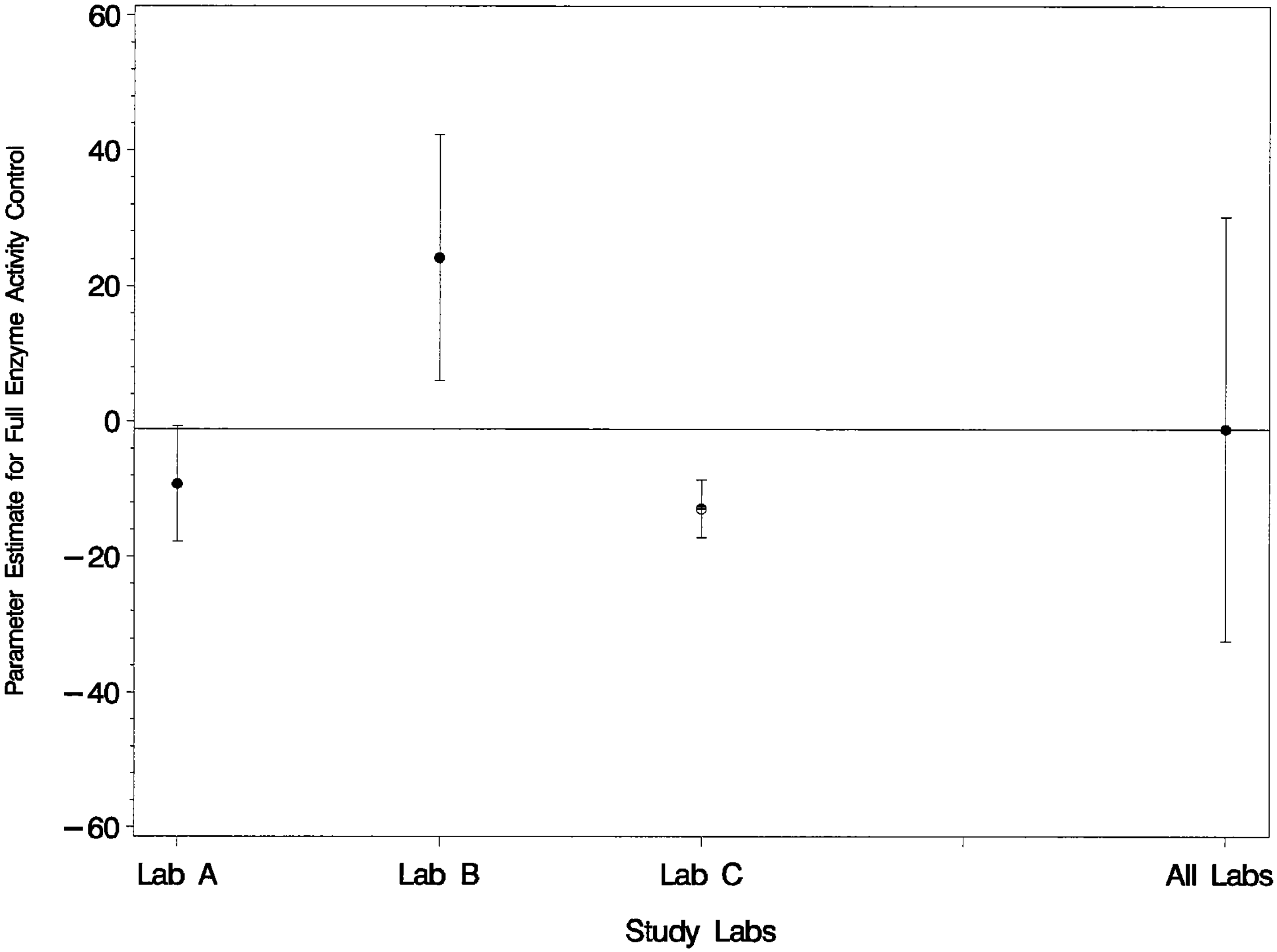

Figure 22. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Full Enzyme Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole. (Lab A is RTI)

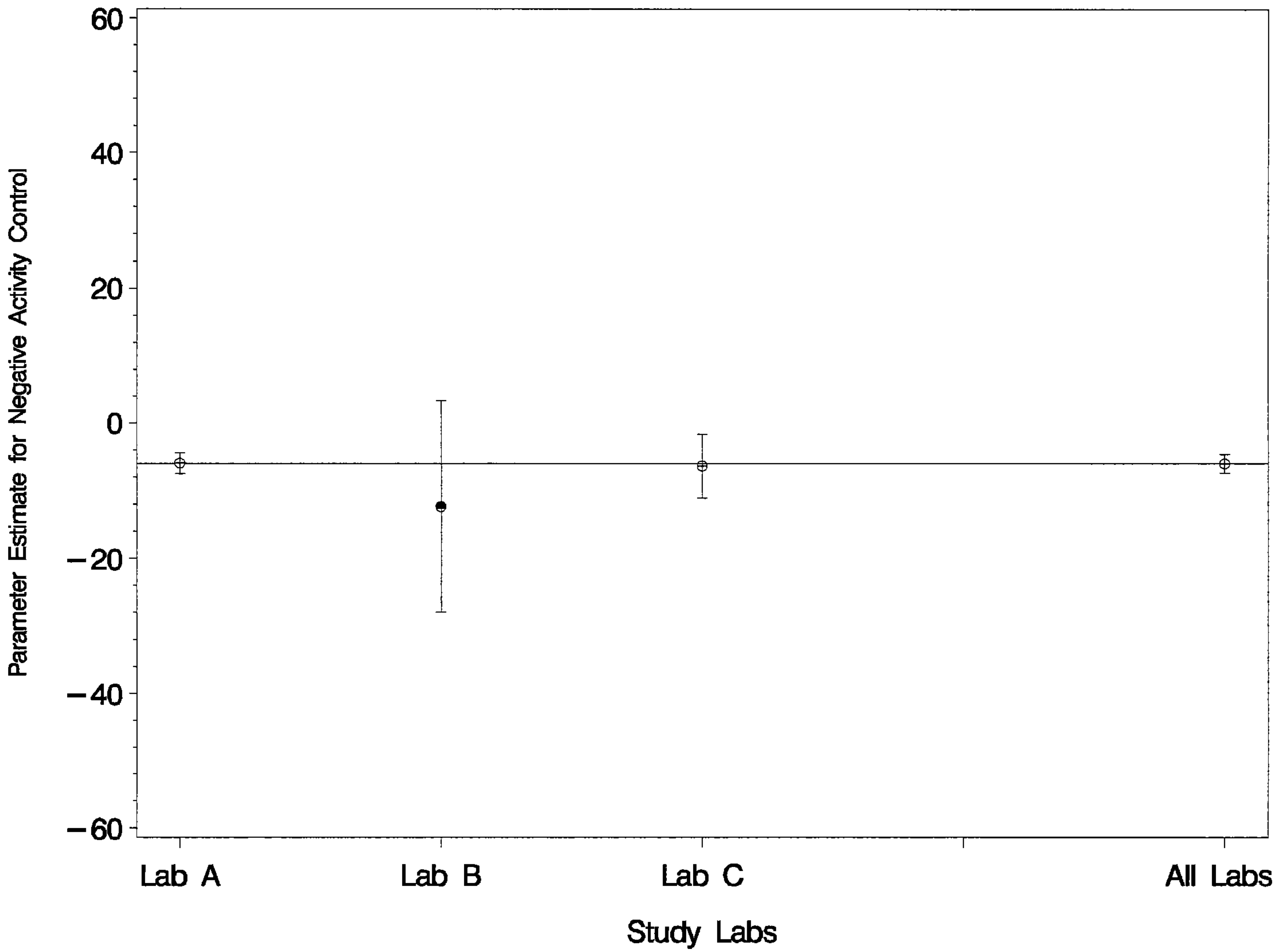

Figure 23. Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Negative Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole. (Lab A is RTI)

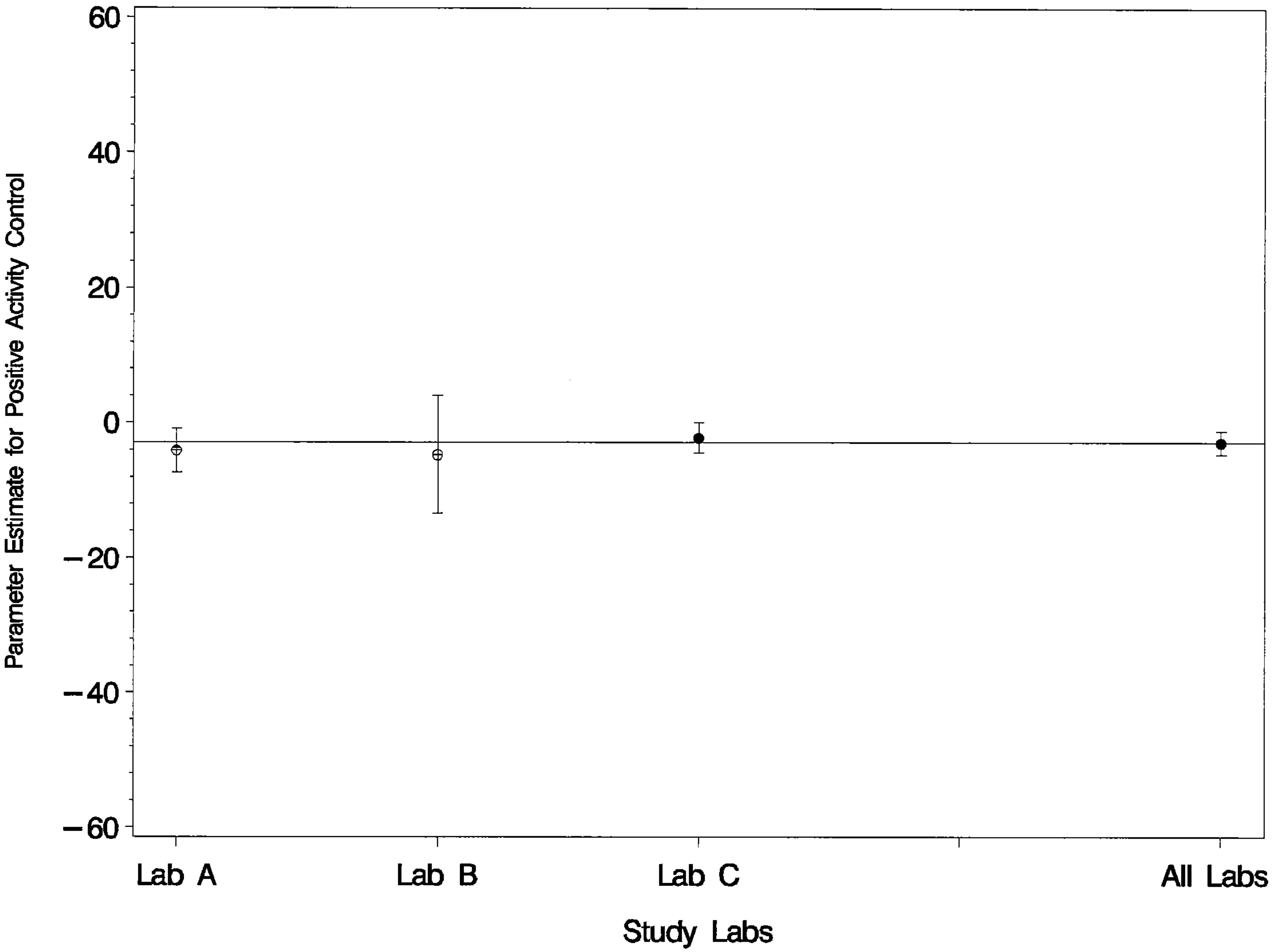

Figure 24. **Parameter Estimates and Their Associated 95% Confidence Intervals for Difference (End Minus Beginning) for Positive Activity Controls in the Placental Aromatase Assay. By Laboratory and Across Laboratories. The Horizontal Reference Line Corresponds to the Average Across Laboratories. Laboratory B Includes Econazole. (Lab A is RTI)**

4.0 DISCUSSION

In this task, the responsiveness of the human placental microsomal aromatase assay to four reference chemicals and positive and negative control test substances (4-OH ASDN and lindane) were evaluated. The assay was conducted by a lead laboratory (RTI) and two participating laboratories using microsomes prepared and distributed by the lead laboratory. As planned, this task provided information about intralaboratory and interlaboratory variability for endpoints such as full enzyme activity and background activity controls, aromatase inhibition (as a percent of control) by the positive and negative controls and aromatase inhibition (percent of control and IC_{50} values) for each of the reference chemicals. In addition, there was some unexpected information that came out of this task such as determining the protein concentration and need for protein QCs, as well as using an appropriate number of parameters to obtain acceptable curve fits to the observed data, which were useful for designing the tasks that followed.

RTI (or the location for the "centrally prepared microsomes") prepared the placental microsomes that were used to conduct the assays in this task. The protein content of the placental microsomal preparation was reported to be 14 mg/mL when the microsomes were first prepared (November 5, 2004; personal communication from S. Black at RTI). Based on the protein concentration determination results in the present task (11.5 ± 0.6, 10.1 ± 0.8, and 11.2 ± 0.4 mg/mL for RTI and Laboratories B and C, respectively), there was a slight decrease in the protein concentration from the time they were first prepared (11/2004) until the time they were used to conduct the present task (2 to 4/2005). If an overall average protein concentration of 11 mg/mL is used for the present task, then the protein concentration decreased 21 percent when compared to the initial results, thereby providing a measure of the protein loss that occurred after 3 to 5 months. Even so, the protein concentration of the microsomal preparation for the present task was sufficient to measure enzyme activity.

During the study, the EPA WAM requested that the protein assay include QC standards at concentrations of 0.125, 0.5, and 1.0 mg/mL. In general, accuracy (% RE) and precision (% CV) were low for the 0.125 mg/mL QC standard. In contrast, accuracy for the laboratories ranged from -4.8 to 15.9 percent and precision for the laboratories ranged from 1.7 to 4.6 percent for the 0.5 and 1.0 mg/mL QC standards. As explained by Dr. J. Matthews and Ms. S. Black (RTI report):

> "The microsomal protein dilutions that were actually measured in the protein assay had a concentration of about 0.23 mg/mL. This concentration falls between the low end of the reliable range (0.25 mg/mL) of the protein standard curve and the low end of the standard curve range (0.13 – 1.5 mg/mL) and therefore may be subject to a large variance from the true value. Furthermore, the microsomal dilution which was used for protein measurement was not the same dilution that was used in the aromatase assay. The dilution used in the aromatase assay was 10-fold more dilute that the one used for protein measurement. This had been the practice because the final dilution for use in the aromatase assay would only contain approximately 0.025 mg/mL, which was below the lower bounds of the protein standard curve. Because the concentration of protein used in the aromatase assay is very low (0.0125 mg/mL) and because the aromatase activity is

> calculated by normalizing the amount of product by the protein content, small variations in protein content can have a large effect on the measured aromatase activity. Therefore, in order to have meaningful comparison of aromatase activity from laboratory to laboratory it is essential to have accurate, reliable protein content determination. For that to be the case, it would be best to measure protein directly in the final microsome dilution (that which is actually used in the assay) and to have the measured values fall in a well defined portion of the protein standard curve (preferably near the middle of the linear range). Efforts are currently underway on a separate work assignment to address these issues."

As a consequence of the results of this task, centrally-prepared QC standards were distributed to the laboratories that participated in later tasks and these standards were used to evaluate day-to-day and laboratory-to-laboratory results. For more information about the preparation of the QC standards for the later tasks, see WA 5-5, Task 15.

The full enzyme activity control value for this assay should be at least 0.03 nmol/mg protein/min according to the acceptance criterium in the study plan. For this task, the overall average full enzyme activity control values were 0.105 ± 0.009, 0.042 ± 0.013, and 0.049 ± 0.003 nmol/mg protein/min for RTI and Laboratories B and C, respectively. The lead laboratory did not have any replicates below the acceptance criterium, whereas one of the participating laboratories had one replicate for aminoglutethimide with full enzyme activity value below the acceptance criterium and the other participating laboratory had two of three replicates for ketoconazole and four of five replicates for econazole below the acceptance criterium. There was no clear association between a replicate with full enzyme activity that was lower than the acceptance criterium and having higher variability (percent CV). For Laboratory B, the enzyme activity began to decrease over time and, for the ketoconazole and econazole analyses, the enzyme activity was very low and repetitions within given replicates were very erratic. Attempts to determine possible causes for the change in enzyme activity did not identify any specific factors that could be used to explain the problem. There was no evidence that the microsomes were damaged during storage or that the assay method was different than before. There was one other consistent anomaly that should be mentioned. There was a decrease in the full enzyme activity when the beginning repetitions were compared to the ending repetitions (also noted in the background and positive/negative controls). This finding occurred for all laboratories and suggested that the time required to analyze the samples decreased the enzyme activity and may have implications on the number of samples that can be analyzed at a given time.

The positive control, 4-OH ASDN, is a known aromatase inhibitor and inhibited aromatase activity in a consistent manner for all laboratories. 4-OH ASDN, at a final concentration of 5×10^{-8} M, resulted in laboratory overall group mean ± SEM inhibition values (as a percent of control) of 47.2 ± 2.3, 54.5 ± 7.1, and 55.9 ± 1.3 percent for RTI and Laboratories B and C, respectively. These results indicated that the target concentration, which was selected to inhibit aromatase approximately 50 percent based on results from previous studies, produced the desired degree of inhibition.

The negative control, lindane, is known to not inhibit aromatase, which it did in a consistent manner for all laboratories. Lindane, at a final concentration of 10^{-6} M, resulted in laboratory overall average ± SEM enzyme activity values of 93.6 ± 4.1, 121.1 ± 24.4, and 99.2 ± 1.0 percent for RTI and Laboratories B and C, respectively. These results indicated that the target concentration, which was selected because it was able to be formulated in concentrations that could be readily tested and not inhibit aromatase activity based on results from previous studies, worked as planned.

The reference chemicals used on this task were aminoglutethimide, chrysin, ketoconazole and econazole. Each was selected for its known effect on aromatase activity and chemical classification. Aminoglutethimide is a non-steroidal aromatase inhibitor; chrysin is a flavonoid with potent aromatase inhibitory effects; econazole is an imidazole with potent aromatase inhibitory effects; and ketoconazole is an imidazole with weak aromatase inhibitory effects. One of the uncertainties of this assay was how well could the laboratories characterize the inhibitory effects of an unknown chemical. In order to evaluate this question, the laboratories were directed to test each of the reference chemicals at eight predefined target concentrations in triplicate repetitions (tubes) per replicate of the assay. Percent of control results from the first replicate of a given reference chemical were reviewed and the target concentrations were adjusted to better define the concentration response curve. Concentrations were selected to define the upper and lower plateaus with intermediate concentrations chosen to distribute as evenly as possible along the slope of the curve. This resulted in some different concentrations being tested in replicate 1 than were tested in replicates 2 and 3. This adjustment in concentrations may also explain some of the differences and variability of the replicates for a given laboratory since the latter replicates were optimized to better characterize the curve and estimate the IC_{50}. Another issue that was identified during this task involved fitting the observed data. As described by Dr. J. Mathews and Ms. S. Black (RTI report),

> "Concentration response curves were fitted to the percent of control values with PRISM software using the following equation:
>
> $$Y = 100/[1 + 10^{(\mu - X)\beta}] + \varepsilon$$
>
> Where: $Y \equiv$ percent of control activity in the inhibitor tube
>
> $X \equiv$ logarithm (base 10) of the concentration
>
> $\beta \equiv$ slope of the concentration response curve (β will be negative)
>
> $\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to 50% of control activity).
>
> DAVG $\equiv$ average DPMs across the repeat tubes with the same reference chemical concentration
>
> $\varepsilon \equiv$ the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts).
>
> The variance was approximated by Y. The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. When the range of the percent of control values was approximately 0 to 100%, the curve fit the data well (r^2 generally >0.99) whereas, in the case of chrysin where the range was abbreviated, the curve fit was not as good (average r^2 =0.9374). The two-parameter model used in this

> work assignment leads to unsatisfactory curve fits where the data do not span the entire 0-100% range. The current model will, however, calculate the IC_{50} properly as the concentration corresponding to 50% inhibition. The alternative four-parameter model, in which the top and bottom plateaus are allowed to vary to fit the data, would result in a more satisfactory curve fit, but the parameter output by PRISM as logEC50 ($\equiv$logIC_{50}) would correspond to (Top + Bottom)/2 and in the case of chrysin, would be approximately (95 + 22)/2 = 58.5% activity. Therefore, careful examination of the PRISM output is necessary and additional calculations may be required to ensure that the actual IC_{50} is reported."

These results led to modifications to the analysis procedure. A four-parameter model was incorporated into the analysis procedure for the follow-on tasks.

The IC_{50} values measured for the four reference chemicals in this study compared well with those found in previous work assignments (WA 2-24, WA 4-10), study plan (WA 4-16), Aromatase Detailed Review Paper (2005), and/or the literature (Chen, et al., 1997; Le Bail, et al., 2001) for aminoglutethimide, chrysin, and ketoconazole but were about 10-fold lower than reported literature values for econazole. For aminoglutethimide, the mean ± SEM IC_{50} values in the present task for RTI and Laboratories B and C were 4.26 ± 0.11, 4.28 ± 0.33, and 4.54 ± 0.94 μM, respectively, with an overall task group mean ± SEM IC_{50} value of 4.36 ± 0.09 μM. These values are in good agreement with a value from a previous work assignment (5.6 μM) and literature values (1.0 to 5.5 μM). For chrysin, the mean ± SEM IC_{50} values for RTI and Laboratories B and C were 4.45 ± 0.54, 3.54 ± 0.33, and 4.17 ± 1.55 μM, respectively with an overall task group mean ± SEM IC_{50} value of 4.05 ± 0.27 μM. These values are in good agreement with a value from a previous work assignment (3.2 μM) and literature values (0.7 to 11 μM). For econazole, the mean ± SEM IC_{50} values for RTI and Laboratory C were 2.19 ± 0.22 and 2.12 ± 0.13 nM, respectively. There was no mean value calculated for Laboratory B. The overall task group mean ± SEM IC_{50} value of 2.10 ± 0.06 nM. These values are in good agreement with a value from a previous work assignment (2.79 nM) but not from reported literature values (30 to 50 nM). For ketoconazole, the mean ± SEM IC_{50} values for RTI and Laboratories B and C were 7.16 ± 0.17, 8.67 ± 1.08, and 6.53 ± 0.66 μM, with an overall task group mean ± SEM IC_{50} value of 7.46 ± 0.64 μM. These values are in good agreement with a value from a previous work assignment (15.0 μM) and from literature values (6 to 60 μM).

5.0 CONCLUSIONS

A lead laboratory and two participating laboratories used human placental microsomes prepared by the lead laboratory to conduct the aromatase assay to test four reference chemicals for aromatase inhibition, which provided sufficient information to make study-to-study and laboratory-to-laboratory comparisons.

Coefficients of variance values for many endpoints were higher for the participating laboratories than for the lead laboratory. There were also incidences when the aromatase activities for the controls or reference chemicals had coefficients of variance that exceeded 20%. One plausible explanation is that this variance was associated with imprecise protein determinations. It is believed that the protein assay accuracy and precision may be improved by using a standard curve that encompasses the protein concentration of the microsomal dilution that is used directly in the aromatase assay (rather than being one dilution removed). The continued use of matched quality control samples in the protein assay are also believed to improve the reliability of the assay and allow for better comparison between laboratories.

IC_{50}s were calculated for each reference chemical using a two parameter equation and each was comparable for values obtained on previous work assignments for these chemicals. There was very little variance in IC_{50} values for aminoglutethimide and ketoconazole. A higher variance was noted for econazole, especially for one of the participating laboratories. For chrysin, the results identified limitations of the two-parameter model for fitting data that do not span the entire 0-100% of control range. Thus, a four-parameter model will need to be developed for handling data of this nature.

6.0 REFERENCES

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APPENDIX A

RTI REPORT

DRAFT TASK REPORT

PLACENTAL AROMATASE VALIDATION STUDY

WA 4-16 Task 5:

Assay to Determine the Effect of Four Reference Chemicals on the Aromatase Activity of Human Placental Microsomes

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

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DRAFT REPORT

Title: **PLACENTAL AROMATASE VALIDATION STUDY WA 4-16 Task 5: Assay to Determine the Effect of Four Reference Chemicals on the Aromatase Activity of Human Placental Microsomes**

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Study Initiation Date: February 1, 2005

Experimental Dates: Feb 3, 2005 – March 9, 2005

Draft Task Report Date: December 13, 2005

RTI Identification Number: 08055.003.032 – Task 5

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1.0 EXECUTIVE SUMMARY

In this task, RTI, as one of four participating laboratories, conducted studies with four potential aromatase inhibitors (reference chemicals) using a single human placental microsomal preparation in order to demonstrate the responsiveness of the assay and to produce data that could be compared with data from other participating laboratories. The reference chemicals were aminoglutethimide, chrysin, ketoconazole, and econazole.

Three (or four) independent replicates were conducted (on separate days) for each reference chemical. Each reference chemical was tested at eight concentrations and there were three (triplicate) repetitions for each concentration of a given replicate. Four types of control samples were included for each replicate. These included:

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

Aromatase activities for each type of control had coefficients of variance on the order of 20% over the twelve accepted assays (the results of two assays were excluded from the analysis) conducted. It is possible that this variance was associated with inaccurate protein determination. The protein assay accuracy may be improved by using a standard curve that encompasses the protein concentration of the microsomal dilution that is used directly in the aromatase assay (rather than being one dilution removed). The continued use of matched quality control samples in the protein assay would also improve the assay and allow for better comparison between laboratories.

IC_{50}s were calculated for each reference chemical using a two-parameter equation and each was comparable to values obtained in previous work assignments for these chemicals. There was very little variance (<5%) in IC_{50} data over three assays for two of the reference chemicals (aminoglutethimide and ketoconazole). A higher variance, mostly associated with data obtained during a single replicate, was noted for econazole. The data obtained with chrysin, however, point out the limitations of the two-parameter model for fitting data that do not span the entire 0-100% of control range. A plan for handling data of this type should be developed for future tasks.

2.0 INTRODUCTION

2.1 Background

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the U. S. Environmental Protection Agency (EPA) to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the 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 Method Validation 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 $P450_{arom}$ 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 also found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is the subject of current research efforts. Aromatase inhibitors have been developed as efficacious therapeutic agents for estrogen-dependent breast cancer to reduce the stimulatory effects of estrogens in the progression of 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 as low as 0.04 mM.

The human placental microsomal aromatase assay was recommended as the in vitro aromatase screening assay to be included in the Tier 1 Screening Battery. This assay will detect environmental contaminants 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 experiments 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.

2.2 Task Description and Objectives

In this task, RTI, as one of four participating laboratories, conducted studies with four potential aromatase inhibitors (reference chemicals) to demonstrate the responsiveness of the assay. Microsomes were prepared by the lead laboratory (RTI International, [RTI]) and supplied to the participating laboratories.

3.0 MATERIALS AND METHODS

3.1 Substrate

The substrate for the aromatase assay was androstenedione (ASDN). Non-radiolabeled and radiolabeled ASDN were used. The non-radiolabeled ASDN (Lot # 024K0809) was obtained from Sigma (St. Louis, MO) by the Sponsor's Chemical Repository and was then distributed to the participating laboratories. The radiolabeled androstenedione ([1-^{3}H]-androstenedione, [^{3}H]ASDN, Lot # 3538496), was obtained from Perkin Elmer Life Science (Boston) 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). The results of this analysis are presented in the report contained in Appendix F.

The substrate solution was prepared fresh each day of assay by combining solutions of [^{3}H]ASDN and non-radiolabeled ASDN in order to prepare a solution containing 2 μM ASDN with ca. 1 μCi/mL. A 1:100 dilution of the radiolabeled [^{3}H]ASDN stock in buffer was prepared fresh each day of assay. A solution (1 mg/mL) of ASDN in ethanol was prepared (fresh each day) and then diluted in buffer to a final concentration of 1 μg/mL. The substrate solution was prepared by combining 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. The addition of 100 μL of the substrate solution to each 2 mL assay volume yielded a final [^{3}H]ASDN concentration of 100 nM with ca. 0.1 μCi/tube.

3.2 Reference and Control Substances

Battelle's Chemical Repository (CR) was responsible for chemistry activities required to perform this study. Their responsibilities included chemical procurement, solubility, formulation stability assessment, formulation preparation, formulation analysis and shipment of stock formulation to the participating laboratories. These chemistry activities and results are described in the Sponsor's Chemistry reports which are appended to this document (Appendix E).

3.2.1 Reference Chemicals

The reference chemical formulations and vehicles were received from Battelle's CR by RTI's Materials Handling Facility (MHF). Each solution and vehicle was assigned an RTI log number. The reference chemical formulations were supplied to the laboratories only after information as to the identity of the chemical was removed from the labels and accompanying custody sheets. In the lab, the reference chemicals were assigned the codes Reference Chemical 1 (RC-1), Reference Chemical 2 (RC-2), etc.

Table 1. Reference Chemicals for Task 5

Reference Chemical	Chemical code	Mfr. Purity (%)	CAS No.	Molecular formula	Molecular weight	Stock Solution ID	Stock Concentration (M)	Vehicle	Storage Conditions
aminoglutethimide	RC 1	>99	125-84-8	$C_{13}H_{16}N_2O_2$	232.3	1-AG-1	0.1	DMSO	Refrigerate
chrysin	RC 2	98.2	480-40-0	$C_{15}H_{10}O_4$	254.2	1-CHRY-1	0.01	DMSO	Refrigerate
ketoconazole	RC 3	>99	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	531.43	1-KET-1	0.01	DMSO	Refrigerate
econazole	RC 4	98	24169-02-6	$C_{18}H_{15}Cl_3N_2O$-HNO_3	444.7	1-ECON-1	0.1	DMSO	Refrigerate

Dilutions of each reference chemical were prepared fresh each day for use in the assay. In each case, the same lot number of vehicle used to prepare the stock solution was used in the preparation of the dilutions. Suppliers and lot numbers for reference chemical vehicles are presented in Table 12 (Section 3.4). The dilution scheme described in Table 2 was used in the preparation of dilutions for the first replicates of aminoglutethimide and econazole (RC-1 and 4, respectively). The scheme described in Table 3 was used for the first replicate of chrysin (RC-2). The scheme described in Table 4 was used for the first replicate of ketoconazole (RC-3). Note that the range of concentrations tested for ketoconazole (replicate 1) was 1E-4 to 1E-11 M rather than the 1E-3 to 1E-10 M specified in the protocol. The concentration of the stock ketoconazole was thought to be 0.1M, when in fact, it was 0.01 M.

Table 2. Dilution Scheme for the First Replicate of Aminoglutethimide and Econazole (RC-1 and 4, respectively)

Dilution ID	μL vehicle	μL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	0	1000	1-AG-1 or 1-ECON-1	1.00E-01	1.00E-03
2	900	100	Diln. 1	1.00E-02	1.00E-04
3	900	100	Diln. 2	1.00E-03	1.00E-05
4	900	100	Diln. 3	1.00E-04	1.00E-06
5	900	100	Diln. 4	1.00E-05	1.00E-07
6	900	100	Diln. 5	1.00E-06	1.00E-08
7	900	100	Diln. 6	1.00E-07	1.00E-09
8	900	100	Diln. 7	1.00E-08	1.00E-10

Table 3. Dilution Scheme for the First Replicate of Chrysin (RC-2)

Dilution ID	µL vehicle	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	0	1000	1-CHRY-1	1.00E-02	1.00E-04
2	900	100	Diln. 1	1.00E-03	1.00E-05
3	900	100	Diln. 2	1.00E-04	1.00E-06
4	500	500	Diln. 3	5.00E-05	5.00E-07
5	900	100	Diln. 3	1.00E-05	1.00E-07
6	900	100	Diln. 5	1.00E-06	1.00E-08
7	900	100	Diln. 6	1.00E-07	1.00E-09
8	900	100	Diln. 7	1.00E-08	1.00E-10

Table 4. Dilution Scheme for the First Replicate of Ketoconazole (RC-3)

Dilution ID	µL vehicle	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	0	1000	1-KET-1	1.00E-02	1.00E-04
2	900	100	Diln. 1	1.00E-03	1.00E-05
3	900	100	Diln. 2	1.00E-04	1.00E-06
4	900	100	Diln. 3	1.00E-05	1.00E-07
5	900	100	Diln. 4	1.00E-06	1.00E-08
6	900	100	Diln. 5	1.00E-07	1.00E-09
7	900	100	Diln. 6	1.00E-08	1.00E-10
8	900	100	Diln. 7	1.00E-09	1.00E-11

The results of each replicate 1 were reviewed and adjustments were made (at the direction of the Study Director) to the concentrations tested in replicates 2 and 3 (and 4) of the assay in order to obtain data that better described the IC_{50} curve. These new dilution schemes are described in Tables 5, 6, 7 and 8 for RC-1, 2, 3, and 4, respectively.

Table 5. Dilution Scheme for the Replicates 2 and 3 of Aminoglutethimide (RC-1)

Dilution ID	µL vehicle	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	0	1000	1-AG-1	1.00E-01	1.00E-03
2	900	100	Diln. 1	1.00E-02	1.00E-04
3	900	100	Diln. 2	1.00E-03	1.00E-05
4	300	300	Diln. 3	5.00E-04	5.00E-06
5	300	100	Diln. 3	2.50E-04	2.50E-06
6	900	100	Diln. 3	1.00E-04	1.00E-06
7	900	100	Diln. 6	1.00E-05	1.00E-07
8	900	100	Diln. 7	1.00E-06	1.00E-08

Table 6. Dilution Scheme for the Replicates 2 and 3 of Chrysin (RC-2)

Dilution ID	µL vehicle	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	0	1000	1-CHRY-1	1.00E-02	1.00E-04
2	900	100	Diln. 1	1.00E-03	1.00E-05
3	300	300	Diln. 2	5.00E-04	5.00E-06
4	300	100	Diln. 2	2.50E-04	2.50E-06
5	900	100	Diln. 2	1.00E-04	1.00E-06
6	500	500	Diln. 5	5.00E-05	5.00E-07
7	900	100	Diln. 5	1.00E-05	1.00E-07
8	900	100	Diln. 7	1.00E-06	1.00E-08

Table 7 Dilution Scheme for the Replicates 2, 3 and 4 of Ketoconazole (RC-3)

Dilution ID	µL vehicle	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	0	1000	1-KET-1	1.00E-02	1.00E-04
2	300	300	Diln. 1	5.00E-03	5.00E-05
3	300	100	Diln. 1	2.50E-03	2.50E-05
4	900	100	Diln. 1	1.00E-03	1.00E-05
5	300	300	Diln. 4	5.00E-04	5.00E-06
6	900	100	Diln. 4	1.00E-04	1.00E-06
7	900	100	Diln. 6	1.00E-05	1.00E-07
8	900	100	Diln. 7	1.00E-06	1.00E-08

Table 8. Dilution Scheme for the Replicates 2, 3 and 4 of Econazole (RC-4)

Dilution ID	µL vehicle	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
Prediln 1[a]	900	100	1-ECON-1	1.00E-02	
Prediln 2[a]	900	100	Prediln 1	1.00E-03	
Prediln 3[a]	900	100	Prediln 2	1.00E-04	
1	900	100	Prediln 3	1.00E-05	1.00E-07
2	900	100	Diln. 1	1.00E-06	1.00E-08
3	300	300	Diln. 2	5.00E-07	5.00E-09
4	300	100	Diln. 2	2.50E-07	2.50E-09
5	900	100	Diln. 2	1.00E-07	1.00E-09
6	300	300	Diln. 5	5.00E-08	5.00E-10
7	300	100	Diln. 5	2.50E-08	2.50E-10
8	900	100	Diln. 5	1.00E-08	1.00E-10

[a]The predilutions were not used in the assay.

3.2.2 Control Chemicals

The known aromatase inhibitor, 4-hydroxyandrostenedione (4-OH ASDN), was used as a positive control and the known aromatase non-inhibitor, lindane, was used as a negative control. Stock solutions (Table 9) were supplied by Battelle's CR and were stored refrigerated. Dilutions were made fresh each day of use in the same vehicle and lot number that was used to prepare the stock solutions (see Table 12, Section 3.4 for details). Tables 10 and 11 describe the dilution scheme for 4-OH ASDN and lindane, respectively.

Table 9. Control Chemicals for Task 5

Test Substance	Mfr Purity	CAS Number	Molecular Formula	Molecular Weight (g/mol)	Stock Solution ID	Stock Formulation Concentration (M)	Vehicle	Storage Conditions
4-OH ASDN	99%	566-48-3	$C_{19}H_{26}O_3$	302.4	2-ASDN-1	0.01	95% ethanol	Refrigerate, Dark
Lindane	99.6%	58-89-9	$C_6H_6Cl_6$	290.8	1-LIN-1	0.1	DMSO	Refrigerate, Dark

Table 10. Dilution Scheme for 4-OH ASDN Control

Dilution ID	µL EtOH	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	9900	100	Stock	1.00E-04	-
2	9500	500	Diln. 1	5.00E-06	5.00E-08

Table 11. Dilution Scheme for Lindane Control

Dilution ID	µL DMSO	µL sample	Sample used	Solution concentration (M)	Final Concentration in assay (M)
1	9900	100	Stock	1.00E-03	-
2	900	100	Diln. 1	1.00E-04	1.00E-06

3.3 Microsomes

Human placental microsomes (Lot # 11343-7, 14 mg protein/mL, prepared at RTI) were used on this task. The microsomes were stored at approximately -70°C. Several vials of the stock microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized using a Potter Elvejhem homogenizer, divided into 160 µL aliquots, refrozen in liquid nitrogen and stored at approximately -70°C. Prior to assay, microsomes were thawed rapidly in a 37 ± 1°C water bath, rehomogenized by brief vortexing and then kept on ice until used. The microsome stock was diluted with buffer (1:50 and then 1:10, for a total 1:500 dilution) and maintained on ice until used. The time between thawing of the microsomes and their use in the assay was limited to less than 2 h and, in most cases was about 30 min.

3.4 Other Assay Components

In addition to substrate, reference or control chemicals or vehicle, and microsomes, the aromatase assay contains β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), propylene glycol and phosphate buffer. Supplier and lot numbers for other aromatase assay components are presented in Table 12.

Table 12. Supplier and Lot Numbers for Aromatase Assay Components

Chemical	Supplier	Lot Number
NADPH	Sigma	103K7046
Propylene glycol	J.T.Baker	Y18600
Sodium phosphate dibasic	J.T.Baker	A08H50
Sodium phosphate monobasic	J.T.Baker	A12H20, X43H15
Vehicle (DMSO) for Reference Chemicals 1 and 3 and Lindane (negative control chemical)	Battelle CR	2969A24437
Vehicle (DMSO) for Reference Chemicals 2 and 4	Battelle CR	2969Y30428
Vehicle (95% EtOH) for 4-OH ASDN (positive control chemical)	Battelle CR	04B1OUB

3.4.1 NADPH

A solution of NADPH (6 mM) in pH 7.4 sodium phosphate buffer (see Section 3.4.2) was prepared fresh each day of assay and was kept on ice until used.

3.4.2 Assay Buffer

The assay buffer was 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) were used in the preparation of the buffer. Solutions of each reagent at 0.1 M were prepared in distilled, deionized water and then the solutions were combined to a final pH of 7.4. The assay buffer was stored for up to one month in the refrigerator (2-8 °C).

3.5 Protein Determination

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

For the protein assays associated with RC-3 and -4, replicates 3 and 4, protein quality control (QC) standards were included in the assay. The QC standards were selected from a set of pre-diluted protein standards (Pierce, Product # 23208, Lot # GA93315); protein standards of 0.125, 0.500 and 1.00 mg/mL nominal concentrations were selected as the low, mid and high concentration QC standards, respectively. For the replicate 4 assays, an additional QC standard of 0.250 mg/mL (from the same set) was added. All QC standards were run in duplicate using the same protocol described above.

3.6 Cytochrome P450 Aromatase (CYP19) Activity

Three (or four) independent replicates were conducted (on separate days) for each reference chemical with all replicates for a given reference chemical conducted by the same technician. Each reference chemical was tested at eight concentrations and there were three (triplicate) repetitions for each concentration of a given replicate. A single replicate study of an example reference chemical is described in Table 13.

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

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

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

The assays were performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH (as applicable) and reference chemical (or control or vehicle), and buffer (0.1 M sodium phosphate buffer, pH 7.4) were combined in the test tubes (total volume 1 mL). The volume of the reference or control chemical solutions or vehicle used was 20 µL (1% of total assay volume). The final concentrations for the assay components are presented in Table 14. The tubes and the microsomal suspension were placed in the 37 ± 1°C 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 was 2 mL, and the tubes were incubated for 15 min. The incubations were stopped by the addition of methylene chloride

(2 mL); the tubes were vortex-mixed for ca. 5 s and placed on ice. The tubes were then vortex-mixed an additional 20-25 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 min at a setting of 1000 rpm to aid in the separation of the organic and aqueous phases. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2 mL). This extraction procedure was performed one additional time, 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. The radiochemical content of each aliquot was determined by using liquid scintillation spectrometry (LSS). Radiolabel found in the aqueous fractions represents ^3H_2O formed. The aromatization of one mole of [^{3}H]ASDN results in the production of one mole of estrone (non-radiolabeled) and one mole of tritiated water.

Table 13. Reference Chemical Study Design

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

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

Table 14. Optimized Aromatase Assay Conditions

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

[a] Final concentrations

3.7 Data Analysis

3.7.1 Aromatase Activity and Percent of Control Calculations

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

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

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

3.7.2 Statistical Analyses

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

$Y \equiv$ percent of control activity in the inhibitor tube

$X \equiv$ logarithm (base 10) of the concentration

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

$\beta \equiv$ slope of the concentration response curve (β will be negative)

$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to 50% of control activity).

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

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

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 was approximated by Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Model fits were carried out using Prism software (Version 3.02). Observed individual percent activity values above 100% were set to 99.5%. Observed individual percent activity values below 0% were set to 0.5%.

Concentration response models were fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate the extent of aromatase inhibition was summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for a reference chemical was a (weighted) geometric mean across the replicates, calculated as described in Appendix G. A mixed effects model was fit to the $\log_{10}(IC_{50})$ and slope estimates from the response curve model fits. The chemical type was assigned as the fixed effect and replicate was assigned as the random effect, with homogeneous covariance parameters within the chemicals. SAS PROC

MIXED was used to obtain estimates of the replicate-to-replicate variation within each chemical by entering the square of the standard errors from the response curve model parameter estimates as the variation estimates for the within replicate (within chemical) variance components. The replicate-to-replicate variation estimates for slope were constrained to be no lower than 1E-30 in order to allow the mixed effects model to converge and provide estimates of the variance components.

3.7.2.2 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits. For each replicate the individual percent of control values were plotted versus logarithm of the reference chemical concentration. The fitted concentration response curve was superimposed on the plot. Individual plots were prepared for each replicate.

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

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

3.7.2.3 Graphical and Analysis of Variance Comparisons of FEAC, BAC, and Positive and Negative Control Percent of Control Across Reference Chemicals and Replicates. The means of the FEAC control values within each replicate and chemical were calculated as the reference value for 100% of control for the replicate and chemical. The percent of control for each repetition within each replicate and chemical was calculated as the ratio of the repetition value divided by the corresponding 100% of control reference value. Graphs of the percent of control values indicating the repetition portion by replicate and chemical for BAC, FEAC, negative and positive controls were prepared. Graphs displaying the differences of the means of the beginning repetitions and the means of the end repetitions within each replicate and chemical were prepared. A mixed effects model was fit to the percent of control values for each control. The fixed effects were assigned as the chemical type, portion, and portion by chemical interaction. The random effects were assigned as replicated within reference chemical and portion by replicate interaction within chemical.

3.7.2.4 Statistical Software. Concentration response curves were fitted to the data using the non-linear regression analysis features in the PRISM statistical analysis package, Version 3.02. Supplemental statistical analyses and displays such as summary tables, graphical displays, analysis of variance, and multiple comparisons were carried out using the SAS statistical analysis system, Version 8.

4.0 RESULTS

4.1 Radiochemical Purity

The [^{3}H] ASDN was found to be 97% radiochemically pure by HPLC analysis. The radiochemical purity report appears in Appendix F.

4.2 Stock Formulation Analysis

The formulation stability and formulation analysis results for all four reference chemicals and the positive and negative control chemicals from the Battelle CR are included in the reports presented in Appendix E. Some of the formulation analysis data are summarized in Table 15.

Table 15. Formulation Analysis Data

Reference Chemical Code	Chemical ID	Stock Solution ID	Manufacturer's Purity (%)	Stock Solution Prep Date	Number of Days Known Stable[a]	Date of Last Use on WA 4-16, Task 5
RC-1	Aminoglutethimide	1-AG-1	>99	1/24/2005	59	2/14/2005
RC-2	Chrysin	1-CHRY-1	98.2	1/25/2005	100	2/14/2005
RC-3	Ketoconazole	1-KET-1	>99	2/4/2005	60	3/9/2005
RC-4	Econazole	1-ECON-1	98	2/7/2005	56	3/9/2005
N/A	Lindane	1-LIN-1	99.6	1/24/2005	168	3/9/2005
N/A	4- OH ASDN	2-ASDN-1	99	1/25/2005	173	3/9/2005

[a] Stability was assessed on formulations stored refrigerated in the dark.

4.3 Protein Analysis

Protein content of the human placental microsomes was measured each day of the aromatase assay as described in Section 3.5. The 1:50 dilution of the stock microsomes was used in the protein assay and then the protein concentration of the stock microsomes was calculated from the data. The measured protein concentration in the human placental microsomes for each day of use is presented in Table 17 (Section 4.4). The average measured protein content of the human placental microsome stock solution was 11.5 ± 2.08 mg/mL (18.1% CV), with a range of 8.95 to 17.0 mg/mL .

In order to better characterize the protein assay, QC standards were included on some runs (see Section 3.5). The QC results are presented in Table 16. The QC results indicate that the protein standard curve reliably correlated with protein concentrations between 0.25 and 1.00 mg/mL but that the correlation was poor at 0.125 mg/mL (percent difference from known of -29%). The average measured protein stock concentration was 11.5 mg/mL. This value was calculated based on the protein assay results obtained using a 1:50 dilution of the stock, so the average concentration of the protein dilution that was assayed was about 0.23 mg/mL (11.49/50). This concentration falls near the low end of the reliable range of the protein standard curve; this may have led to the wide day-to-day variance found in the protein concentration of the placental microsomes.

Table 16. Protein QC Table

Known Concentration (mg/mL)	Measured Concentration (mg/mL)		Mean	SD	SEM	% CV	% Difference from Known
	Assay Date:						
	2/24/2005	3/9/2005					
0.123	0.073	0.095					
	0.090	0.091					
Average	0.081	0.093	0.087	0.010	0.005	11.32	-29.15
0.252	N/A	0.296					
	N/A	0.283					
Average	N/A	0.289	0.289	0.009	0.006	3.13	14.82
0.502	0.524	0.514					
	0.546	0.569					
Average	0.535	0.541	0.538	0.025	0.012	4.59	7.19
1.014	1.01	1.03					
	0.992	1.01					
Average	1.00	1.02	1.01	0.016	0.008	1.55	-0.23

4.4 Aromatase Activity

Aromatase activity was measured in the presence of eight concentrations of each of four reference chemicals. Each assay set also included four types of controls (see Section 3.6). Three (or four) replicates were run of each reference chemical. Information regarding assay dates, technicians, protein concentration, substrate specific activity and tested reference chemical concentration ranges is presented in Table 17. Radioactivity remaining in each assay tube after extraction of the unreacted substrate represented product estrone. The aromatase activity was calculated by normalizing the radioactivity present in each tube by the amount of microsomal protein and the reaction time and has the units nmol/mg protein/min.

Table 17. Aromatase Assay Summary

Reference Chemical Code	Reference Chemical ID	Replicate	Assay Date	Technician	Protein Stock Concentration (measured) mg/mL	Substrate Soln SA µCi/µg ASDN	Reference Chemical Concentration Range (M)
RC-1	Aminoglutethimide	1	2/4/05	1	9.37	1.57	1.00E-03 to 1.00E-10
RC-1	Aminoglutethimide	2	2/8/05	1	11.9	1.35	1.00E-03 to 1.00E-08
RC-1	Aminoglutethimide	3	2/14/05	1	10.1	1.48	1.00E-03 to 1.00E-08
RC-2	Chrysin	1	2/4/05	2	8.95	1.63	1.00E-04 to 1.00E-10
RC-2	Chrysin	2	2/8/05	2	9.84	1.61	1.00E-04 to 1.00E-08
RC-2	Chrysin	3	2/14/05	2	12.4	1.51	1.00E-04 to 1.00E-08
RC-3	Ketoconazole	1	2/18/05	1	11.0	1.50	1.00E-04 to 1.00E-11
RC-3	Ketoconazole	2	2/22/05	1	12.3	1.36	1.00E-04 to 1.00E-08
RC-3	Ketoconazole	3	2/24/05	1	13.9	1.35	1.00E-04 to 1.00E-08
RC-3	Ketoconazole	4	3/9/05	1	11.7	0.718*	1.00E-04 to 1.00E-08
RC-4	Econazole	1	2/18/05	2	10.4	1.56	1.00E-03 to 1.00E-10
RC-4	Econazole	2	2/22/05	2	17.0	1.37	1.00E-07 to 1.00E-10
RC-4	Econazole	3	2/24/05	2	11.7	1.52	1.00E-07 to 1.00E-10
RC-4	Econazole	4	3/9/05	2	10.3	1.48	1.00E-07 to 1.00E-10

*Inadvertently used less of the [^{3}H]ASDN in the preparation of this substrate solution than was called for. There was still sufficient radioactivity in the samples for analysis.

4.4.1 Control Results

Each replicate set for each chemical included four types of controls, each run in quadruplicate. The control types were full aromatase activity, background activity, positive and negative controls. The positive control tubes contained the known aromatase inhibitor, 4-OH

ASDN, at a concentration of 5×10^{-8} M and the negative control tubes contained the known aromatase non-inhibitor, lindane, at a concentration of 1×10^{-6} M. The control tubes were divided so that two of each type were run at the beginning of the set and two were run at the end of the set. In general, there was about a 6 minute interval between the beginning and the end of each replicate set. The aromatase activity in the full aromatase activity controls represented 100% activity and since all aromatase activities were corrected for background, the background activity controls necessarily were set to 0%. The mean activities for each type of control (except background) for the beginning and end groups and the overall means, SD, SEM and CVs across replicates and across chemicals are presented in Table 18. Figure 1 contains graphical representations of the beginning and end percent of control values for each type of control for each reference chemical and replicate. The percent of control values for background controls are fairly consistent and close to 0% of control across the replicates and chemicals except for ketoconazole replicate 2 and econazole replicates 2 and 4. The percent of control values for full aromatase activity and negative controls are fairly consistent and close to 100% of control across the replicates and chemicals except for econazole replicate 4. The percent of control values of the positive control are fairly consistent and close to 45% of control except for ketoconazole replicate 2 and econazole replicate 4.

Table 18. Control Activities[a]

Reference Chemical	Replicate	Mean Beginning Control	Mean End Control	Overall Mean	SD	SEM	%CV	Overall (by chemical)			
								Mean	±SD	SEM	%CV
				Full Aromatase Activity Controls							
Aminoglutethimide	1	0.1358	0.1292	0.1325	0.0046	0.0023	3.50	0.1206	0.0190	0.0110	15.73
	2	0.1027	0.0947	0.0987	0.0047	0.0023	4.72				
	3	0.1352	0.1259	0.1305	0.0054	0.0027	4.13				
Chrysin	1	0.1104	0.1067	0.1085	0.0060	0.0030	5.48	0.1019	0.0181	0.0104	17.71
	2	0.1183	0.1132	0.1157	0.0034	0.0017	2.90				
	3	0.0835	0.0795	0.0815	0.0027	0.0013	3.26				
Ketoconazole	1	0.1237	0.1204	0.1221	0.0024	0.0012	1.96	0.1174	0.0129	0.0075	11.02
	2	0.1062	0.0993	0.1027	0.0046	0.0023	4.48				
	3*	0.1045	0.0978	0.1012	0.0041	0.0021	4.08				
	4	0.1320	0.1226	0.1273	0.0055	0.0028	4.34				
Econazole	1*	0.0764	0.0606	0.0685	0.0110	0.0055	16.07	0.0803	0.0146	0.0084	18.19
	2	0.0667	0.0605	0.0636	0.0038	0.0019	6.02				
	3	0.0936	0.0882	0.0909	0.0033	0.0017	3.66				
	4[b]	0.1059	0.0667	0.0863	0.0433	0.0216	50.14				
Overall								0.1050	0.0217	0.063	20.66

(continued)

Table 18. Control Activities (continued)

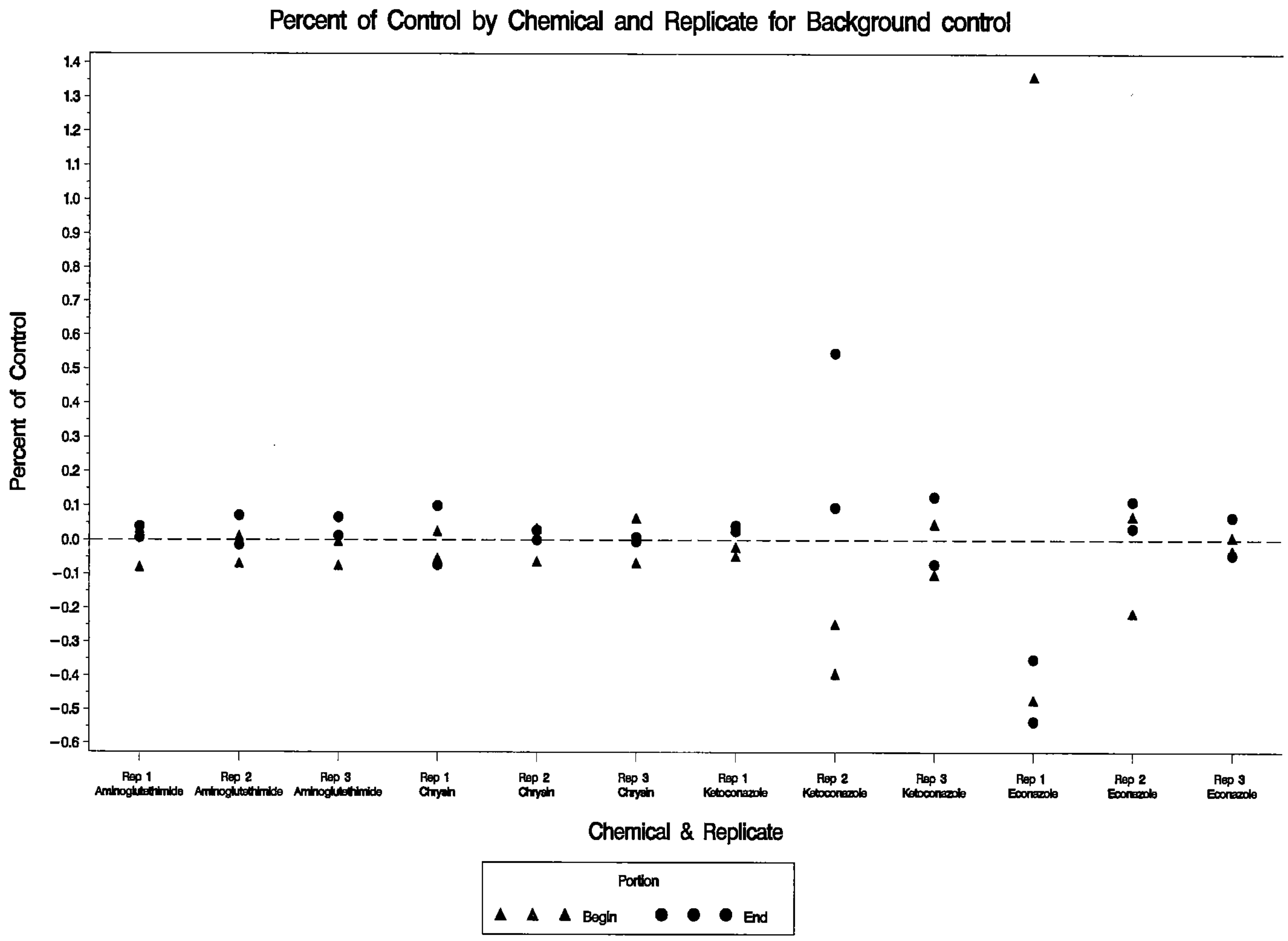
Reference Chemical	Replicate	Mean Beginning Control	Mean End Control	Overall Mean	SD	SEM	%CV	Overall (by chemical)			
								Mean	±SD	SEM	%CV
				Positive Controls							
aminoglutethimide	1	0.0566	0.0522	0.0544	0.0031	0.0016	5.73	0.0539	0.0069	0.0040	12.75
	2	0.0480	0.0455	0.0468	0.0016	0.0008	3.45				
	3	0.0638	0.0571	0.0605	0.0040	0.0020	6.60				
chrysin	1	0.0507	0.0564	0.0535	0.0034	0.0017	6.28	0.0485	0.0091	0.0052	18.68
	2	0.0549	0.0532	0.0540	0.0010	0.0005	1.86				
	3	0.0396	0.0365	0.0381	0.0019	0.0010	5.10				
ketoconazole	1	0.0579	0.0544	0.0561	0.0025	0.0013	4.46	0.0505	0.0092	0.0053	18.22
	2	0.0489	0.0309	0.0399	0.0151	0.0075	37.85				
	3*	0.0490	0.0447	0.0468	0.0030	0.0015	6.31				
	4	0.0576	0.0534	0.0555	0.0026	0.0013	4.60				
econazole	1*	0.0354	0.0307	0.0330	0.0027	0.0014	8.23	0.0429	0.0123	0.0071	28.62
	2	0.0317	0.0287	0.0302	0.0019	0.0009	6.22				
	3	0.0456	0.0420	0.0438	0.0041	0.0021	9.39				
	4	0.0574	0.0521	0.0547	0.0057	0.0028	10.34				
Overall								0.0490	0.0091	0.0026	18.68
				Negative Controls							
aminoglutethimide	1	0.1348	0.1261	0.1304	0.0054	0.0027	4.14	0.1175	0.0176	0.0101	14.95
	2	0.1007	0.0943	0.0975	0.0039	0.0020	4.03				
	3	0.1304	0.1187	0.1245	0.0072	0.0036	5.80				
chrysin	1	0.1061	0.1050	0.1055	0.0034	0.0017	3.18	0.0996	0.0191	0.0110	19.17
	2	0.1200	0.1101	0.1150	0.0072	0.0036	6.24				
	3	0.0794	0.0770	0.0782	0.0014	0.0007	1.77				
ketoconazole	1	0.1247	0.1164	0.1205	0.0052	0.0026	4.32	0.1150	0.0124	0.0071	10.76
	2	0.1057	0.0960	0.1008	0.0056	0.0028	5.56				
	3*	0.1024	0.0943	0.0984	0.0050	0.0025	5.11				
	4	0.1278	0.1195	0.1237	0.0053	0.0027	4.30				
econazole	1*	0.0756	0.0595	0.0675	0.0095	0.0048	14.09	0.0650	0.0235	0.0135	36.10
	2	0.0648	0.0628	0.0638	0.0015	0.0007	2.33				
	3	0.0916	0.0864	0.0890	0.0030	0.0015	3.36				
	4	0.0445	0.0397	0.0421	0.0028	0.0014	6.73				
Overall								0.0993	0.0270	0.0078	27.20

[a] Units are nmol/mg protein/min

* Data from this replicate was excluded from calculation of overall mean, SD, SEM and %CV.

[b] The aromatase activities for the 4 tubes in this set were: 0.1016, 0.1103, 0.1116 and 0.0217. When the last value is excluded from the calculations the mean activity (over that replicate) is 0.1078 ± 0.0054, SEM 0.0031 and 5.05 %CV.

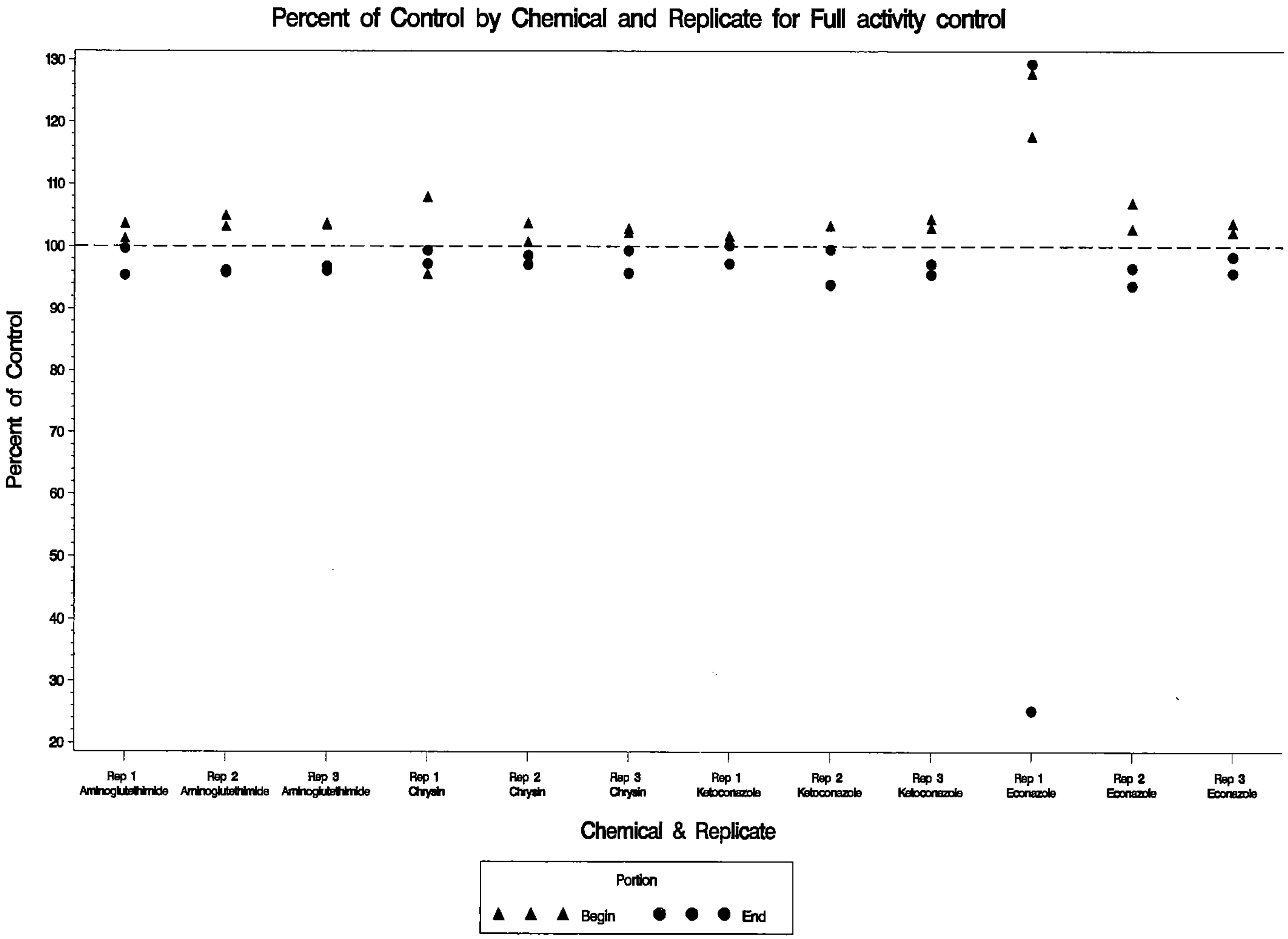
There was a consistent and statistically significant decrease in activity of about 5-10% for all controls (except background) between the beginning and end control tubes. This difference in beginning and end control activities is presented graphically in the statisticians report in Appendix G. The coefficient of variance for the full aromatase activity and positive controls across all chemicals and replicates was approximately 20%, while that for the negative controls was slightly higher at 27%. There was a larger variance in all controls for the econazole sets and the reason for this is not clear. It does not appear to be technician related since the same technician ran the chrysin and econazole assays. The large variance in negative control activities from replicate to replicate within econazole likely contributed to the finding of statistically significant differences between replicates within a reference chemical for that control type.



RTI Project 08055.003.032: WA 4−16 Task 5, control_analysis.sas (11MAY05)

Figure 1. Graphs of Percent of Control Data by Chemical and Replicate

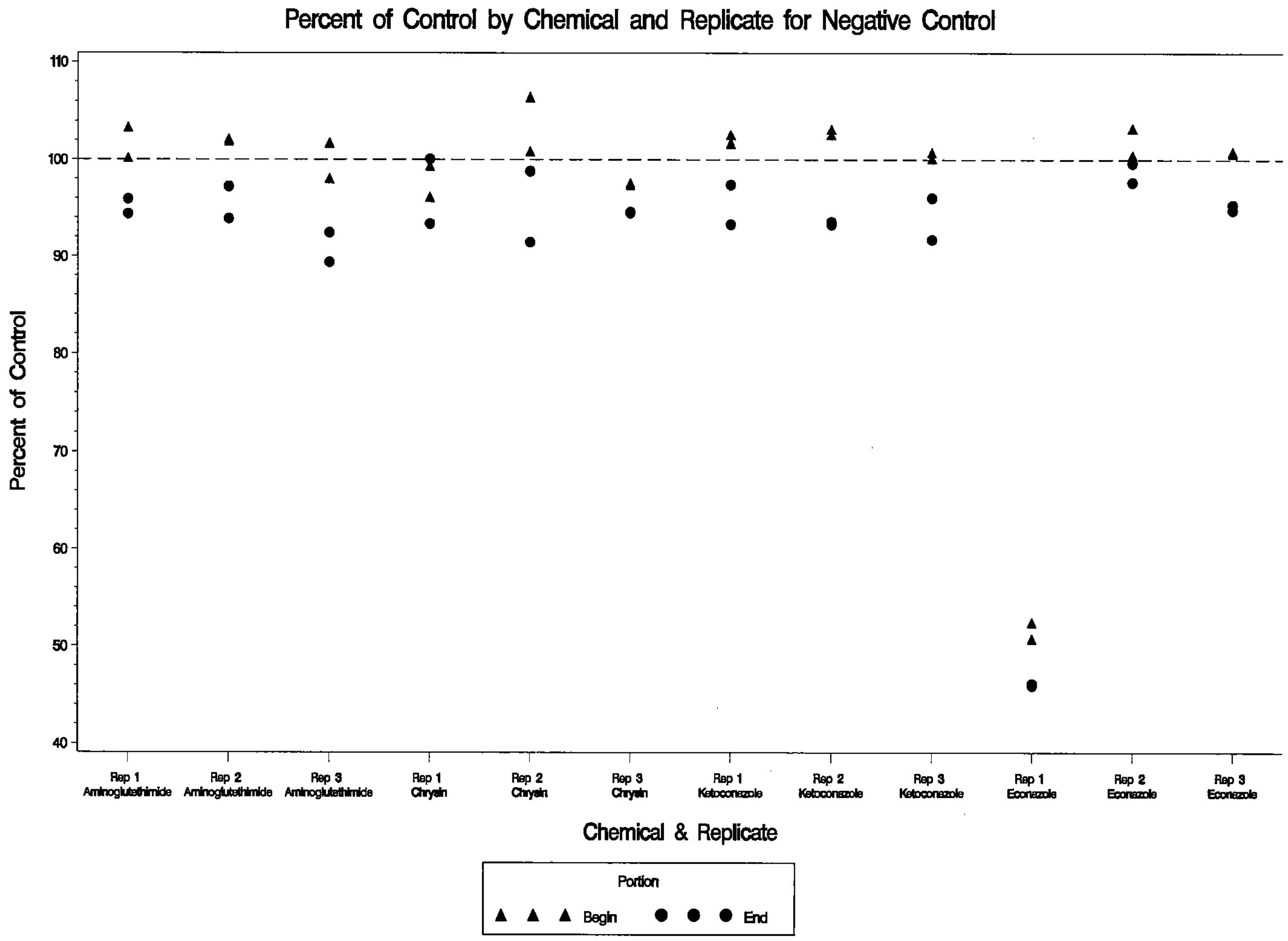
Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Figure 1 (continued). Graphs of Percent of Control Data by Chemical and Replicate

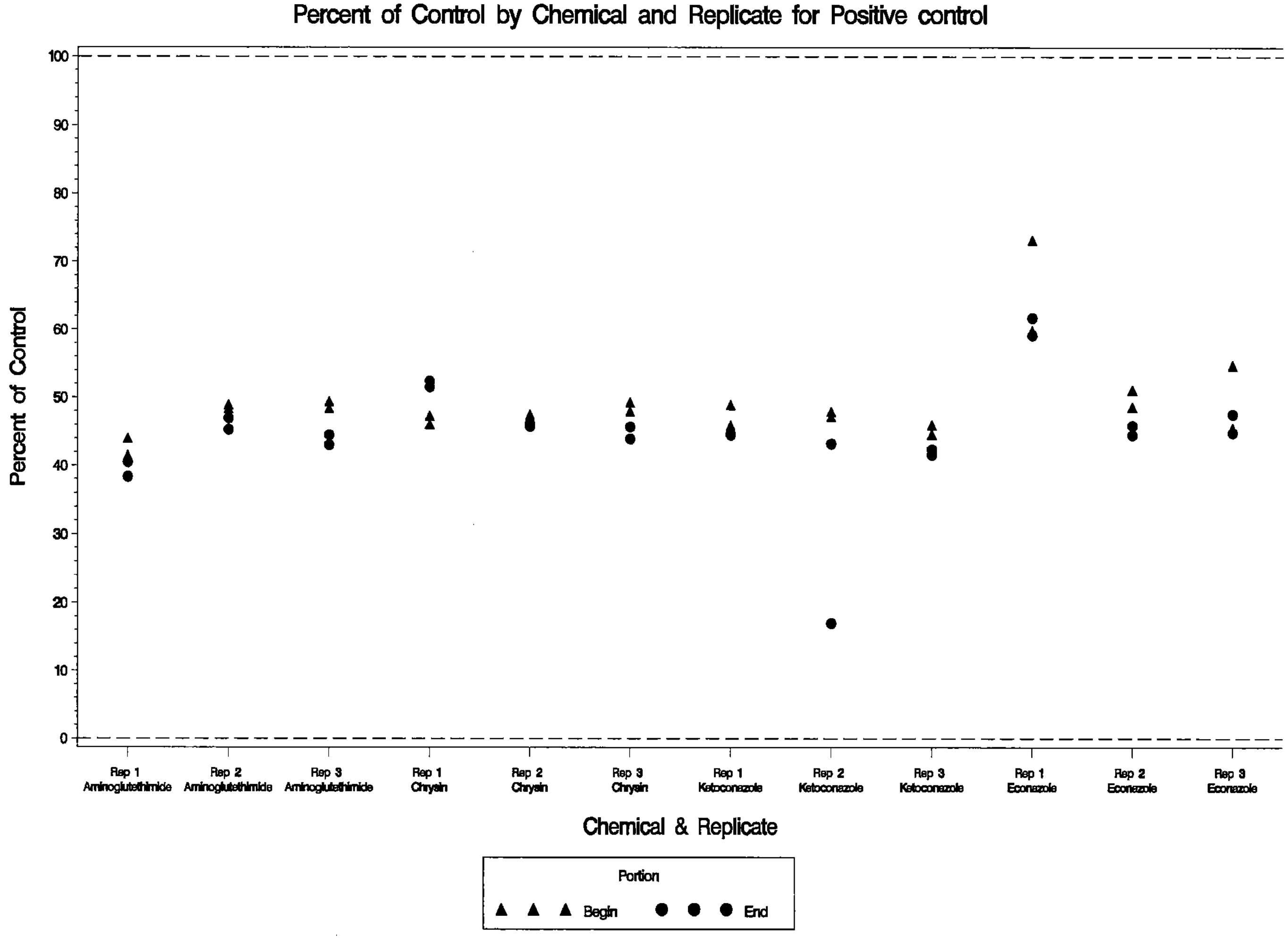
Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Figure 1 (continued). Graphs of Percent of Control Data by Chemical and Replicate

Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



RTI Project 08055.003.032: WA 4–16 Task 5, control_analysis.sas (11MAY05)

Figure 1 (continued). Graphs of Percent of Control Data by Chemical and Replicate

Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole

4.4.2 Percent of Control Values

The aromatase activity found in each assay tube was normalized to percent of control by dividing by the average full enzyme activity for the replicate. The percent of control values for each reference chemical replicate and tube along with the mean, SD, SEM and %CV of the percent of control across tubes within a replicate are presented in Table 19. Note that in replicate 3 of ketoconazole the percent of control values found for log[ketoconazole] of -4.6 and -5.0 were well out of range of the values found at those levels in the other replicates. It appears that there was an error in the preparation of these tubes, so the data from this replicate was excluded from further analysis and a fourth replicate was conducted. In the case of replicate 1 of econazole, the percent of control values ranged only from -0.06 to 24.99%, which is in contrast with the nearly 0-100% range found with the other replicates, so a fourth replicate of econazole was conducted and replicate 1 was excluded from further analysis.

Table 19. Percent of Control Values

Reference Chemical (RC)	Replicate	Log[RC] (M)	Percent of Control			Mean	±SD	SEM	%CV
			Tube 1	Tube 2	Tube 3				
Aminoglutethimide	1	-3.00	0.37	0.32	0.40	0.36	0.04	0.02	11.30
		-4.00	4.40	4.30	4.35	4.35	0.05	0.03	1.11
		-5.00	31.04	29.56	29.59	30.06	0.85	0.49	2.82
		-6.00	79.26	79.73	77.34	78.78	1.26	0.73	1.61
		-7.00	99.00	95.62	93.44	96.02	2.80	1.62	2.92
		-8.00	100.23	98.99	98.57	99.26	0.86	0.50	0.87
		-9.00	98.74	98.04	98.35	98.38	0.35	0.20	0.36
		-10.00	100.25	94.61	94.63	96.49	3.25	1.88	3.37
	2	-3.00	0.43	0.37	0.26	0.35	0.09	0.05	24.93
		-4.00	4.50	4.11	4.17	4.26	0.21	0.12	4.98
		-5.00	32.48	31.69	31.54	31.91	0.50	0.29	1.58
		-5.30	47.57	47.07	47.71	47.45	0.33	0.19	0.70
		-5.60	63.11	61.59	62.17	62.29	0.77	0.44	1.24
		-6.00	70.10	78.46	77.30	75.28	4.53	2.61	6.02
		-7.00	97.82	93.55	91.72	94.36	3.13	1.81	3.32
		-8.00	102.40	95.32	96.37	98.03	3.82	2.20	3.90
	3	-3.00	0.33	0.32	0.39	0.35	0.04	0.02	10.89
		-4.00	4.56	4.58	4.44	4.53	0.08	0.04	1.66
		-5.00	32.38	30.63	29.40	30.80	1.50	0.87	4.87
		-5.30	47.02	48.65	48.36	48.01	0.87	0.50	1.82
		-5.60	65.17	64.06	62.97	64.07	1.10	0.64	1.72
		-6.00	82.04	80.44	78.11	80.20	1.97	1.14	2.46
		-7.00	98.13	93.71	93.57	95.13	2.59	1.50	2.73
		-8.00	99.23	97.41	92.88	96.51	3.27	1.89	3.39

(continued)

Table 19. Percent of Control Values (continued)

Reference Chemical (RC)	Replicate	Log[RC] (M)	Percent of Control			Mean	±SD	SEM	%CV
			Tube 1	Tube 2	Tube 3				
Chrysin	1	-4.00	19.84	23.36	22.98	22.06	1.93	1.11	8.75
		-5.00	26.76	25.06	26.33	26.05	0.89	0.51	3.40
		-6.00	74.91	77.16	76.22	76.10	1.13	0.65	1.49
		-6.30	84.74	84.18	83.83	84.25	0.46	0.27	0.55
		-7.00	95.65	92.64	91.01	93.10	2.35	1.36	2.53
		-8.00	96.12	96.16	95.69	95.99	0.26	0.15	0.28
		-9.00	96.20	93.24	91.45	93.63	2.40	1.38	2.56
		-10.00	94.89	96.98	96.99	96.29	1.21	0.70	1.26
	2	-4.00	26.04	28.01	24.64	26.23	1.69	0.98	6.45
		-5.00	30.43	32.12	30.28	30.94	1.02	0.59	3.31
		-5.30	46.05	45.97	47.26	46.43	0.72	0.42	1.56
		-5.60	64.90	62.92	66.35	64.72	1.72	0.99	2.66
		-6.00	82.81	82.12	81.21	82.05	0.80	0.46	0.98
		-6.30	89.61	89.00	87.64	88.75	1.01	0.58	1.14
		-7.00	96.96	98.07	96.11	97.05	0.98	0.57	1.01
		-8.00	97.87	96.52	96.46	96.95	0.80	0.46	0.82
	3	-4.00	19.85	24.03	24.10	22.66	2.43	1.41	10.74
		-5.00	28.01	26.86	27.97	27.62	0.65	0.38	2.36
		-5.30	43.45	44.08	44.36	43.96	0.46	0.27	1.06
		-5.60	59.62	60.02	59.91	59.85	0.20	0.12	0.34
		-6.00	76.57	75.00	77.81	76.46	1.41	0.81	1.84
		-6.30	83.53	86.83	83.76	84.71	1.84	1.06	2.18
		-7.00	97.42	94.38	95.90	95.90	1.52	0.88	1.58
		-8.00	94.47	98.00	98.68	97.05	2.26	1.30	2.33
Ketoconazole	1	-4.00	6.12	6.25	5.87	6.08	0.20	0.11	3.23
		-5.00	42.09	43.00	42.97	42.69	0.52	0.30	1.22
		-6.00	87.74	89.29	87.43	88.16	1.00	0.58	1.13
		-7.00	100.39	102.24	98.44	100.36	1.90	1.10	1.90
		-8.00	102.63	100.19	98.70	100.51	1.99	1.15	1.98
		-9.00	101.07	98.61	99.67	99.78	1.24	0.71	1.24
		-10.00	100.71	101.95	99.38	100.68	1.28	0.74	1.28
		-11.00	100.59	100.02	93.80	98.13	3.77	2.17	3.84
	2	-4.00	6.17	5.96	5.74	5.96	0.22	0.13	3.64
		-4.30	12.39	12.39	11.72	12.17	0.38	0.22	3.16
		-4.60	23.14	22.55	21.96	22.55	0.59	0.34	2.62
		-5.00	43.25	42.12	38.94	41.43	2.23	1.29	5.39
		-5.30	58.02	57.61	55.72	57.12	1.23	0.71	2.15
		-6.00	86.67	86.99	83.79	85.82	1.76	1.02	2.05
		-7.00	99.49	97.33	93.49	96.77	3.04	1.75	3.14
		-8.00	99.66	101.12	91.05	97.28	5.44	3.14	5.60
	3*	-4.00	5.91	6.61	6.56	6.36	0.39	0.22	6.08
		-4.30	13.08	13.09	12.52	12.90	0.32	0.19	2.51
		-4.60	111.90	107.50	107.20	108.87	2.63	1.52	2.42
		-5.00	17.41	17.54	17.10	17.35	0.23	0.13	1.31
		-5.30	59.47	58.87	57.18	58.50	1.19	0.69	2.03
		-6.00	88.97	85.24	86.10	86.77	1.95	1.13	2.25
		-7.00	101.19	96.92	91.70	96.61	4.75	2.74	4.92
		-8.00	100.84	96.11	88.20	95.05	6.38	3.69	6.72

(continued)

Table 19. Percent of Control Values (continued)

Reference Chemical (RC)	Replicate	Log[RC] (M)	Percent of Control			Mean	±SD	SEM	%CV
			Tube 1	Tube 2	Tube 3				
Ketoconazole	4	-4.00	6.23	6.07	6.05	6.12	0.10	0.06	1.64
		-4.30	14.10	13.11	13.72	13.64	0.50	0.29	3.68
		-4.60	23.41	23.22	22.46	23.03	0.51	0.29	2.20
		-5.00	43.33	43.07	40.58	42.33	1.52	0.88	3.59
		-5.30	60.16	57.95	58.04	58.72	1.25	0.72	2.13
		-6.00	87.14	86.09	83.98	85.74	1.61	0.93	1.88
		-7.00	100.18	98.50	94.79	97.82	2.76	1.59	2.82
		-8.00	100.74	100.12	94.96	98.61	3.17	1.83	3.22
Econazole	1*	-3.00	-0.10	-0.07	-0.02	-0.06	0.04	0.02	-59.14
		-4.00	0.16	0.00	-0.08	0.02	0.12	0.07	510.69
		-5.00	-0.04	0.04	0.02	0.01	0.04	0.03	567.10
		-6.00	0.27	0.22	0.30	0.26	0.04	0.02	14.52
		-7.00	1.58	1.26	1.36	1.40	0.16	0.09	11.68
		-8.00	3.51	1.68	4.22	3.14	1.31	0.76	41.79
		-9.00	12.64	9.85	20.55	14.35	5.55	3.20	38.68
		-10.00	22.74	24.56	27.68	24.99	2.50	1.44	10.00
	2	-7.00	1.88	1.90	1.92	1.90	0.02	0.01	1.12
		-8.00	16.00	15.71	17.20	16.31	0.79	0.46	4.84
		-8.30	29.70	29.90	27.90	29.17	1.10	0.64	3.78
		-8.60	44.95	45.02	44.99	44.98	0.04	0.02	0.08
		-9.00	70.20	70.29	67.35	69.28	1.67	0.97	2.41
		-9.30	84.80	83.45	83.47	83.91	0.78	0.45	0.92
		-9.60	90.45	86.88	85.10	87.48	2.73	1.57	3.12
		-10.00	93.68	95.26	94.05	94.33	0.83	0.48	0.88
	3	-7.00	1.82	1.72	1.65	1.73	0.08	0.05	4.90
		-8.00	15.08	15.19	15.01	15.09	0.09	0.05	0.61
		-8.30	26.49	25.83	26.33	26.22	0.34	0.20	1.31
		-8.60	42.69	42.21	41.30	42.07	0.70	0.41	1.67
		-9.00	68.91	66.61	65.29	66.94	1.83	1.06	2.74
		-9.30	78.55	77.08	79.22	78.28	1.09	0.63	1.40
		-9.60	90.82	87.16	90.04	89.34	1.93	1.11	2.16
		-10.00	90.92	91.66	95.51	92.70	2.46	1.42	2.66
	4	-7.00	2.43	2.07	2.37	2.29	0.19	0.11	8.45
		-8.00	23.81	22.68	23.52	23.34	0.58	0.34	2.50
		-8.30	42.02	21.56	27.16	30.25	10.57	6.10	34.96
		-8.60	63.76	62.14	33.97	53.29	16.75	9.67	31.43
		-9.00	89.95	86.79	58.91	78.55	17.08	9.86	21.74
		-9.30	106.25	102.45	105.75	104.82	2.06	1.19	1.97
		-9.60	115.48	104.27	68.88	96.21	24.32	14.04	25.28
		-10.00	108.59	125.37	119.59	117.85	8.53	4.92	7.23

*Data from these replicates was not used in the calculation of IC_{50} results.

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

The mean percent of control values across tubes for each replicate and the overall mean, SD, SEM and %CV across replicates are presented in Table 20. The %CV at each tested concentration for aminoglutethimide, chrysin, and ketoconazole is less than 10%. The %CV found for econazole ranged from 5.05 to 24.4%; while there was little variance between replicates 2 and 3, the data from replicate 4 varied somewhat from that of the other two replicates.

Table 20. Mean Percent of Control per Replicate and Percent of Control across Replicates

Reference Chemical (RC)	Log[RC] (M)	Mean Percent of Control				Overall			
		Rep 1	Rep 2	Rep 3	Rep 4	Mean	±SD	SEM	%CV
Aminogluthethimide	-3.00	0.36	0.35	0.35	[a]	0.35	0.01	0.01	2.51
	-4.00	4.35	4.26	4.53		4.38	0.14	0.08	3.14
	-5.00	30.06	31.91	30.80		30.92	0.93	0.54	3.00
	-5.30	NA	47.45	48.01		47.73	0.40	0.28	0.83
	-5.60	NA	62.29	64.07		63.18	1.26	0.89	1.99
	-6.00	78.78	75.28	80.20		78.09	2.53	1.46	3.24
	-7.00	96.02	94.36	95.13		95.17	0.83	0.48	0.87
	-8.00	99.26	98.03	96.51		97.93	1.38	0.80	1.41
	-9.00	98.38	NA	NA		NC	NC	NC	NC
	-10.00	96.49	NA	NA		NC	NC	NC	NC
Chrysin	-4.00	22.06	26.23	22.66	[a]	23.65	2.25	1.30	9.53
	-5.00	26.05	30.94	27.62		28.20	2.50	1.44	8.86
	-5.30	NA	46.43	43.96		45.20	1.74	1.23	3.85
	-5.60	NA	64.72	59.85		62.29	3.45	2.44	5.53
	-6.00	76.10	82.05	76.46		78.20	3.34	1.93	4.27
	-6.30	84.25	88.75	84.71		85.90	2.48	1.43	2.88
	-7.00	93.10	97.05	95.90		95.35	2.03	1.17	2.13
	-8.00	95.99	96.95	97.05		96.66	0.58	0.34	0.60
	-9.00	93.63	NA	NA		NC	NC	NC	NC
	-10.00	96.29	NA	NA		NC	NC	NC	NC
Ketoconazole	-4.00	6.08	5.96	6.36[b]	6.12	6.05	0.08	0.05	1.40
	-4.30	NA	12.17	12.90	13.64	12.90	1.04	0.74	8.09
	-4.60	NA	22.55	108.87	23.03	22.79	0.34	0.24	1.48
	-5.00	42.69	41.43	17.35	42.33	42.15	0.64	0.37	1.53
	-5.30	NA	57.12	58.50	58.72	57.92	1.13	0.80	1.95
	-6.00	88.16	85.82	86.77	85.74	86.57	1.37	0.79	1.59
	-7.00	100.36	96.77	96.61	97.82	98.32	1.84	1.06	1.88
	-8.00	100.51	97.28	95.05	98.61	98.80	1.62	0.94	1.64
	-9.00	99.78	NA	NA	NA	NC	NC	NC	NC
	-10.00	100.68	NA	NA	NA	NC	NC	NC	NC
	-11.00	98.13	NA	NA	NA	NC	NC	NC	NC
Econazole	-3.00	-0.06[c]	NA	NA	NA	NC	NC	NC	NC
	-4.00	0.02	NA	NA	NA	NC	NC	NC	NC
	-5.00	0.01	NA	NA	NA	NC	NC	NC	NC
	-6.00	0.26	NA	NA	NA	NC	NC	NC	NC
	-7.00	1.40	1.90	1.73	2.29	1.97	0.29	0.17	14.69
	-8.00	3.14	16.31	15.09	23.34	18.24	4.45	2.57	24.40
	-8.30	NA	29.17	26.22	30.25	28.54	2.09	1.20	7.31
	-8.60	NA	44.98	42.07	53.29	46.78	5.82	3.36	12.45
	-9.00	14.35	69.28	66.94	78.55	71.59	6.14	3.54	8.58
	-9.30	NA	83.91	78.28	104.82	89.00	13.98	8.07	15.71
	-9.60	NA	87.48	89.34	96.21	91.01	4.60	2.66	5.05
	-10.00	24.99	94.33	92.70	117.85	101.63	14.08	8.13	13.85

NA Not applicable – this test concentration was not assayed in this replicate

NC Not calculated – only one data point – no mean, SD, SEM or CV calculated.

[a] There was no replicate 4 for aminoglutethimide or chrysin.

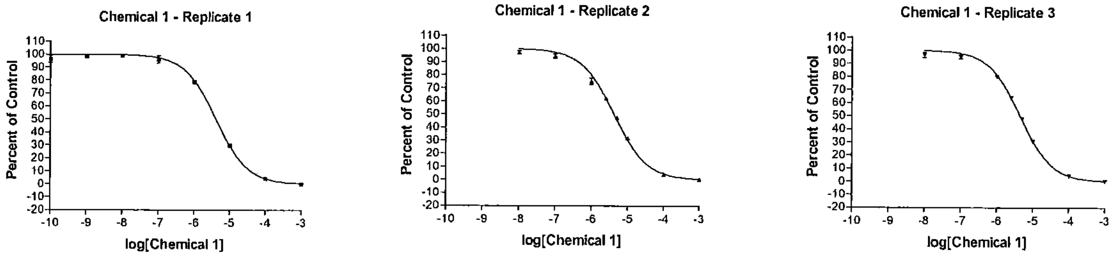
[b] Data from replicate 3 of ketoconazole are excluded from the calculation of mean, SD, SEM and %CV

[c] Data from replicate 1 of econazole are excluded from the calculation of mean, SD, SEM and %CV

4.4.3 IC_{50} Values

For each reference chemical and replicate the percent of control values were fitted to the equation presented in Section 3.7.2.1 using Prism 3.02 and the IC_{50} and slope were calculated. The concentration response curves for aminoglutethimide, chrysin, ketoconazole, and econazole are presented in Figures 2-5, respectively.

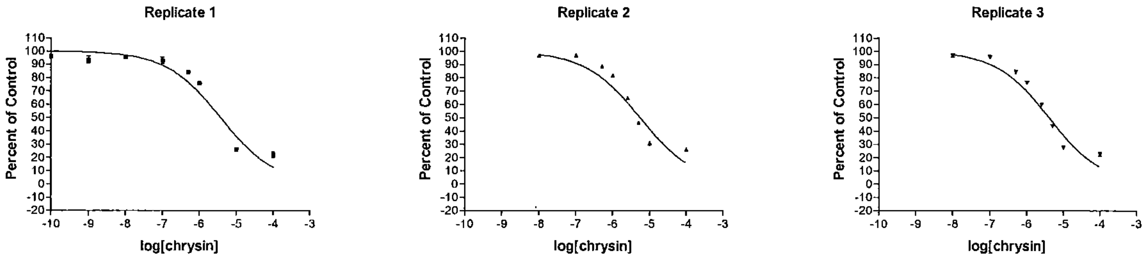
For aminoglutethimide, ketoconazole, and econazole, the percent of control values for the tested concentrations essentially span the range 0-100%. The model fits the data well for each of these reference chemicals. In the case of chrysin, however, where the percent of control data for the tested concentrations ranges from approximately 22-100%, the model does not fit the data as well. The poor fit arises due to the constraint inherent in the equation for the top and bottom plateaus to be 100 and 0%, respectively. In the current case, with the model constrained so that the top and bottom plateaus are 100 and 0%, PRISM estimates the $\log IC_{50}$ value as the log concentration corresponding to 50% activity, even when the data do not fully span the 0-100 % range.



	Replicate 1	Replicate 2	Replicate 3
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-5.389	-5.374	-5.351
HILLSLOPE	-0.9702	-0.9578	-0.9904
EC50	4.0860e-006	4.2250e-006	4.4600e-006
Std. Error			
LOGEC50	0.01560	0.01590	0.01073
HILLSLOPE	0.01865	0.02456	0.01854
95% Confidence Intervals			
LOGEC50	-5.421 to -5.356	-5.407 to -5.341	-5.373 to -5.328
HILLSLOPE	-1.009 to -0.9315	-1.009 to -0.9069	-1.029 to -0.9520
EC50	3.7930e-006 to 4.4020e-006	3.9160e-006 to 4.5580e-006	4.2370e-006 to 4.6940e-006
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9976	0.9934	0.9964
Weighted Sum of Squares (1/Y)	1.206	3.153	1.516
Absolute Sum of Squares	96.52	196.4	110.7
Sy.x	2.095	2.988	2.243
Data			
Number of X values	10	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	6	0	0

Figure 2. Fitted Concentration Response Curves for Aminoglutethimide[a]

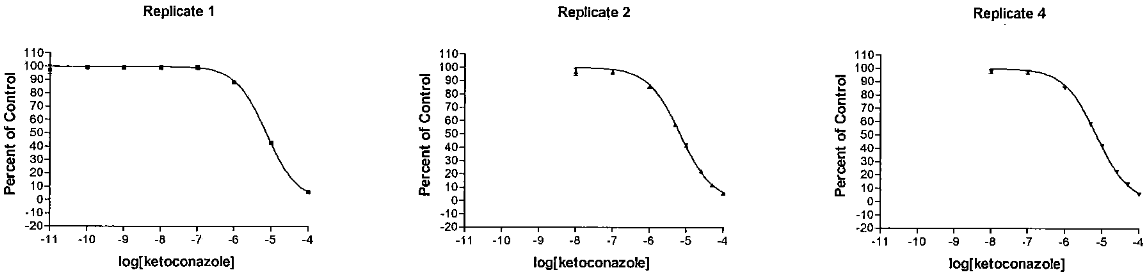
[a]Units are as follows: Bottom and Top: percent; Log(EC50) and EC50: M. EC50 = IC_{50}.



	Replicate 1	Replicate 2	Replicate 3
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-5.427	-5.260	-5.388
HILLSLOPE	-0.5892	-0.5774	-0.5976
EC50	3.7450e-006	5.4980e-006	4.0890e-006
Std. Error			
LOGEC50	0.08528	0.07054	0.06225
HILLSLOPE	0.06366	0.06615	0.06115
95% Confidence Intervals			
LOGEC50	-5.603 to -5.250	-5.406 to -5.114	-5.517 to -5.259
HILLSLOPE	-0.7212 to -0.4571	-0.7146 to -0.4402	-0.7244 to -0.4708
EC50	2.4920e-006 to 5.6270e-006	3.9250e-006 to 7.7000e-006	3.0370e-006 to 5.5040e-006
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9454	0.9213	0.9456
Weighted Sum of Squares (1/Y)	30.11	32.62	27.98
Absolute Sum of Squares	1121	1392	1005
Sy.x	7.138	7.953	6.759
Data			
Number of X values	10	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	6	0	0

Figure 3. Fitted Concentration Response Curves for Chrysin[a]

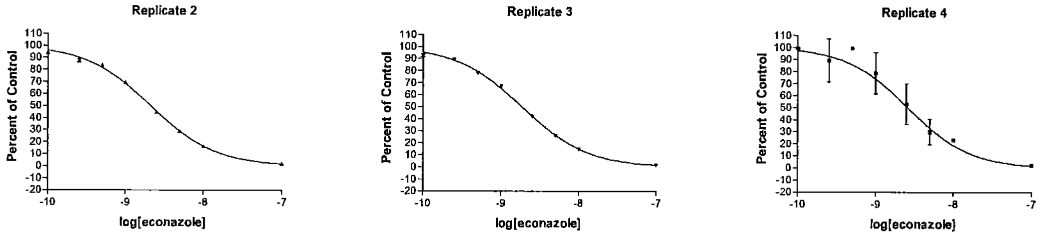
[a]Units are as follows: Bottom and Top: percent; Log(EC50) and EC50: M. EC50 = IC_{50}.



	Replicate 1	Replicate 2	Replicate 4
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-5.128	-5.164	-5.143
HILLSLOPE	-1.047	-0.9929	-0.9865
EC50	7.4400e-006	6.8530e-006	7.1990e-006
Std. Error			
LOGEC50	0.01013	0.01360	0.01103
HILLSLOPE	0.01686	0.02162	0.01757
95% Confidence Intervals			
LOGEC50	-5.149 to -5.107	-5.192 to -5.136	-5.166 to -5.120
HILLSLOPE	-1.082 to -1.012	-1.038 to -0.9481	-1.023 to -0.9501
EC50	7.0890e-006 to 7.8090e-006	6.4230e-006 to 7.3130e-006	6.8290e-006 to 7.5880e-006
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9981	0.9954	0.9977
Weighted Sum of Squares (1/Y)	0.5664	1.988	1.316
Absolute Sum of Squares	50.44	136.8	67.62
Sy.x	1.514	2.494	1.753
Data			
Number of X values	11	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	9	0	0

Figure 4. Fitted Concentration Response Curves for Ketoconazole[a]

[a] Units are as follows: Bottom and Top: percent; Log(EC50) and EC50: M. EC50 = IC_{50}.



	Replicate 4	Replicate 2	Replicate 3
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-8.584	-8.676	-8.732
HILLSLOPE	-1.081	-1.037	-1.023
EC50	2.6090e-009	2.1090e-009	1.8520e-009
Std. Error			
LOGEC50	0.05389	0.008105	0.007136
HILLSLOPE	0.1224	0.01570	0.01321
95% Confidence Intervals			
LOGEC50	-8.695 to -8.472	-8.693 to -8.659	-8.747 to -8.718
HILLSLOPE	-1.334 to -0.8267	-1.069 to -1.004	-1.050 to -0.9951
EC50	2.0170e-009 to 3.3740e-009	2.0290e-009 to 2.1920e-009	1.7900e-009 to 1.9160e-009
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9123	0.9972	0.9976
Weighted Sum of Squares (1/Y)	48.83	1.038	0.7921
Absolute Sum of Squares	2788	73.93	62.03
Sy.x	11.26	1.833	1.679
Data			
Number of X values	8	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	0	0	0

Figure 5. Fitted Concentration Response Curves for Econazole[a]

[a]Units are as follows: Bottom and Top: percent; Log(EC50) and EC50: M. EC50 = IC_{50}.

Figure 6 shows the concentration response curve fits of each replicate per reference chemical superimposed on one plot. It is clear that the replicate to replicate variation in curve fit is low, especially for aminoglutethimide and ketoconazole. There is also little variance in the chrysin data and curves from replicate to replicate. As mentioned before, the curve fit is poor for chrysin since the data do not span the entire 0-100% range. There is a higher degree of replicate to replicate variance for econazole, most of which is associated with replicate 4 in which large tube to tube variation was noted.

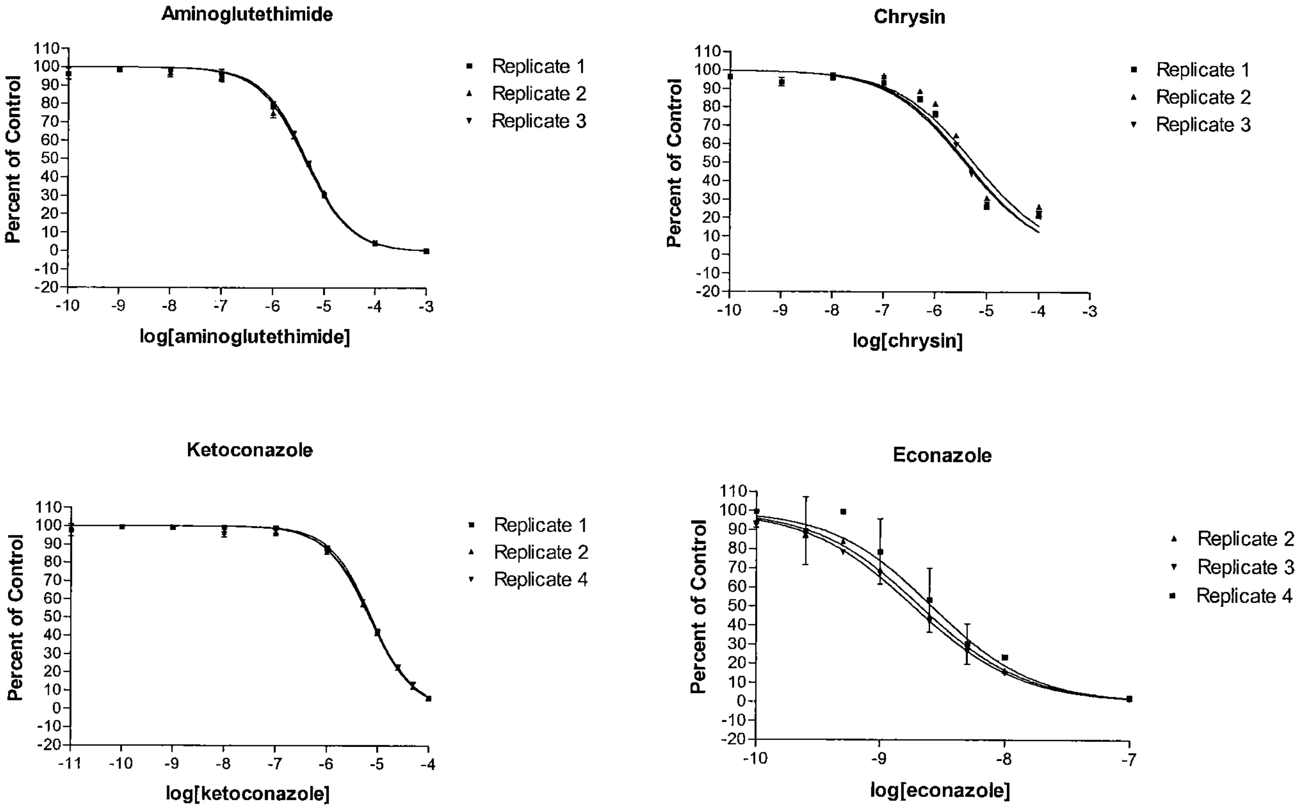


Figure 6. Concentration Response Curve Fits – Overlay of Individual Replicate Results

Figure 7 presents the results of curve fitting across the replicates for each reference chemical. The mean percent of control data for the repetitions within a replicate (as presented in Table 20) were fit to the model.

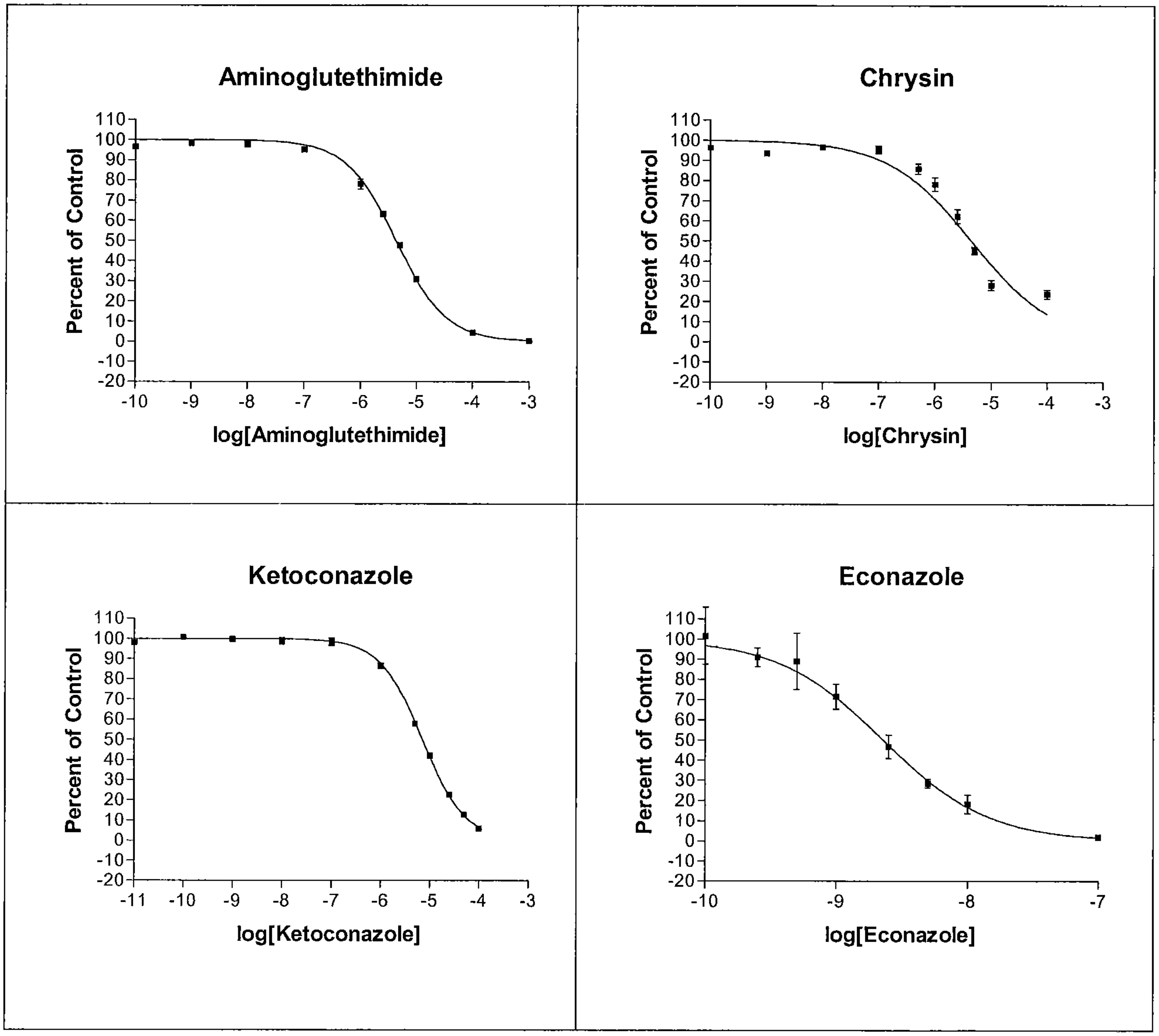


Figure 7. Average Concentration Response Curve Fits (across replicates)

The IC_{50} and slope data are summarized in Table 21 by chemical and replicate. The status of each response is also indicated in the table. The concentration response curve for aminoglutethimide, ketoconazole, and econazole are characterized as “Complete (C)” since the percent of control data essentially spanned the 0-100% range. The curve for chrysin is characterized as "Incomplete-Interpolated (II)" since the percent of control data ranged from ca. 22-100%. The standard errors of the parameter estimates of log$_{10}$(IC50) for aminoglutethimide and ketoconazole are fairly consistent across the replicates. The standard errors of estimates of log$_{10}$(IC50) for chrysin show a decreasing value across the replicates. The standard errors for replicates 2 and 3 for the estimates of log$_{10}$(IC50) of econazole are similar, showing consistent variation in the estimates, however, the standard error for replicate 4 is very large. For the response curves estimates of the slope, the standard errors of the slope estimates for aminoglutethimide, chrysin, and ketoconazole are fairly consistent within the chemicals. The standard errors for the slope estimates of econazole in replicates 2 and 3 are similar; however, the standard error for replicate 4 is very large.

The overall means, SE and 95% confidence intervals for the IC$_{50}$ and slope for each chemical (across replicates) are presented in Table 22. The standard error for the mean of log$_{10}$(IC$_{50}$) for chrysin is about 4 times larger than the standard errors for aminoglutethimide and ketoconazole. The slope estimates for aminoglutethimide, ketoconazole, and econazole are similar in value but the standard errors of aminoglutethimide and econazole are about half the size of the standard error for ketoconazole. The slope estimate for chrysin is about half the value of the other slope estimates and has a standard error more than 3 times the size of the standard errors for the slope estimates of the other chemicals. Graphs of the IC$_{50}$ and slope means, standard errors and confidence intervals are included in statistician's report in Appendix G.

Table 21. Summary of LogIC$_{50}$ and Slope and Their Associated SE

Reference Chemical	Chemical Code	Replicate	Log IC$_{50}$	SE LogIC$_{50}$	Slope	SE Slope	Status	IC$_{50}$
Aminoglutethimide	1	1	-5.389	0.01560	-0.9702	0.01865	C	4.09 µM
		2	-5.374	0.01590	-0.9578	0.02456	C	4.23 µM
		3	-5.351	0.01073	-0.9904	0.01854	C	4.46 µM
Chrysin	2	1	-5.427	0.08528	-0.5892	0.06366	II	3.75 µM
		2	-5.260	0.07054	-0.5774	0.06615	II	5.50 µM
		3	-5.388	0.06225	-0.5976	0.06115	II	4.09 µM
Ketoconazole	3	1	-5.128	0.01013	-1.047	0.01686	C	7.44 µM
		2	-5.164	0.01360	-0.9929	0.02162	C	6.85 µM
		4	-5.143	0.01103	-0.9865	0.01757	C	7.20 µM
Econazole	4	2	-8.676	0.00811	-1.037	0.01570	C	2.11 nM
		3	-8.732	0.00714	-1.023	0.01321	C	1.85 nM
		4	-8.584	0.05389	-1.081	0.1224	C	2.61 nM

Status = codes as described in the statistical analysis section that describe the curve fit that lead to the IC$_{50}$ calculation.

Table 22. Summary Results

Chemical	dependent	GeoMean IC_{50}	Mean	Standard Error	Lower	Upper
Aminoglutethimide	Log_IC_{50}	4.27519E-06	-5.36904	0.01184	-5.39634	-5.34175
Aminoglutethimide	Slope	NA	-0.97533	0.01159	-1.00207	-0.94860
Chrysin	Log_IC_{50}	4.42135E-06	-5.35445	0.04879	-5.46696	-5.24193
Chrysin	Slope	NA	-0.58859	0.03669	-0.67321	-0.50398
Ketoconazole	Log_IC_{50}	7.18761E-06	-5.14342	0.01004	-5.16656	-5.12027
Ketoconazole	Slope	NA	-1.00976	0.01987	-1.05557	-0.96394
Econazole	Log_IC_{50}	2.09650E-09	-8.67850	0.03632	-8.76227	-8.59474
Econazole	Slope	NA	-1.02916	0.01007	-1.05239	-1.00593

NA = Not applicable

5.0 DISCUSSION

This WA 4-16, Task 5 involved testing the response of human placental microsome aromatase activity to inhibition by four reference chemicals. This task was performed in the four aromatase validation laboratories (RTI, Battelle, WIL and In Vitro) and only the results obtained by RTI are presented here.

The microsomes used in this task were prepared at RTI on a previous task of this WA. At the time of preparation, the protein content of the microsomal suspension was determined to be 14 mg/mL. For Task 5, the aromatase assay was performed a total of 14 times and the protein content of the microsomal preparation was measured each day the aromatase assay was conducted. The average measured protein content of the microsome stock was 11.5 ± 2.1 mg/mL (18% CV), with a range of 9.0 to 17.0 mg/mL. It should be noted, though, that the 17.0 mg/mL value was somewhat of an outlier – the next highest measured value was 13.9 mg/mL.

In order to better characterize the protein assay, QC standards were included on some runs. The data show acceptable correlation (<15% variance from known) of calculated and known amounts for the QC standards in the 0.25 to 1 mg/mL range. However, at 0.123 mg/mL the variance from known was nearly 30%. The microsomal protein dilutions that were actually measured in the protein assay had a concentration of about 0.23 mg/mL. This concentration falls between the low end of the reliable range (0.25 mg/mL) of the protein standard curve and the low end of the standard curve range (0.13 – 1.5 mg/mL) and therefore may be subject to a large variance from the true value. Furthermore, the microsomal dilution which was used for protein measurement was not the same dilution that was used in the aromatase assay. The dilution used in the aromatase assay was 10-fold more dilute that the one used for protein measurement. This had been the practice because the final dilution for use in the aromatase assay would only contain approximately 0.025 mg/mL, which was below the lower bounds of the protein standard curve.

Because the concentration of protein used in the aromatase assay is very low (0.0125 mg/mL) and because the aromatase activity is calculated by normalizing the amount of product by the protein content, small variations in protein content can have a large effect on the measured aromatase activity. Therefore, in order to have meaningful comparison of aromatase activity from laboratory to laboratory it is essential to have accurate, reliable protein content determination. For that to be the case, it would be best to measure protein directly in the final microsome dilution (that which is actually used in the assay) and to have the measured values fall in a well defined portion of the protein standard curve (preferably near the middle of the linear range). Efforts are currently underway on a separate work assignment to address these issues.

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

All calculated aromatase activities were corrected for the radioactivity measured in the background activity controls, so the average background control activity for a replicate necessarily was zero. The average full aromatase activity across all twelve accepted runs of the aromatase assay was 0.1050 ± 0.0217 nmol/mg/min (20.66 % CV). The positive control activity across the 12 runs averaged 0.0490 ± 0.0091 nmol/mg/min (18.68 %CV) and the negative control activity averaged 0.0993 ± 0.0270 nmol/mg/min (27.20 %CV). For all controls, a decrease in aromatase activity was noted between the controls run at the beginning of each replicate and those run at the end of the replicate. While these differences were small (generally less than 5%CV), the trend to lower activity between the beginning and end of the assay was extremely consistent and may indicate an issue with decreasing aromatase activity over time. The beginning vs. end percent of control activities were statistically significant ($p<0.05$) for full activity, positive and negative controls, though this may be a function of the tightness of the data within each portion (beginning and end).

Aromatase activities were expressed as percent of control after normalizing by the average full enzyme activity for a given replicate. The background and full enzyme activity controls were set at 0 and 100% by definition. The positive and negative control percent of control activities generally ranged from 40-50% and 90-100%, respectively, across all runs of the assay. There was an unexplained higher degree of variance in all controls in the econazole replicate sets; this led to a statistically significant difference between replicates within a reference chemical for negative control values.

The reference chemicals used on this task were aminoglutethimide, chrysin, ketoconazole and econazole. Each of the four reference chemicals was added to the aromatase assay at eight predefined concentrations in triplicate repetitions (tubes) per replicate of the assay. Data from the first replicate of each reference chemical were reviewed and the target concentrations were adjusted to better define the concentration response curve. Concentrations were selected to define the upper and lower plateaus with intermediate concentrations chosen to distribute as evenly as possible along the slope of the curve. The aromatase assay was conducted in triplicate for each reference chemical and all replicates of a given reference chemical were conducted by a given technician. For ketoconazole and econazole, one of the original three replicates was deemed unacceptable and was excluded from further analysis and a fourth replicate was conducted as a replacement. The percent of control values generally ranged from <10% to >95% for aminoglutethimide, ketoconazole and econazole. For chrysin, the percent of control values ranged from about 22% to >95%. Concentration response curves were fitted to the percent of control values with PRISM software using the following equation:

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

Where: $Y \equiv$ percent of control activity in the inhibitor tube

$X \equiv$ logarithm (base 10) of the concentration

$\beta \equiv$ slope of the concentration response curve (β will be negative)

$\mu \equiv \log_{10}IC_{50}$ (IC_{50} is the concentration corresponding to 50% of control activity).

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

$\varepsilon \equiv$ the variation among repetitions, distributed with mean 0 and variance proportional to DAVG (based on Poisson distribution theory for radiation counts).

The variance was approximated by Y.

The response curve was fitted by weighted least squares nonlinear regression analysis with weights equal to 1/Y. Where the range of the percent of control values was approximately 0 to 100%, the curve fit the data well (r^2 generally >0.99) whereas, in the case of chrysin where the range was abbreviated, the curve fit was not as good (average r^2 =0.9374). The two-parameter model used in this work assignment leads to unsatisfactory curve fits where the data do not span the entire 0-100% range. The current model will, however, calculate the IC_{50} properly as the concentration corresponding to 50% inhibition. The alternative four-parameter model, in which the top and bottom plateaus are allowed to vary to fit the data, would result in a more satisfactory curve fit, but the parameter output by PRISM as logEC50 ($\equiv \log IC_{50}$) would correspond to (Top + Bottom)/2 and in the case of chrysin, would be approximately (95 + 22)/2 = 58.5% activity. Therefore, careful examination of the PRISM output is necessary and additional calculations may be required to ensure that the actual IC_{50} is reported. This issue is currently under review and may lead to revisions in the analysis method for future tasks on this WA and WA 4-17.

Replicate to replicate variation in IC_{50} was very low (<5%CV) for aminoglutethimide and ketoconazole while that for chrysin (20.9%) and econazole (17.6%) was somewhat higher. The IC_{50} values measured for the four reference chemicals in this study compared well with those found in previous work assignments.

6.0 CONCLUSION

In this WA 4-16 Task 5, the performance of the aromatase assay was assessed using a single human placental microsome preparation and four reference chemicals. The data reported here will be compared with data obtained from other participating laboratories using the same microsomal preparation and reference chemicals.

Aromatase activities for each type of control had coefficients of variance on the order of 20% over the twelve assays conducted. It is possible that this variance is associated with imprecise protein determination. The protein assay accuracy and precision may be improved by using a standard curve that encompasses the protein concentration of the microsomal dilution that is used directly in the aromatase assay (rather than being one dilution removed). The continued use of matched quality control samples in the protein assay would also improve the assay and allow for better comparison between laboratories.

IC_{50}s were calculated for each reference chemical using a two parameter equation and each was comparable for values obtained on previous work assignments for these chemicals. There was very little variance (<5%) in IC_{50} data over three assays for two of the reference chemicals (aminoglutethimide and ketoconazole). A higher variance, mostly associated with data obtained during a single replicate, was noted for econazole. The data obtained with chrysin, however, point out the limitations of the two-parameter model for fitting data that do not span the entire 0-100% of control range. A plan for handling data of this type should be developed for future tasks.

Appendix A

Study Protocol with Two Amendments and Protocol Deviation

EPA Contract No.:	68-W-01-023
EPA Work Assignment No.:	4-16
Task Number:	5
RTI Study Code:	An05-928

TITLE: Assay to Determine the Effect of Four Reference Chemicals on the Aromatase Activity of Human Placental Microsomes

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PROPOSED EXPERIMENTAL START DATE: February 2, 2005
PROPOSED EXPERIMENTAL END DATE: February 28, 2005

AMENDMENTS:

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

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Appendices

1.0 OBJECTIVES

Task 5: Conduct Studies with Centrally Prepared Microsomes

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

1.1 Justification for Test System

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

1.2 Test Method

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

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

2.0 MATERIALS RECEIPT AND/OR PREPARATION

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

2.1 Substrate

2.1.1 Substrate Name/Supplier

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

2.1.2 Radiochemical Purity

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 -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 ultraviolet (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 Reference Chemicals

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

Table 1. Reference Chemicals for Task 5

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

2.2.1 Reference Chemical Formulation and Analysis

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

2.3 Control Substances

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

Table 2. Control Substances

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

2.3.1 Control Substance Formulation and Analysis

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

2.4 Microsomes

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

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

On the day of use, microsomes are thawed quickly in a 37 ± 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 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 2 h before proceeding with the assay or microsomal enzyme activity may be decreased.

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

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

2.5 Other Assay Components

2.5.1 Buffer

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

2.5.2 Propylene Glycol

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

2.5.3 NADPH

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

3.0 PROTEIN ASSAY

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

4.0 AROMATASE ASSAY METHOD

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Each test tube will be uniquely identified by applying a label or writing directly on the test tube. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The final concentrations for the assay components are presented in Table 3. The tubes and the microsomal suspension will be placed at 37 ± 1°C in the water bath for five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes are then vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

Table 3. Optimized Aromatase Assay Conditions

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

[a] Final concentrations

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

5.0 DETERMINATION OF THE RESPONSE OF AROMATASE ACTIVITY TO REFERENCE CHEMICALS

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

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

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

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

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

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

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

Table 4. Reference Chemical Study Design

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

Sample type	Repetitions (test tubes)	Description	Test Chemical concentration (M final)
		Chemical added	
Reference Chemical Concentration 5	3	Complete assay with Reference Chemical added	1 x 10^{-7}
Reference Chemical Concentration 6	3	Complete assay with Reference Chemical added	1 x 10^{-8}
Reference Chemical Concentration 7	3	Complete assay with Reference Chemical added	1 x 10^{-9}
Reference Chemical Concentration 8	3	Complete assay with Reference Chemical added	1 x 10^{-10}

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

6.0 DATA ANALYSIS

6.1 *Aromatase Activity and Percent of Control Calculations*

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

6.2 *Statistical Analyses*

6.2.1 Concentration Response Fits for the Reference Chemicals

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

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

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

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

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

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

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

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

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

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

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

For each reference chemical and replicate the estimated $\log_{10}IC_{50}$ (μ), the within replicate standard error of μ, the IC_{50}, the slope (β), the within replicate standard error of β, and the "Status" of each response curve will be displayed in a table. The "Status" of each response curve is indicated as:

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

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

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

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

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

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

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

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

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

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

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

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

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

The random effects will be

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

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

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

6.2.4 Statistical Software

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

6.2.5 Interlaboratory Statistical Analysis

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

7.0 RETENTION OF RECORDS

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

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

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

9.0 REPORTS

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

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

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

10.0 STUDY RECORDS TO BE MAINTAINED

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

Appendix A

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

Substrate Specific Activity Worksheet

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

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

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

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

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

Protein Worksheet

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

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

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

Microsome and Chemical Dilutions Worksheet

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

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

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

Activity Calculation Worksheet

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

The function of each section is described below:

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

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

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

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

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

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

Output: Percent conversion to product.

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

Data input: None.

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

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

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

Data output: nmol ^3H_2O formed.

Section 5 (Columns O-R)

This section calculates aromatase activity in each tube.

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

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

Results Summary Worksheet

This worksheet summarizes the results.

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

Data input: None.

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

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

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

Data input: None.

Output: Log[test inhibitor].

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

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

Data input: None.

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

EPA Contract No.:	68-W-01-023
EPA Work Assignment No.:	4-16
Task Number:	5
RTI Study Code:	An05-928

Amendment 1

TITLE: Assay to Determine the Effect of Four Reference Chemicals on the Aromatase Activity of Human Placental Microsomes

SPONSOR: Battelle
505 King Ave.
Columbus, OH 43201

TESTING FACILITY: RTI International*
DMPK
3040 Cornwallis Rd.
Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: February 2, 2005
PROPOSED EXPERIMENTAL END DATE: February 28, 2005

**RTI International is a trade name of Research Triangle Institute.*

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RTI Study Director

Jerry D. Johnson 2-11-05
Jerry Johnson, Ph.D. DABT Date
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J. Thomas McClintock, ~~Ph.D.~~ Date
EPA Quality Assurance Manager ①

① EPA signatures not required on amendment per QMP.
TW 2-16-05

Item 1

Section 2.4 Microsomes, fourth paragraph, which read:

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

Is hereby amended as follows:

1. It is moved to precede the current third paragraph.
2. Text is changed to the following.

Human placental microsomes (6 tubes of ca. 500 µL each) will be thawed quickly in a 37 ± 1°C water bath and then are immediately transferred to an ice bath. The microsomes will be pooled and rehomogenized using a Potter-Elvejhem homogenizer (about 5–10 passes). The pooled sample will be aliquoted into portions appropriate for use in a single experiments (ca. 160 µL) and the samples will be flash frozen and stored at -70 to -80°C for future use. Each tube will provide enough protein for a single experiment and any excess thawed microsomal preparation will be discarded.

Justification

This change is made in response to concerns of a reviewer with regard to the effect of multiple freeze/thaw cycles on microsome activity. This change will standardize the number of freeze/thaw cycles experienced by the microsomes used in this task to 2. The first freeze was at microsome preparation, the first thaw and second freeze will occur during the repackaging described above and the second thaw will occur at assay.

Item 2

Section 2.4 Microsomes, third paragraph, which read:

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 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. It is recommended that microsomes not be left on ice for longer than approximately 2 h before proceeding with the assay or microsomal enzyme activity may be decreased.

Is hereby amended as follows:

1. It is moved to follow the current fourth paragraph
2. The text is changed as indicated in **bold** type:

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 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. **Microsomes are not be left on ice for longer than approximately 2 h before proceeding with the assay. Appropriate documentation of time from thaw to use must be maintained.**

Justification

This change is made in response to concerns of a reviewer with regard to the effect of the length of time microsomes are stored on ice on aromatase activity. This change limits that time to approximately 2 h.

Item 3

Section 5.0 Determination of the response of aromatase activity to reference chemicals, second paragraph, which read:

Four types of control samples will be included for each replicate. These include:
- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)
- background activity controls (all components that are in the full aromatase activity controls, except NADPH)
- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at a single concentration)
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at a single concentration).

Is hereby amended as follows (changes in **bold**)

Four types of control samples will be included for each replicate. These include:
- full enzyme (aromatase) activity controls (substrate, NADPH, propylene glycol, buffer, vehicle [used for preparation of test substance solutions] and microsomes)
- background activity controls (all components that are in the full aromatase activity controls, except NADPH)
- positive controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of 4-OH ASDN at **5E-8M**)
- negative controls (all components that are in the full aromatase activity controls, except vehicle, and with the addition of lindane at **1E-6M**).

Justification

This change adds the concentrations of the positive and negative controls to the text.

Item 4

Sections 6.2.1 and 6.2.3. are hereby amended as follows:

The use of the abbreviations FEAC (for full enzyme activity control) and BAC (for background activity control) is discontinued for all protocols and reports. The laboratory personnel may still use these abbreviations for labeling samples and on data collection forms.

Justification

This change made at the recommendation of a reviewer.

EPA Contract No.:	68-W-01-023
EPA Work Assignment No.:	4-16
Task Number:	5
RTI Study Code:	An05-928

Amendment 2

TITLE: Assay to Determine the Effect of Four Reference Chemicals on the Aromatase Activity of Human Placental Microsomes

SPONSOR: Battelle
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Research Triangle Park, NC 27709

PROPOSED EXPERIMENTAL START DATE: February 2, 2005
PROPOSED EXPERIMENTAL END DATE: February 28, 2005

---

*RTI International is a trade name of Research Triangle Institute.

Approved By:

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Terri Y Pollock 2-16-05
Terri L. Pollock, B.A. Date
EDSP Quality Assurance Manager, Battelle

① EPA signatures not required on amendment per QMP.
TP 2-16-05

Item 1

Section 2.4 Microsomes, fourth paragraph, which read (as amended previously):

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 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. Microsomes are not be left on ice for longer than approximately 2 h before proceeding with the assay. Appropriate documentation of time from thaw to use must be maintained.

Is hereby amended as follows:

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. **Vortex the microsomes about 5 seconds in the microcentrifuge tubes in which they are stored and then remove an aliquot directly from that tube for dilution for use in the assay**. The microsomes are diluted in buffer (serial dilutions may be necessary) to an approximate protein concentration of 0.025 mg/mL. The addition of 1 mL of that microsome dilution will result in a final approximate protein concentration of 0.0125 mg/mL in the assay tubes. All microsome samples must be kept on ice until they are placed in the water bath just prior to their addition to the aromatase assay. Microsomes are not be left on ice for longer than approximately 2 h before proceeding with the assay. Appropriate documentation of time from thaw to use must be maintained.

Justification

There have been problems with poor recovery of microsomal suspension from the Potter Elvejhem homogenizers because of the tendency of the preparation to coat the tube and pestle. Preliminary data show good recovery and consistent aromatase activity in samples mixed by vortexing prior to removal of an aliquot for dilution.

Protocol Deviation 1

ORIGINAL DOCUMENT SPECIFICATIONS:
Page 10, Section 5.0 Determination of the Response of Aromatase Activity to Reference Chemicals, Table 4:

The Reference Chemical concentrations to be tested range from 10^{-3} to 10^{-10} M.

DEVIATION:

Because the supplied ketoconazole solution was 0.01M, the highest concentration that could be tested in the assay was 10^{-4}M. NO adjustment for this lower stock concentration was made in the first replicate of the assay, so the lowest concentration tested in that replicate was 10^{-11}M.

REASON/IMPACT OF CHANGE:

This deviation is expected to have no adverse effect on the data; in fact, the IC_{50} calculated from this replicate is very similar to that calculated from other replicates. Appropriate adjustments to the tested concentrations were made per instructions in the protocol for replicates 2-4 of the assay.

J Mott
Study Director

1-24-06
Date

Protocol Deviation 2

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 13, Section 6.2.1 Concentration Response Fits for the Reference Chemicals

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

DEVIATION:

Concentration response models were fitted for each replicate test within each reference chemical. Based on the results of the fit within each replicate the extent of aromatase inhibition was summarized as IC_{50} (10^{μ}) and slope (β). The estimated IC_{50} for a reference chemical was a (weighted) geometric mean across the replicates, calculated as described in Appendix G. **A mixed effects model was fit to the $\log_{10}(IC_{50})$ and slope estimates from the response curve model fits. The chemical type was assigned as the fixed effect and replicate was assigned as the random effect, with homogeneous covariance parameters within the chemicals. SAS PROC MIXED was used to obtain estimates of the replicate-to-replicate variation within each chemical by entering the square of the standard errors from the response curve model parameter estimates as the variation estimates for the within replicate (within chemical) variance components. The replicate-to-replicate variation estimates for slope were constrained to be no lower than 1E-30 in order to allow the mixed effects model to converge and provide estimates of the variance components.**

REASON/IMPACT OF CHANGE:

The model was changed to a mixed effects analysis of variance model since there weren't enough degrees of freedom to fit models by chemical with replicate as a random effect term. See email from Margie Byron, dated 5/9/2005, in the record. No adverse effect is expected as a result of this change.

Study Director

1-24-06

Date

Appendix B

QAPP

1.0 TITLE AND APPROVAL

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

Task 5 -Conduct Multiple Chemical Studies with Centrally Prepared Microsomes

for

EPA CONTRACT NUMBER 68-W-01-023

January 24, 2005

SIGNATURE PAGE

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

Concurrences and Approvals

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2.0 TABLE OF CONTENTS

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4.0 PROJECT ORGANIZATION

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

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

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

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

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

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

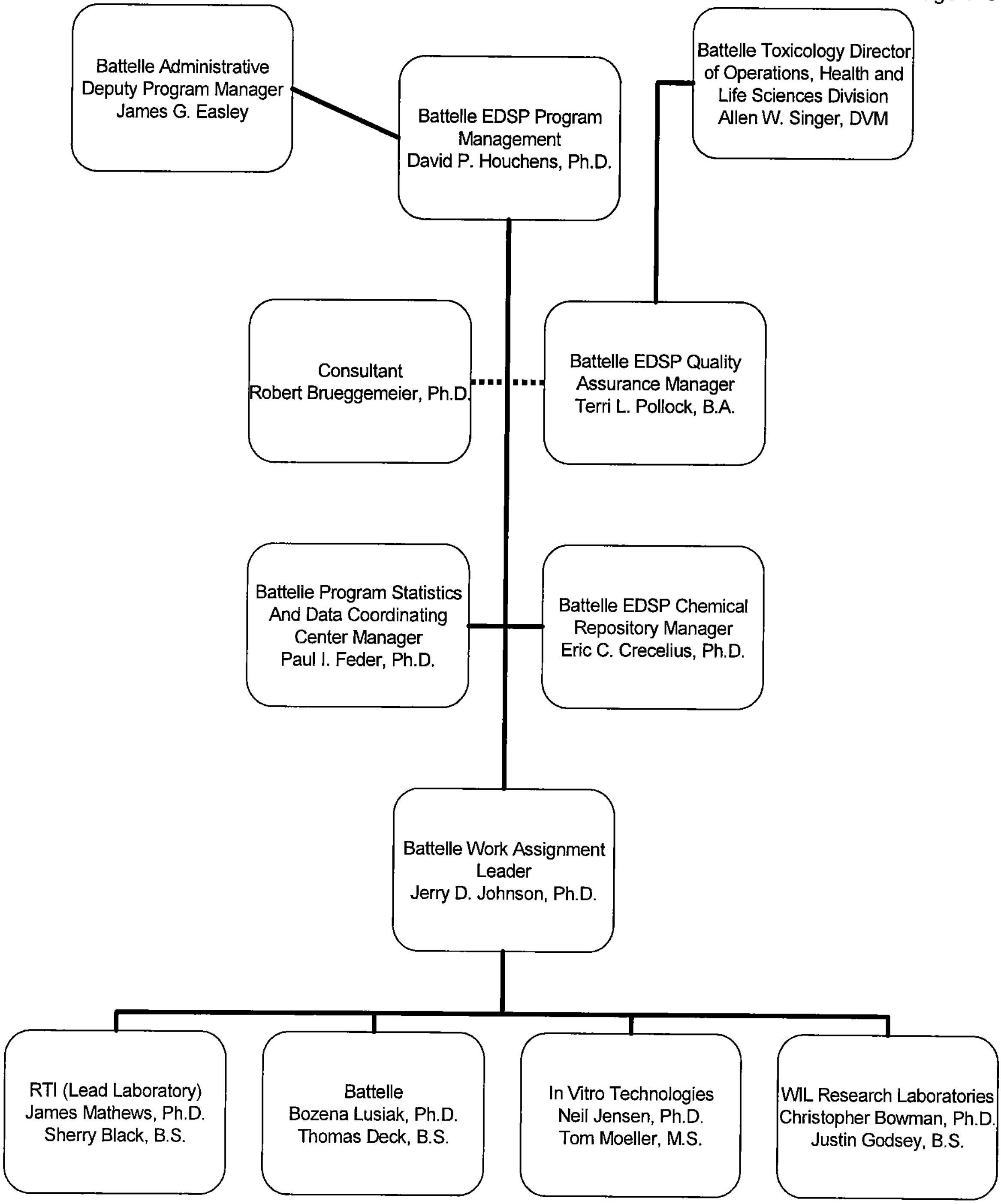


Figure 1. WA 4-16 Project Organization Overview

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

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

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

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

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

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

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

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

5.0 PROBLEM DEFINITION/BACKGROUND

5.1 Problem Definition

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

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

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

Table 1. Validation Study Plan Experiments

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

*EDMVAC = Endodrine Disruptor Method Validation Committee

5.2 Background

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

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

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

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

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

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

6.0 PROJECT/TASK DESCRIPTION

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

Task 4: Conduct Positive Control Studies in the Participating Laboratories

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

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

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

Task 5: Conduct Studies with Centrally Prepared Microsomes

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

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

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

Task 6: Prepare Microsomes in Two Participating Laboratories

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

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

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

Task 7: Conduct Studies with Microsomes Prepared in Participating Laboratories

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

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

7.0 QUALITY OBJECTIVES AND CRITERIA

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

7.1 Data Quality Indicators

7.1.1 Precision

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

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

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

7.1.2 Bias

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

7.1.3 Accuracy

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

8.0 SPECIAL TRAINING/CERTIFICATION

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

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

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

9.0 DOCUMENTS AND RECORDS

9.1 Retention of Specimens and Records

Archiving procedures will be specified in the individual protocols.

9.2 Quality Assurance Project Plan

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

Version 1
Month, Year
Page 1 of 1

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

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

9.3 Data Forms

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

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

9.4 Microsome Storage Conditions

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

9.5 Reports

9.5.1 Interim Data Summary, and Draft and Final Reports

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

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

Each final task report will include:

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

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

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

9.5.2 QA Assessment Reports

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

9.5.3 Status Reports

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

10.0 SAMPLING PROCESS DESIGN (EXPERIMENTAL DESIGN

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

11.0 SAMPLING METHODS

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

Each test and standard chemical will be supplied to the participating laboratories by Battelle as a stock solution at the highest concentration necessary for use in the assay. These solutions will be well-mixed prior to the preparation of dilutions of these stock solutions by the individual participating laboratories.

12.0 SAMPLE HANDLING AND CUSTODY

12.1 Test and Reference Chemical Solutions

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

12.2 Sample Collection Documentation

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

13.0 ANALYTICAL METHODS

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

14.0 QUALITY CONTROL

14.1 Methods

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

14.2 Data Collection

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

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

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

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

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

15.0 INSTRUMENT/EQUIPMENT TESTING, INSPECTION, AND MAINTENANCE

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

16.0 INSTRUMENT/EQUIPMENT CALIBRATION AND FREQUENCY

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

Scintillation Counters will be calibrated using procedures described in the relevant SOPs. Calibration of pH meters occurs as specified in relevant SOPs. The water bath, pipettes, spectrophotometer, and HPLC equipment will be calibrated using the procedures and schedule in applicable SOPs. Any equipment or instrument that does not meet acceptance criteria as described in the relevant SOP will not be used for this work assignment.

17.0 INSPECTION/ACCEPTANCE OF SUPPLIES AND CONSUMABLES

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

18.0 NON-DIRECT MEASUREMENTS

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

19.0 DATA MANAGEMENT

19.1 Data Management Overview

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

19.2 Data Transfer

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

20.0 ASSESSMENTS AND RESPONSE ACTIONS

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

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

20.1 Technical Systems Audits

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

20.2 Type, Scheduling, and Performance of Technical Systems Audits

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

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

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

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

20.3 Audits of Data Quality

An ADQ is a process by which the accuracy of data calculations and reporting will be assessed to ensure that the reported results are of high quality and accurately reflect the raw data and accurately describe the materials used in the study. The acceptance criteria for the ADQ are that data collection, analysis, and reporting must meet the requirements of the applicable facility and program SOPs, the WA protocols and QAPP, QMP, and the FIFRA GLPs, or be explained and evaluated in a deviation report, as previously described.

20.4 Scheduling and Performance of Audits of Data Quality

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

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

20.5 Audit Report Format

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

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

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

20.6 Response Actions and Resolution of Issues

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

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

20.7 Independent Assessments

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

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

21.0 REPORTS TO MANAGEMENT

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

22.0 DATA REVIEW, VERIFICATION, AND VALIDATION

The data produced under this work assignment will be reviewed by the technical personnel for the validation process and by EDSP QA team members for the verification process (see section 23). The criteria used for validation depend on the type of data. For dose solution sample data, information regarding the condition of the containers and whether or not samples were compromised is recorded in the sample chain-of-custody records. Compromised samples are not analyzed. The criteria for validating data are those found in Section 7 (Data Quality Objectives).

23.0 VERIFICATION AND VALIDATION METHODS

23.1 Chain of Custody for Data

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

23.2 Data Validation

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

23.3 Data Verification

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

24.0 RECONCILIATION AND USER REQUIREMENTS

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

25.0 REFERENCES

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

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

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

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

APPENDIX A

TEMPLATE PROTOCOL FOR TASK 5

Appendix C

Data Tables

Table C-1. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 1

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	9674	19348	18901	37802	0.1	199840	18.92	37665	0.0377	1	0.009	15	0.1342
			0.5	2	9227	18454			0.1								
	2	2	0.5	1	9785	19570	19341	38682	0.1	199840	19.36	38545	0.0386	1	0.009	15	0.1374
			0.5	2	9556	19112			0.1								
	3	2	0.5	1	8939	17878	17798	35596	0.1	199840	17.81	35459	0.0355	1	0.009	15	0.1264
			0.5	2	8859	17718			0.1								
	4	2	0.5	1	9337	18674	18596	37192	0.1	199840	18.61	37055	0.0371	1	0.009	15	0.1321
			0.5	2	9259	18518			0.1								
Background control	1	2	0.5	1	26	52	54	108	0.1	199840	0.05	-30	0.0000	1	0.009	15	-0.0001
			0.5	2	28	56			0.1								
	2	2	0.5	1	42	84	75	150	0.1	199840	0.08	13	0.0000	1	0.009	15	0.0000
			0.5	2	33	66			0.1								
	3	2	0.5	1	44	88	76	152	0.1	199840	0.08	15	0.0000	1	0.009	15	0.0001
			0.5	2	32	64			0.1								
	4	2	0.5	1	30	60	70	140	0.1	199840	0.07	3	0.0000	1	0.009	15	0.0000
			0.5	2	40	80			0.1								
Positive control	1	2	0.5	1	4224	8448	8250	16500	0.1	199840	8.26	16363	0.0164	1	0.009	15	0.0583
			0.5	2	4026	8052			0.1								
	2	2	0.5	1	3975	7950	7775	15550	0.1	199840	7.78	15413	0.0154	1	0.009	15	0.0549
			0.5	2	3800	7600			0.1								
	3	2	0.5	1	3609	7218	7194	14388	0.1	199840	7.20	14251	0.0143	1	0.009	15	0.0508
			0.5	2	3585	7170			0.1								
	4	2	0.5	1	3831	7662	7595	15190	0.1	199840	7.60	15053	0.0151	1	0.009	15	0.0536
			0.5	2	3764	7528			0.1								
Negative Control	1	2	0.5	1	9728	19456	19278	38556	0.1	199840	19.29	38419	0.0385	1	0.009	15	0.1369
			0.5	2	9550	19100			0.1								
	2	2	0.5	1	9529	19058	18687	37374	0.1	199840	18.70	37237	0.0373	1	0.009	15	0.1327
			0.5	2	9158	18316			0.1								
	3	2	0.5	1	9126	18252	17899	35798	0.1	199840	17.91	35661	0.0357	1	0.009	15	0.1271
			0.5	2	8773	17546			0.1								
	4	2	0.5	1	8982	17964	17614	35228	0.1	199840	17.63	35091	0.0351	1	0.009	15	0.1251

Table C-1. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 1 (Con't)

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
Negative Control (Con't)																	
			0.5	2	8632	17264			0.1								
11343-26A Chem 1	1-1	2	0.5	1	68	136	138	276	0.1	199840	0.14	139	0.0001	1	0.009	15	0.0005
			0.5	2	70	140			0.1								
	1-2	2	0.5	1	66	132	128	256	0.1	199840	0.13	119	0.0001	1	0.009	15	0.0004
			0.5	2	62	124			0.1								
	1-3	2	0.5	1	72	144	143	286	0.1	199840	0.14	149	0.0001	1	0.009	15	0.0005
			0.5	2	71	142			0.1								
	2-1	2	0.5	1	466	932	886	1772	0.1	199840	0.89	1635	0.0016	1	0.009	15	0.0058
			0.5	2	420	840			0.1								
	2-2	2	0.5	1	436	872	868	1736	0.1	199840	0.87	1599	0.0016	1	0.009	15	0.0057
			0.5	2	432	864			0.1								
	2-3	2	0.5	1	450	900	877	1754	0.1	199840	0.88	1617	0.0016	1	0.009	15	0.0058
			0.5	2	427	854			0.1								
	3-1	2	0.5	1	2950	5900	5839	11678	0.1	199840	5.84	11541	0.0116	1	0.009	15	0.0411
			0.5	2	2889	5778			0.1								
	3-2	2	0.5	1	2878	5756	5564	11128	0.1	199840	5.57	10991	0.0110	1	0.009	15	0.0392
			0.5	2	2686	5372			0.1								
	3-3	2	0.5	1	2831	5662	5569	11138	0.1	199840	5.57	11001	0.0110	1	0.009	15	0.0392
			0.5	2	2738	5476			0.1								
	4-1	2	0.5	1	7651	15302	14803	29606	0.1	199840	14.81	29469	0.0295	1	0.009	15	0.1050
			0.5	2	7152	14304			0.1								
	4-2	2	0.5	1	7489	14978	14891	29782	0.1	199840	14.90	29645	0.0297	1	0.009	15	0.1057
			0.5	2	7402	14804			0.1								
	4-3	2	0.5	1	7320	14640	14447	28894	0.1	199840	14.46	28757	0.0288	1	0.009	15	0.1025
			0.5	2	7127	14254			0.1								
	5-1	2	0.5	1	9248	18496	18474	36948	0.1	199840	18.49	36811	0.0369	1	0.009	15	0.1312
			0.5	2	9226	18452			0.1								
	5-2	2	0.5	1	8958	17916	17844	35688	0.1	199840	17.86	35551	0.0356	1	0.009	15	0.1267
			0.5	2	8886	17772			0.1								
	5-3	2	0.5	1	8804	17608	17440	34880	0.1	199840	17.45	34743	0.0348	1	0.009	15	0.1238
			0.5	2	8636	17272			0.1								

Table C-1. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 1 (Con't)

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
Negative Control (Con't)																	
	6-1	2	0.5	1	9449	18898	18701	37402	0.1	199840	18.72	37265	0.0373	1	0.009	15	0.1328
			0.5	2	9252	18504			0.1								
	6-2	2	0.5	1	9374	18748	18472	36944	0.1	199840	18.49	36807	0.0369	1	0.009	15	0.1312
			0.5	2	9098	18196			0.1								
	6-3	2	0.5	1	9353	18706	18393	36786	0.1	199840	18.41	36649	0.0367	1	0.009	15	0.1306
			0.5	2	9040	18080			0.1								
	7-1	2	0.5	1	9456	18912	18424	36848	0.1	199840	18.44	36711	0.0368	1	0.009	15	0.1308
			0.5	2	8968	17936			0.1								
	7-2	2	0.5	1	9261	18522	18294	36588	0.1	199840	18.31	36451	0.0365	1	0.009	15	0.1299
			0.5	2	9033	18066			0.1								
	7-3	2	0.5	1	9366	18732	18353	36706	0.1	199840	18.37	36569	0.0366	1	0.009	15	0.1303
			0.5	2	8987	17974			0.1								
	8-1	2	0.5	1	9497	18994	18705	37410	0.1	199840	18.72	37273	0.0373	1	0.009	15	0.1328
			0.5	2	9208	18416			0.1								
	8-2	2	0.5	1	9001	18002	17657	35314	0.1	199840	17.67	35177	0.0352	1	0.009	15	0.1254
			0.5	2	8656	17312			0.1								
	8-3	2	0.5	1	8830	17660	17660	35320	0.1	199840	17.67	35183	0.0352	1	0.009	15	0.1254
			0.5	2	8830	17660			0.1								

Table C-2. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 2

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	7910	15820	15689	31378	0.1	181726	17.27	31244	0.0363	1	0.012	15	0.1018
			0.5	2	7779	15558											
	2	2	0.5	1	7979	15958	15957	31914	0.1	181726	17.56	31780	0.0369	1	0.012	15	0.1036
			0.5	2	7978	15956											
	3	2	0.5	1	7226	14452	14572	29144	0.1	181726	16.04	29010	0.0337	1	0.012	15	0.0945
			0.5	2	7346	14692											
	4	2	0.5	1	7229	14458	14627	29254	0.1	181726	16.10	29120	0.0338	1	0.012	15	0.0949
			0.5	2	7398	14796											
Background control	1	2	0.5	1	33	66	69	138	0.1	181726	0.08	4	0.0000	1	0.012	15	0.0000
			0.5	2	36	72											
	2	2	0.5	1	23	46	57	114	0.1	181726	0.06	-21	0.0000	1	0.012	15	-0.0001
			0.5	2	34	68											
	3	2	0.5	1	35	70	78	156	0.1	181726	0.09	22	0.0000	1	0.012	15	0.0001
			0.5	2	43	86											
	4	2	0.5	1	33	66	65	130	0.1	181726	0.07	-5	0.0000	1	0.012	15	0.0000
			0.5	2	32	64											
Positive control	1	2	0.5	1	3688	7376	7477	14954	0.1	181726	8.23	14820	0.0172	1	0.012	15	0.0483
			0.5	2	3789	7578											
	2	2	0.5	1	3669	7338	7389	14778	0.1	181726	8.13	14644	0.0170	1	0.012	15	0.0477
			0.5	2	3720	7440											
	3	2	0.5	1	3525	7050	7174	14348	0.1	181726	7.90	14214	0.0165	1	0.012	15	0.0463
			0.5	2	3649	7298											
	4	2	0.5	1	3424	6848	6922	13844	0.1	181726	7.62	13710	0.0159	1	0.012	15	0.0447
			0.5	2	3498	6996											

Table C-2. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 2 (Con't)

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
Negative Control	1	2	0.5	1	7700	15400	15530	31060	0.1	181726	17.09	30926	0.0359	1	0.012	15	0.1008
			0.5	2	7830	15660											
	2	2	0.5	1	7704	15408	15502	31004	0.1	181726	17.06	30870	0.0359	1	0.012	15	0.1006
			0.5	2	7798	15596											
	3	2	0.5	1	7491	14982	14787	29574	0.1	181726	16.27	29440	0.0342	1	0.012	15	0.0959
			0.5	2	7296	14592											
	4	2	0.5	1	7114	14228	14284	28568	0.1	181726	15.72	28434	0.0330	1	0.012	15	0.0927
			0.5	2	7170	14340											
11343-26A	1-1	2	0.5	1	71	142	132	264	0.1	181726	0.15	130	0.0002	1	0.012	15	0.0004
			0.5	2	61	122											
	1-2	2	0.5	1	66	132	124	248	0.1	181726	0.14	114	0.0001	1	0.012	15	0.0004
			0.5	2	58	116											
	1-3	2	0.5	1	48	96	106	212	0.1	181726	0.12	78	0.0001	1	0.012	15	0.0003
			0.5	2	58	116											
	2-1	2	0.5	1	393	786	749	1498	0.1	181726	0.82	1364	0.0016	1	0.012	15	0.0044
			0.5	2	356	712											
	2-2	2	0.5	1	357	714	689	1378	0.1	181726	0.76	1244	0.0014	1	0.012	15	0.0041
			0.5	2	332	664											
	2-3	2	0.5	1	355	710	699	1398	0.1	181726	0.77	1264	0.0015	1	0.012	15	0.0041
			0.5	2	344	688											
	3-1	2	0.5	1	2479	4958	4986	9972	0.1	181726	5.49	9838	0.0114	1	0.012	15	0.0321
			0.5	2	2507	5014											
	3-2	2	0.5	1	2445	4890	4867	9734	0.1	181726	5.36	9600	0.0111	1	0.012	15	0.0313
			0.5	2	2422	4844											

Table C-2. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 2 (Con't)

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
11343-26A (con't)																	
	3-3	2	0.5	1	2395	4790	4844	9688	0.1	181726	5.33	9554	0.0111	1	0.012	15	0.0311
			0.5	2	2449	4898											
	4-1	2	0.5	1	3648	7296	7271	14542	0.1	181726	8.00	14408	0.0167	1	0.012	15	0.0470
			0.5	2	3623	7246											
	4-2	2	0.5	1	3575	7150	7196	14392	0.1	181726	7.92	14258	0.0166	1	0.012	15	0.0465
			0.5	2	3621	7242											
	4-3	2	0.5	1	3600	7200	7292	14584	0.1	181726	8.03	14450	0.0168	1	0.012	15	0.0471
			0.5	2	3692	7384											
	5-1	2	0.5	1	4872	9744	9625	19250	0.1	181726	10.59	19116	0.0222	1	0.012	15	0.0623
			0.5	2	4753	9506											
	5-2	2	0.5	1	4705	9410	9394	18788	0.1	181726	10.34	18654	0.0217	1	0.012	15	0.0608
			0.5	2	4689	9378											
	5-3	2	0.5	1	4687	9374	9482	18964	0.1	181726	10.44	18830	0.0219	1	0.012	15	0.0614
			0.5	2	4795	9590											
	6-1	2	0.5	1	5455	10910	10683	21366	0.1	181726	11.76	21232	0.0247	1	0.012	15	0.0692
			0.5	2	5228	10456											
	6-2	2	0.5	1	5981	11962	11949	23898	0.1	181726	13.15	23764	0.0276	1	0.012	15	0.0774
			0.5	2	5968	11936											
	6-3	2	0.5	1	5814	11628	11773	23546	0.1	181726	12.96	23412	0.0272	1	0.012	15	0.0763
			0.5	2	5959	11918											
	7-1	2	0.5	1	7390	14780	14881	29762	0.1	181726	16.38	29628	0.0344	1	0.012	15	0.0965
			0.5	2	7491	14982											

Table C-2. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 2 (Con't)

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
11343-26A (con't)																	
	7-2	2	0.5	1	6923	13846	14234	28468	0.1	181726	15.67	28334	0.0329	1	0.012	15	0.0923
			0.5	2	7311	14622											
	7-3	2	0.5	1	7129	14258	13957	27914	0.1	181726	15.36	27780	0.0323	1	0.012	15	0.0905
			0.5	2	6828	13656											
	8-1	2	0.5	1	7684	15368	15574	31148	0.1	181726	17.14	31014	0.0360	1	0.012	15	0.1011
			0.5	2	7890	15780											
	8-2	2	0.5	1	7172	14344	14502	29004	0.1	181726	15.96	28870	0.0335	1	0.012	15	0.0941
			0.5	2	7330	14660											
	8-3	2	0.5	1	7289	14578	14662	29324	0.1	181726	16.14	29190	0.0339	1	0.012	15	0.0951
			0.5	2	7373	14746											

Table C-3. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 3

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	9649	19298	19284	38568	0.1	196680	19.61	38441	0.0410	1	0.010	15	0.1351
			0.5	2	9635	19270			0.1					1		15	
	2	2	0.5	1	9652	19304	19318	38636	0.1	196680	19.64	38509	0.0410	1	0.010	15	0.1353
			0.5	2	9666	19332			0.1					1		15	
	3	2	0.5	1	8588	17176	17908	35816	0.1	196680	18.21	35689	0.0380	1	0.010	15	0.1254
			0.5	2	9320	18640			0.1					1		15	
	4	2	0.5	1	9049	18098	18045	36090	0.1	196680	18.35	35963	0.0383	1	0.010	15	0.1263
			0.5	2	8996	17992			0.1					1		15	
Background control	1	2	0.5	1	21	42	50	100	0.1	196680	0.05	-28	0.0000	1	0.010	15	-0.0001
			0.5	2	29	58			0.1					1		15	
	2	2	0.5	1	35	70	63	126	0.1	196680	0.06	-2	0.0000	1	0.010	15	0.0000
			0.5	2	28	56			0.1					1		15	
	3	2	0.5	1	36	72	66	132	0.1	196680	0.07	5	0.0000	1	0.010	15	0.0000
			0.5	2	30	60			0.1					1		15	
	4	2	0.5	1	34	68	76	152	0.1	196680	0.08	25	0.0000	1	0.010	15	0.0001
			0.5	2	42	84			0.1					1		15	
Positive control	1	2	0.5	1	4619	9238	9238	18476	0.1	196680	9.39	18349	0.0196	1	0.010	15	0.0645
			0.5	2	4619	9238			0.1					1		15	
	2	2	0.5	1	4534	9068	9052	18104	0.1	196680	9.20	17977	0.0192	1	0.010	15	0.0632
			0.5	2	4518	9036			0.1					1		15	
	3	2	0.5	1	3944	7888	8054	16108	0.1	196680	8.19	15981	0.0170	1	0.010	15	0.0561
			0.5	2	4110	8220			0.1					1		15	
	4	2	0.5	1	4189	8378	8325	16650	0.1	196680	8.47	16523	0.0176	1	0.010	15	0.0580
			0.5	2	4136	8272			0.1					1		15	

Table C-3. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 3 (Con't)

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
Negative Control	1	2	0.5	1	9354	18708	18271	36542	0.1	196680	18.58	36415	0.0388	1	0.010	15	0.1279
			0.5	2	8917	17834			0.1					1		15	
	2	2	0.5	1	9478	18956	18961	37922	0.1	196680	19.28	37795	0.0403	1	0.010	15	0.1328
			0.5	2	9483	18966			0.1					1		15	
	3	2	0.5	1	8627	17254	17235	34470	0.1	196680	17.53	34343	0.0366	1	0.010	15	0.1207
			0.5	2	8608	17216			0.1					1		15	
	4	2	0.5	1	8375	16750	16666	33332	0.1	196680	16.95	33205	0.0354	1	0.010	15	0.1167
			0.5	2	8291	16582			0.1					1		15	
11343-26A	1-1	2	0.5	1	67	134	125	250	0.1	196680	0.13	123	0.0001	1	0.010	15	0.0004
			0.5	2	58	116			0.1					1		15	
	1-2	2	0.5	1	72	144	123	246	0.1	196680	0.13	119	0.0001	1	0.010	15	0.0004
			0.5	2	51	102			0.1					1		15	
	1-3	2	0.5	1	68	136	136	272	0.1	196680	0.14	145	0.0002	1	0.010	15	0.0005
			0.5	2	68	136			0.1					1		15	
	2-1	2	0.5	1	460	920	911	1822	0.1	196680	0.93	1695	0.0018	1	0.010	15	0.0060
			0.5	2	451	902			0.1					1		15	
	2-2	2	0.5	1	470	940	915	1830	0.1	196680	0.93	1703	0.0018	1	0.010	15	0.0060
			0.5	2	445	890			0.1					1		15	
	2-3	2	0.5	1	416	832	889	1778	0.1	196680	0.90	1651	0.0018	1	0.010	15	0.0058
			0.5	2	473	946			0.1					1		15	
	3-1	2	0.5	1	3016	6032	6078	12156	0.1	196680	6.18	12029	0.0128	1	0.010	15	0.0423
			0.5	2	3062	6124			0.1					1		15	
	3-2	2	0.5	1	2965	5930	5753	11506	0.1	196680	5.85	11379	0.0121	1	0.010	15	0.0400
			0.5	2	2788	5576			0.1					1		15	

Table C-3. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 3 (Con't)

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
11343-26A (con't)																	
	3-3	2	0.5	1	2768	5536	5524	11048	0.1	196680	5.62	10921	0.0116	1	0.010	15	0.0384
			0.5	2	2756	5512			0.1					1		15	
	4-1	2	0.5	1	4596	9192	8797	17594	0.1	196680	8.95	17467	0.0186	1	0.010	15	0.0614
			0.5	2	4201	8402			0.1					1		15	
	4-2	2	0.5	1	4549	9098	9101	18202	0.1	196680	9.25	18075	0.0193	1	0.010	15	0.0635
			0.5	2	4552	9104			0.1					1		15	
	4-3	2	0.5	1	4582	9164	9047	18094	0.1	196680	9.20	17967	0.0192	1	0.010	15	0.0631
			0.5	2	4465	8930			0.1					1		15	
	5-1	2	0.5	1	6064	12128	12170	24340	0.1	196680	12.38	24213	0.0258	1	0.010	15	0.0851
			0.5	2	6106	12212			0.1					1		15	
	5-2	2	0.5	1	5955	11910	11962	23924	0.1	196680	12.16	23797	0.0254	1	0.010	15	0.0836
			0.5	2	6007	12014			0.1					1		15	
	5-3	2	0.5	1	5863	11726	11761	23522	0.1	196680	11.96	23395	0.0249	1	0.010	15	0.0822
			0.5	2	5898	11796			0.1					1		15	
	6-1	2	0.5	1	7610	15220	15302	30604	0.1	196680	15.56	30477	0.0325	1	0.010	15	0.1071
			0.5	2	7692	15384			0.1					1		15	
	6-2	2	0.5	1	7510	15020	15005	30010	0.1	196680	15.26	29883	0.0319	1	0.010	15	0.1050
			0.5	2	7495	14990			0.1					1		15	
	6-3	2	0.5	1	7108	14216	14573	29146	0.1	196680	14.82	29019	0.0309	1	0.010	15	0.1020
			0.5	2	7465	14930			0.1					1		15	
	7-1	2	0.5	1	9182	18364	18291	36582	0.1	196680	18.60	36455	0.0389	1	0.010	15	0.1281
			0.5	2	9109	18218			0.1					1		15	

Table C-3. Data for the Calculation of Aromatase Activity: Aminoglutethimide, Replicate 3 (Con't)

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
11343-26A (con't)																	
	7-2	2	0.5	1	8841	17682	17470	34940	0.1	196680	17.76	34813	0.0371	1	0.010	15	0.1223
			0.5	2	8629	17258			0.1					1		15	
	7-3	2	0.5	1	8628	17256	17444	34888	0.1	196680	17.74	34761	0.0371	1	0.010	15	0.1221
			0.5	2	8816	17632			0.1					1		15	
	8-1	2	0.5	1	9288	18576	18495	36990	0.1	196680	18.81	36863	0.0393	1	0.010	15	0.1295
			0.5	2	9207	18414			0.1					1		15	
	8-2	2	0.5	1	9178	18356	18158	36316	0.1	196680	18.46	36189	0.0386	1	0.010	15	0.1271
			0.5	2	8980	17960			0.1					1		15	
	8-3	2	0.5	1	8634	17268	17316	34632	0.1	196680	17.61	34505	0.0368	1	0.010	15	0.1212
			0.5	2	8682	17364			0.1					1		15	

Table C-4. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 1

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	7407	14814	14509	29018	0.1	201134	14.43	28846	0.0279	1	0.009	15	0.1037
			0.5	2	7102	14204			0.1					1		15	
	2	2	0.5	1	8201	16402	16367	32734	0.1	201134	16.27	32562	0.0315	1	0.009	15	0.1171
			0.5	2	8166	16332			0.1					1		15	
	3	2	0.5	1	7485	14970	14752	29504	0.1	201134	14.67	29332	0.0283	1	0.009	15	0.1055
			0.5	2	7267	14534			0.1					1		15	
	4	2	0.5	1	7582	15164	15076	30152	0.1	201134	14.99	29980	0.0290	1	0.009	15	0.1078
			0.5	2	7494	14988			0.1					1		15	
Background control	1	2	0.5	1	41	82	90	180	0.1	201134	0.09	8	0.0000	1	0.009	15	0.0000
			0.5	2	49	98			0.1					1		15	
	2	2	0.5	1	49	98	78	156	0.1	201134	0.08	-16	0.0000	1	0.009	15	-0.0001
			0.5	2	29	58			0.1					1		15	
	3	2	0.5	1	39	78	75	150	0.1	201134	0.07	-22	0.0000	1	0.009	15	-0.0001
			0.5	2	36	72			0.1					1		15	
	4	2	0.5	1	42	84	101	202	0.1	201134	0.10	30	0.0000	1	0.009	15	0.0001
			0.5	2	59	118			0.1					1		15	
Positive control	1	2	0.5	1	3599	7198	7220	14440	0.1	201134	7.18	14268	0.0138	1	0.009	15	0.0513
			0.5	2	3621	7242			0.1					1		15	
	2	2	0.5	1	3533	7066	7040	14080	0.1	201134	7.00	13908	0.0134	1	0.009	15	0.0500
			0.5	2	3507	7014			0.1					1		15	
	3	2	0.5	1	4030	8060	7990	15980	0.1	201134	7.94	15808	0.0153	1	0.009	15	0.0569
			0.5	2	3960	7920			0.1					1		15	
	4	2	0.5	1	3940	7880	7856	15712	0.1	201134	7.81	15540	0.0150	1	0.009	15	0.0559
			0.5	2	3916	7832			0.1					1		15	

Table C-4. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 1 (Con't)

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
Negative Control	1	2	0.5	1	7538	15076	15077	30154	0.1	201134	14.99	29982	0.0290	1	0.009	15	0.1078
			0.5	2	7539	15078			0.1					1		15	
	2	2	0.5	1	7322	14644	14591	29182	0.1	201134	14.51	29010	0.0280	1	0.009	15	0.1043
			0.5	2	7269	14538			0.1					1		15	
	3	2	0.5	1	7039	14078	14173	28346	0.1	201134	14.09	28174	0.0272	1	0.009	15	0.1013
			0.5	2	7134	14268			0.1					1		15	
	4	2	0.5	1	7835	15670	15183	30366	0.1	201134	15.10	30194	0.0292	1	0.009	15	0.1086
			0.5	2	7348	14696			0.1					1		15	
11343-26B	1-1	2	0.5	1	1518	3036	3080	6160	0.1	201134	3.06	5988	0.0058	1	0.009	15	0.0215
			0.5	2	1562	3124			0.1					1		15	
	1-2	2	0.5	1	1775	3550	3611	7222	0.1	201134	3.59	7050	0.0068	1	0.009	15	0.0254
			0.5	2	1836	3672			0.1					1		15	
	1-3	2	0.5	1	1784	3568	3553	7106	0.1	201134	3.53	6934	0.0067	1	0.009	15	0.0249
			0.5	2	1769	3538			0.1					1		15	
	2-1	2	0.5	1	2059	4118	4124	8248	0.1	201134	4.10	8076	0.0078	1	0.009	15	0.0290
			0.5	2	2065	4130			0.1					1		15	
	2-2	2	0.5	1	1950	3900	3867	7734	0.1	201134	3.85	7562	0.0073	1	0.009	15	0.0272
			0.5	2	1917	3834			0.1					1		15	
	2-3	2	0.5	1	2024	4048	4059	8118	0.1	201134	4.04	7946	0.0077	1	0.009	15	0.0286
			0.5	2	2035	4070			0.1					1		15	
	3-1	2	0.5	1	5764	11528	11390	22780	0.1	201134	11.33	22608	0.0218	1	0.009	15	0.0813
			0.5	2	5626	11252			0.1					1		15	
	3-2	2	0.5	1	5918	11836	11730	23460	0.1	201134	11.66	23288	0.0225	1	0.009	15	0.0838
			0.5	2	5812	11624			0.1					1		15	

Table C-4. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 1 (Con't)

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
11343-26B (con't)																	
	3-3	2	0.5	1	5738	11476	11587	23174	0.1	201134	11.52	23002	0.0222	1	0.009	15	0.0827
			0.5	2	5849	11698			0.1					1		15	
	4-1	2	0.5	1	6434	12868	12874	25748	0.1	201134	12.80	25576	0.0247	1	0.009	15	0.0920
			0.5	2	6440	12880			0.1					1		15	
	4-2	2	0.5	1	6429	12858	12789	25578	0.1	201134	12.72	25406	0.0245	1	0.009	15	0.0914
			0.5	2	6360	12720			0.1					1		15	
	4-3	2	0.5	1	6379	12758	12736	25472	0.1	201134	12.66	25300	0.0244	1	0.009	15	0.0910
			0.5	2	6357	12714			0.1					1		15	
	5-1	2	0.5	1	7299	14598	14519	29038	0.1	201134	14.44	28866	0.0279	1	0.009	15	0.1038
			0.5	2	7220	14440			0.1					1		15	
	5-2	2	0.5	1	7046	14092	14066	28132	0.1	201134	13.99	27960	0.0270	1	0.009	15	0.1006
			0.5	2	7020	14040			0.1					1		15	
	5-3	2	0.5	1	6912	13824	13819	27638	0.1	201134	13.74	27466	0.0265	1	0.009	15	0.0988
			0.5	2	6907	13814			0.1					1		15	
	6-1	2	0.5	1	7289	14578	14591	29182	0.1	201134	14.51	29010	0.0280	1	0.009	15	0.1043
			0.5	2	7302	14604			0.1					1		15	
	6-2	2	0.5	1	7309	14618	14597	29194	0.1	201134	14.51	29022	0.0280	1	0.009	15	0.1044
			0.5	2	7288	14576			0.1					1		15	
	6-3	2	0.5	1	7287	14574	14525	29050	0.1	201134	14.44	28878	0.0279	1	0.009	15	0.1039
			0.5	2	7238	14476			0.1					1		15	
	7-1	2	0.5	1	7261	14522	14602	29204	0.1	201134	14.52	29032	0.0280	1	0.009	15	0.1044
			0.5	2	7341	14682			0.1					1		15	
	7-2	2	0.5	1	7101	14202	14156	28312	0.1	201134	14.08	28140	0.0272	1	0.009	15	0.1012
			0.5	2	7055	14110			0.1					1		15	

Table C-4. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 1 (Con't)

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
11343-26B (con't)																	
	7-3	2	0.5	1	6798	13596	13886	27772	0.1	201134	13.81	27600	0.0267	1	0.009	15	0.0993
			0.5	2	7088	14176			0.1					1		15	
	8-1	2	0.5	1	7134	14268	14405	28810	0.1	201134	14.32	28638	0.0277	1	0.009	15	0.1030
			0.5	2	7271	14542			0.1					1		15	
	8-2	2	0.5	1	7365	14730	14721	29442	0.1	201134	14.64	29270	0.0283	1	0.009	15	0.1053
			0.5	2	7356	14712			0.1					1		15	
	8-3	2	0.5	1	7348	14696	14722	29444	0.1	201134	14.64	29272	0.0283	1	0.009	15	0.1053
			0.5	2	7374	14748			0.1					1		15	

Table C-5. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 2

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	8949	17898	17743	35486	0.1	203136	17.47	35310	0.0344	1	0.010	15	0.1166
			0.5	2	8794	17588			0.1					1		15	
	2	2	0.5	1	9201	18402	18269	36538	0.1	203136	17.99	36362	0.0354	1	0.010	15	0.1200
			0.5	2	9068	18136			0.1					1		15	
	3	2	0.5	1	8664	17328	17100	34200	0.1	203136	16.84	34024	0.0332	1	0.010	15	0.1123
			0.5	2	8436	16872			0.1					1		15	
	4	2	0.5	1	8808	17616	17359	34718	0.1	203136	17.09	34542	0.0337	1	0.010	15	0.1140
			0.5	2	8551	17102			0.1					1		15	
Background control	1	2	0.5	1	33	66	77	154	0.1	203136	0.08	-22	0.0000	1	0.010	15	-0.0001
			0.5	2	44	88			0.1					1		15	
	2	2	0.5	1	54	108	94	188	0.1	203136	0.09	12	0.0000	1	0.010	15	0.0000
			0.5	2	40	80			0.1					1		15	
	3	2	0.5	1	46	92	88	176	0.1	203136	0.09	0	0.0000	1	0.010	15	0.0000
			0.5	2	42	84			0.1					1		15	
	4	2	0.5	1	48	96	93	186	0.1	203136	0.09	10	0.0000	1	0.010	15	0.0000
			0.5	2	45	90			0.1					1		15	
Positive control	1	2	0.5	1	4320	8640	8390	16780	0.1	203136	8.26	16604	0.0162	1	0.010	15	0.0548
			0.5	2	4070	8140			0.1					1		15	
	2	2	0.5	1	4206	8412	8409	16818	0.1	203136	8.28	16642	0.0162	1	0.010	15	0.0549
			0.5	2	4203	8406			0.1					1		15	
	3	2	0.5	1	4086	8172	8107	16214	0.1	203136	7.98	16038	0.0156	1	0.010	15	0.0529
			0.5	2	4021	8042			0.1					1		15	
	4	2	0.5	1	4132	8264	8176	16352	0.1	203136	8.05	16176	0.0158	1	0.010	15	0.0534
			0.5	2	4044	8088			0.1					1		15	

Table C-5. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 2 (Con't)

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
Negative Control	1	2	0.5	1	8957	17914	17774	35548	0.1	203136	17.50	35372	0.0345	1	0.010	15	0.1168
			0.5	2	8817	17634			0.1					1		15	
	2	2	0.5	1	9606	19212	18749	37498	0.1	203136	18.46	37322	0.0364	1	0.010	15	0.1232
			0.5	2	9143	18286			0.1					1		15	
	3	2	0.5	1	8730	17460	17406	34812	0.1	203136	17.14	34636	0.0338	1	0.010	15	0.1143
			0.5	2	8676	17352			0.1					1		15	
	4	2	0.5	1	8128	16256	16121	32242	0.1	203136	15.87	32066	0.0313	1	0.010	15	0.1058
			0.5	2	7993	15986			0.1					1		15	
11343-26B	1-1	2	0.5	1	2306	4612	4652	9304	0.1	203136	4.58	9128	0.0089	1	0.010	15	0.0301
			0.5	2	2346	4692			0.1					1		15	
	1-2	2	0.5	1	2544	5088	4998	9996	0.1	203136	4.92	9820	0.0096	1	0.010	15	0.0324
			0.5	2	2454	4908			0.1					1		15	
	1-3	2	0.5	1	2179	4358	4408	8816	0.1	203136	4.34	8640	0.0084	1	0.010	15	0.0285
			0.5	2	2229	4458			0.1					1		15	
	2-1	2	0.5	1	2720	5440	5422	10844	0.1	203136	5.34	10668	0.0104	1	0.010	15	0.0352
			0.5	2	2702	5404			0.1					1		15	
	2-2	2	0.5	1	2846	5692	5719	11438	0.1	203136	5.63	11262	0.0110	1	0.010	15	0.0372
			0.5	2	2873	5746			0.1					1		15	
	2-3	2	0.5	1	2697	5394	5396	10792	0.1	203136	5.31	10616	0.0103	1	0.010	15	0.0350
			0.5	2	2699	5398			0.1					1		15	
	3-1	2	0.5	1	4087	8174	8161	16322	0.1	203136	8.03	16146	0.0157	1	0.010	15	0.0533
			0.5	2	4074	8148			0.1					1		15	
	3-2	2	0.5	1	4096	8192	8146	16292	0.1	203136	8.02	16116	0.0157	1	0.010	15	0.0532
			0.5	2	4050	8100			0.1					1		15	

Table C-5. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 2 (Con't)

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
11343-26B (con't)																	
	3-3	2	0.5	1	4183	8366	8373	16746	0.1	203136	8.24	16570	0.0161	1	0.010	15	0.0547
			0.5	2	4190	8380			0.1					1		15	
	4-1	2	0.5	1	5718	11436	11465	22930	0.1	203136	11.29	22754	0.0222	1	0.010	15	0.0751
			0.5	2	5747	11494			0.1					1		15	
	4-2	2	0.5	1	5586	11172	11118	22236	0.1	203136	10.95	22060	0.0215	1	0.010	15	0.0728
			0.5	2	5532	11064			0.1					1		15	
	4-3	2	0.5	1	5914	11828	11719	23438	0.1	203136	11.54	23262	0.0227	1	0.010	15	0.0768
			0.5	2	5805	11610			0.1					1		15	
	5-1	2	0.5	1	7321	14642	14605	29210	0.1	203136	14.38	29034	0.0283	1	0.010	15	0.0958
			0.5	2	7284	14568			0.1					1		15	
	5-2	2	0.5	1	7199	14398	14484	28968	0.1	203136	14.26	28792	0.0281	1	0.010	15	0.0950
			0.5	2	7285	14570			0.1					1		15	
	5-3	2	0.5	1	7214	14428	14324	28648	0.1	203136	14.10	28472	0.0277	1	0.010	15	0.0940
			0.5	2	7110	14220			0.1					1		15	
	6-1	2	0.5	1	7992	15984	15796	31592	0.1	203136	15.55	31416	0.0306	1	0.010	15	0.1037
			0.5	2	7804	15608			0.1					1		15	
	6-2	2	0.5	1	7860	15720	15689	31378	0.1	203136	15.45	31202	0.0304	1	0.010	15	0.1030
			0.5	2	7829	15658			0.1					1		15	
	6-3	2	0.5	1	7678	15356	15451	30902	0.1	203136	15.21	30726	0.0299	1	0.010	15	0.1014
			0.5	2	7773	15546			0.1					1		15	
	7-1	2	0.5	1	8558	17116	17085	34170	0.1	203136	16.82	33994	0.0331	1	0.010	15	0.1122
			0.5	2	8527	17054			0.1					1		15	

Table C-5. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 2 (Con't)

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
11343-26B (con't)																	
	7-2	2	0.5	1	8586	17172	17280	34560	0.1	203136	17.01	34384	0.0335	1	0.010	15	0.1135
			0.5	2	8694	17388			0.1					1		15	
	7-3	2	0.5	1	8502	17004	16936	33872	0.1	203136	16.67	33696	0.0328	1	0.010	15	0.1112
			0.5	2	8434	16868			0.1					1		15	
	8-1	2	0.5	1	8539	17078	17245	34490	0.1	203136	16.98	34314	0.0334	1	0.010	15	0.1133
			0.5	2	8706	17412			0.1					1		15	
	8-2	2	0.5	1	8519	17038	17008	34016	0.1	203136	16.75	33840	0.0330	1	0.010	15	0.1117
			0.5	2	8489	16978			0.1					1		15	
	8-3	2	0.5	1	8559	17118	16997	33994	0.1	203136	16.73	33818	0.0330	1	0.010	15	0.1116
			0.5	2	8438	16876			0.1					1		15	

Table C-6. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 3

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	7524	15048	15045	30090	0.1	190100	15.83	29939	0.0312	1	0.012	15	0.0838
			0.5	2	7521	15042			0.1					1		15	
	2	2	0.5	1	7393	14786	14939	29878	0.1	190100	15.72	29727	0.0309	1	0.012	15	0.0832
			0.5	2	7546	15092			0.1					1		15	
	3	2	0.5	1	7156	14312	14527	29054	0.1	190100	15.28	28903	0.0301	1	0.012	15	0.0809
			0.5	2	7371	14742			0.1					1		15	
	4	2	0.5	1	6966	13932	13999	27998	0.1	190100	14.73	27847	0.0290	1	0.012	15	0.0780
			0.5	2	7033	14066			0.1					1		15	
Background control	1	2	0.5	1	28	56	66	132	0.1	190100	0.07	-20	0.0000	1	0.012	15	-0.0001
			0.5	2	38	76			0.1					1		15	
	2	2	0.5	1	42	84	85	170	0.1	190100	0.09	19	0.0000	1	0.012	15	0.0001
			0.5	2	43	86			0.1					1		15	
	3	2	0.5	1	35	70	75	150	0.1	190100	0.08	-2	0.0000	1	0.012	15	0.0000
			0.5	2	40	80			0.1					1		15	
	4	2	0.5	1	36	72	77	154	0.1	190100	0.08	3	0.0000	1	0.012	15	0.0000
			0.5	2	41	82			0.1					1		15	
Positive control	1	2	0.5	1	3703	7406	7247	14494	0.1	190100	7.62	14343	0.0149	1	0.012	15	0.0402
			0.5	2	3544	7088			0.1					1		15	
	2	2	0.5	1	3525	7050	7056	14112	0.1	190100	7.42	13961	0.0145	1	0.012	15	0.0391
			0.5	2	3531	7062			0.1					1		15	
	3	2	0.5	1	3186	6372	6467	12934	0.1	190100	6.80	12783	0.0133	1	0.012	15	0.0358
			0.5	2	3281	6562			0.1					1		15	
	4	2	0.5	1	3336	6672	6723	13446	0.1	190100	7.07	13295	0.0138	1	0.012	15	0.0372
			0.5	2	3387	6774			0.1					1		15	

Table C-6. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 3 (Con't)

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
Negative Control	1	2	0.5	1	7021	14042	14274	28548	0.1	190100	15.02	28397	0.0295	1	0.012	15	0.0795
			0.5	2	7253	14506			0.1					1		15	
	2	2	0.5	1	7207	14414	14249	28498	0.1	190100	14.99	28347	0.0295	1	0.012	15	0.0794
			0.5	2	7042	14084			0.1					1		15	
	3	2	0.5	1	6934	13868	13842	27684	0.1	190100	14.56	27533	0.0287	1	0.012	15	0.0771
			0.5	2	6908	13816			0.1					1		15	
	4	2	0.5	1	6868	13736	13824	27648	0.1	190100	14.54	27497	0.0286	1	0.012	15	0.0770
			0.5	2	6956	13912			0.1					1		15	
11343-26B	1-1	2	0.5	1	1471	2942	2964	5928	0.1	190100	3.12	5777	0.0060	1	0.012	15	0.0162
			0.5	2	1493	2986			0.1					1		15	
	1-2	2	0.5	1	1780	3560	3573	7146	0.1	190100	3.76	6995	0.0073	1	0.012	15	0.0196
			0.5	2	1793	3586			0.1					1		15	
	1-3	2	0.5	1	1771	3542	3582	7164	0.1	190100	3.77	7013	0.0073	1	0.012	15	0.0196
			0.5	2	1811	3622			0.1					1		15	
	2-1	2	0.5	1	2056	4112	4152	8304	0.1	190100	4.37	8153	0.0085	1	0.012	15	0.0228
			0.5	2	2096	4192			0.1					1		15	
	2-2	2	0.5	1	1974	3948	3985	7970	0.1	190100	4.19	7819	0.0081	1	0.012	15	0.0219
			0.5	2	2011	4022			0.1					1		15	
	2-3	2	0.5	1	2088	4176	4146	8292	0.1	190100	4.36	8141	0.0085	1	0.012	15	0.0228
			0.5	2	2058	4116			0.1					1		15	
	3-1	2	0.5	1	3207	6414	6399	12798	0.1	190100	6.73	12647	0.0132	1	0.012	15	0.0354
			0.5	2	3192	6384			0.1					1		15	
	3-2	2	0.5	1	3255	6510	6490	12980	0.1	190100	6.83	12829	0.0133	1	0.012	15	0.0359
			0.5	2	3235	6470			0.1					1		15	

Table C-6. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 3 (Con't)

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
11343-26B (con't)	3-3	2	0.5	1	3236	6472	6531	13062	0.1	190100	6.87	12911	0.0134	1	0.012	15	0.0362
			0.5	2	3295	6590			0.1					1		15	
	4-1	2	0.5	1	4438	8876	8752	17504	0.1	190100	9.21	17353	0.0181	1	0.012	15	0.0486
			0.5	2	4314	8628			0.1					1		15	
	4-2	2	0.5	1	4479	8958	8810	17620	0.1	190100	9.27	17469	0.0182	1	0.012	15	0.0489
			0.5	2	4331	8662			0.1					1		15	
	4-3	2	0.5	1	4370	8740	8793	17586	0.1	190100	9.25	17435	0.0181	1	0.012	15	0.0488
			0.5	2	4423	8846			0.1					1		15	
	5-1	2	0.5	1	5534	11068	11218	22436	0.1	190100	11.80	22285	0.0232	1	0.012	15	0.0624
			0.5	2	5684	11368			0.1					1		15	
	5-2	2	0.5	1	5514	11028	10990	21980	0.1	190100	11.56	21829	0.0227	1	0.012	15	0.0611
			0.5	2	5476	10952			0.1					1		15	
	5-3	2	0.5	1	5691	11382	11399	22798	0.1	190100	11.99	22647	0.0236	1	0.012	15	0.0634
			0.5	2	5708	11416			0.1					1		15	
	6-1	2	0.5	1	6080	12160	12231	24462	0.1	190100	12.87	24311	0.0253	1	0.012	15	0.0681
			0.5	2	6151	12302			0.1					1		15	
	6-2	2	0.5	1	6467	12934	12711	25422	0.1	190100	13.37	25271	0.0263	1	0.012	15	0.0708
			0.5	2	6244	12488			0.1					1		15	
	6-3	2	0.5	1	6059	12118	12264	24528	0.1	190100	12.90	24377	0.0254	1	0.012	15	0.0683
			0.5	2	6205	12410			0.1					1		15	
	7-1	2	0.5	1	7182	14364	14252	28504	0.1	190100	14.99	28353	0.0295	1	0.012	15	0.0794
			0.5	2	7070	14140			0.1					1		15	
	7-2	2	0.5	1	6878	13756	13810	27620	0.1	190100	14.53	27469	0.0286	1	0.012	15	0.0769
			0.5	2	6932	13864			0.1					1		15	

Table C-6. Data for the Calculation of Aromatase Activity: Chrysin, Replicate 3 (Con't)

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
11343-26B (con't)	7-3	2	0.5	1	6932	13864	14031	28062	0.1	190100	14.76	27911	0.0290	1	0.012	15	0.0782
			0.5	2	7099	14198			0.1					1		15	
	8-1	2	0.5	1	6590	13180	13823	27646	0.1	190100	14.54	27495	0.0286	1	0.012	15	0.0770
			0.5	2	7233	14466			0.1					1		15	
	8-2	2	0.5	1	7173	14346	14336	28672	0.1	190100	15.08	28521	0.0297	1	0.012	15	0.0799
			0.5	2	7163	14326			0.1					1		15	
	8-3	2	0.5	1	7217	14434	14435	28870	0.1	190100	15.19	28719	0.0299	1	0.012	15	0.0804
			0.5	2	7218	14436			0.1					1		15	

Table C-7. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 1

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	9634	19268	19412	38824	0.1	192759	20.14	38716	0.0407	1	0.011	15	0.1233
			0.5	2	9778	19556											
	2	2	0.5	1	9731	19462	19534	39068	0.1	192759	20.27	38960	0.0410	1	0.011	15	0.1241
			0.5	2	9803	19606											
	3	2	0.5	1	9577	19154	19241	38482	0.1	192759	19.96	38374	0.0403	1	0.011	15	0.1222
			0.5	2	9664	19328											
	4	2	0.5	1	9378	18756	18683	37366	0.1	192759	19.38	37258	0.0392	1	0.011	15	0.1187
			0.5	2	9305	18610											
Background control	1	2	0.5	1	23	46	50	100	0.1	192759	0.05	-8	0.0000	1	0.011	15	0.0000
			0.5	2	27	54											
	2	2	0.5	1	24	48	45	90	0.1	192759	0.05	-18	0.0000	1	0.011	15	-0.0001
			0.5	2	21	42											
	3	2	0.5	1	30	60	59	118	0.1	192759	0.06	10	0.0000	1	0.011	15	0.0000
			0.5	2	29	58											
	4	2	0.5	1	29	58	62	124	0.1	192759	0.06	16	0.0000	1	0.011	15	0.0001
			0.5	2	33	66											
Positive control	1	2	0.5	1	4691	9382	9428	18856	0.1	192759	9.78	18748	0.0197	1	0.011	15	0.0597
			0.5	2	4737	9474											
	2	2	0.5	1	4380	8760	8851	17702	0.1	192759	9.18	17594	0.0185	1	0.011	15	0.0560
			0.5	2	4471	8942											
	3	2	0.5	1	4328	8656	8577	17154	0.1	192759	8.90	17046	0.0179	1	0.011	15	0.0543
			0.5	2	4249	8498											
	4	2	0.5	1	4295	8590	8612	17224	0.1	192759	8.94	17116	0.0180	1	0.011	15	0.0545
			0.5	2	4317	8634											

Table C-7. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 1 (Con't)

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
Negative Control	1	2	0.5	1	9826	19652	19544	39088	0.1	192759	20.28	38980	0.0410	1	0.011	15	0.1241
			0.5	2	9718	19436											
	2	2	0.5	1	9719	19438	19708	39416	0.1	192759	20.45	39308	0.0413	1	0.011	15	0.1252
			0.5	2	9989	19978											
	3	2	0.5	1	9521	19042	18721	37442	0.1	192759	19.42	37334	0.0392	1	0.011	15	0.1189
			0.5	2	9200	18400											
	4	2	0.5	1	9055	18110	17934	35868	0.1	192759	18.61	35760	0.0376	1	0.011	15	0.1139
			0.5	2	8879	17758											
11343-29A Ref Chem 3	1-1	2	0.5	1	623	1246	1227	2454	0.1	192759	1.27	2346	0.0025	1	0.011	15	0.0075
			0.5	2	604	1208											
	1-2	2	0.5	1	643	1286	1252	2504	0.1	192759	1.30	2396	0.0025	1	0.011	15	0.0076
			0.5	2	609	1218											
	1-3	2	0.5	1	613	1226	1178	2356	0.1	192759	1.22	2248	0.0024	1	0.011	15	0.0072
			0.5	2	565	1130											
	2-1	2	0.5	1	4026	8052	8119	16238	0.1	192759	8.42	16130	0.0170	1	0.011	15	0.0514
			0.5	2	4093	8186											
	2-2	2	0.5	1	4257	8514	8295	16590	0.1	192759	8.61	16482	0.0173	1	0.011	15	0.0525
			0.5	2	4038	8076											
	2-3	2	0.5	1	4168	8336	8288	16576	0.1	192759	8.60	16468	0.0173	1	0.011	15	0.0524
			0.5	2	4120	8240											
	3-1	2	0.5	1	8530	17060	16869	33738	0.1	192759	17.50	33630	0.0354	1	0.011	15	0.1071
			0.5	2	8339	16678											
	3-2	2	0.5	1	8698	17396	17166	34332	0.1	192759	17.81	34224	0.0360	1	0.011	15	0.1090
			0.5	2	8468	16936											

Table C-7. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 1 (Con't)

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
11343-29A Ref Chem 3 (con't)	3-3	2	0.5	1	8482	16964	16808	33616	0.1	192759	17.44	33508	0.0352	1	0.011	15	0.1067
			0.5	2	8326	16652											
	4-1	2	0.5	1	9895	19790	19292	38584	0.1	192759	20.02	38476	0.0404	1	0.011	15	0.1225
			0.5	2	9397	18794											
	4-2	2	0.5	1	9920	19840	19647	39294	0.1	192759	20.39	39186	0.0412	1	0.011	15	0.1248
			0.5	2	9727	19454											
	4-3	2	0.5	1	9415	18830	18918	37836	0.1	192759	19.63	37728	0.0397	1	0.011	15	0.1202
			0.5	2	9503	19006											
	5-1	2	0.5	1	9856	19712	19722	39444	0.1	192759	20.46	39336	0.0414	1	0.011	15	0.1253
			0.5	2	9866	19732											
	5-2	2	0.5	1	9725	19450	19254	38508	0.1	192759	19.98	38400	0.0404	1	0.011	15	0.1223
			0.5	2	9529	19058											
	5-3	2	0.5	1	9280	18560	18968	37936	0.1	192759	19.68	37828	0.0398	1	0.011	15	0.1205
			0.5	2	9688	19376											
	6-1	2	0.5	1	9649	19298	19423	38846	0.1	192759	20.15	38738	0.0407	1	0.011	15	0.1234
			0.5	2	9774	19548											
	6-2	2	0.5	1	9612	19224	18951	37902	0.1	192759	19.66	37794	0.0397	1	0.011	15	0.1204
			0.5	2	9339	18678											
	6-3	2	0.5	1	9626	19252	19154	38308	0.1	192759	19.87	38200	0.0402	1	0.011	15	0.1217
			0.5	2	9528	19056											
	7-1	2	0.5	1	9645	19290	19353	38706	0.1	192759	20.08	38598	0.0406	1	0.011	15	0.1229
			0.5	2	9708	19416											

Table C-7. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 1 (Con't)

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
11343-29A Ref Chem 3 (con't)	7-2	2	0.5	1	9818	19636	19591	39182	0.1	192759	20.33	39074	0.0411	1	0.011	15	0.1244
			0.5	2	9773	19546											
	7-3	2	0.5	1	9496	18992	19099	38198	0.1	192759	19.82	38090	0.0400	1	0.011	15	0.1213
			0.5	2	9603	19206											
	8-1	2	0.5	1	9618	19236	19330	38660	0.1	192759	20.06	38552	0.0405	1	0.011	15	0.1228
			0.5	2	9712	19424											
	8-2	2	0.5	1	9567	19134	19221	38442	0.1	192759	19.94	38334	0.0403	1	0.011	15	0.1221
			0.5	2	9654	19308											
	8-3	2	0.5	1	9041	18082	18029	36058	0.1	192759	18.71	35950	0.0378	1	0.011	15	0.1145
			0.5	2	8988	17976											

Table C-8. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 2

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	8616	17232	17052	34104	0.1	181626	18.78	33873	0.0392	1	0.012	15	0.1062
			0.5	2	8436	16872											
	2	2	0.5	1	8618	17236	17053	34106	0.1	181626	18.78	33875	0.0392	1	0.012	15	0.1062
			0.5	2	8435	16870											
	3	2	0.5	1	8293	16586	16422	32844	0.1	181626	18.08	32613	0.0377	1	0.012	15	0.1022
			0.5	2	8129	16258											
	4	2	0.5	1	7633	15266	15498	30996	0.1	181626	17.07	30765	0.0356	1	0.012	15	0.0964
			0.5	2	7865	15730											
Background control	1	2	0.5	1	30	60	75	150	0.1	181626	0.08	-81	-0.0001	1	0.012	15	-0.0003
			0.5	2	45	90											
	2	2	0.5	1	26	52	51	102	0.1	181626	0.06	-129	-0.0001	1	0.012	15	-0.0004
			0.5	2	25	50											
	3	2	0.5	1	61	122	131	262	0.1	181626	0.14	31	0.0000	1	0.012	15	0.0001
			0.5	2	70	140											
	4	2	0.5	1	92	184	205	410	0.1	181626	0.23	179	0.0002	1	0.012	15	0.0006
			0.5	2	113	226											
Positive control	1	2	0.5	1	4012	8024	7971	15942	0.1	181626	8.78	15711	0.0182	1	0.012	15	0.0492
			0.5	2	3959	7918											
	2	2	0.5	1	3977	7954	7850	15700	0.1	181626	8.64	15469	0.0179	1	0.012	15	0.0485
			0.5	2	3873	7746											
	3	2	0.5	1	1501	3002	2903	5806	0.1	181626	3.20	5575	0.0065	1	0.012	15	0.0175
			0.5	2	1402	2804											
	4	2	0.5	1	3652	7304	7198	14396	0.1	181626	7.93	14165	0.0164	1	0.012	15	0.0444
			0.5	2	3546	7092											

Table C-8. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 2 (Con't)

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
Negative Control	1	2	0.5	1	8586	17172	16932	33864	0.1	181626	18.64	33633	0.0389	1	0.012	15	0.1054
			0.5	2	8346	16692											
	2	2	0.5	1	8605	17210	17025	34050	0.1	181626	18.75	33819	0.0391	1	0.012	15	0.1060
			0.5	2	8420	16840											
	3	2	0.5	1	7709	15418	15455	30910	0.1	181626	17.02	30679	0.0355	1	0.012	15	0.0961
			0.5	2	7746	15492											
	4	2	0.5	1	7737	15474	15408	30816	0.1	181626	16.97	30585	0.0354	1	0.012	15	0.0958
			0.5	2	7671	15342											
11343-29a Chemical 3	1-1	2	0.5	1	569	1138	1127	2254	0.1	181626	1.24	2023	0.0023	1	0.012	15	0.0063
			0.5	2	558	1116											
	1-2	2	0.5	1	577	1154	1092	2184	0.1	181626	1.20	1953	0.0023	1	0.012	15	0.0061
			0.5	2	515	1030											
	1-3	2	0.5	1	538	1076	1056	2112	0.1	181626	1.16	1881	0.0022	1	0.012	15	0.0059
			0.5	2	518	1036											
	2-1	2	0.5	1	1053	2106	2146	4292	0.1	181626	2.36	4061	0.0047	1	0.012	15	0.0127
			0.5	2	1093	2186											
	2-2	2	0.5	1	1054	2108	2146	4292	0.1	181626	2.36	4061	0.0047	1	0.012	15	0.0127
			0.5	2	1092	2184											
	2-3	2	0.5	1	1047	2094	2037	4074	0.1	181626	2.24	3843	0.0044	1	0.012	15	0.0120
			0.5	2	990	1980											
	3-1	2	0.5	1	1925	3850	3909	7818	0.1	181626	4.30	7587	0.0088	1	0.012	15	0.0238
			0.5	2	1984	3968											
	3-2	2	0.5	1	1914	3828	3812	7624	0.1	181626	4.20	7393	0.0086	1	0.012	15	0.0232
			0.5	2	1898	3796											

Table C-8. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 2 (Con't)

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
11343-29a Chemical 3 (con't)	3-3	2	0.5	1	1887	3774	3715	7430	0.1	181626	4.09	7199	0.0083	1	0.012	15	0.0226
			0.5	2	1828	3656											
	4-1	2	0.5	1	3662	7324	7204	14408	0.1	181626	7.93	14177	0.0164	1	0.012	15	0.0444
			0.5	2	3542	7084											
	4-2	2	0.5	1	3509	7018	7019	14038	0.1	181626	7.73	13807	0.0160	1	0.012	15	0.0433
			0.5	2	3510	7020											
	4-3	2	0.5	1	3282	6564	6498	12996	0.1	181626	7.16	12765	0.0148	1	0.012	15	0.0400
			0.5	2	3216	6432											
	5-1	2	0.5	1	4846	9692	9626	19252	0.1	181626	10.60	19021	0.0220	1	0.012	15	0.0596
			0.5	2	4780	9560											
	5-2	2	0.5	1	4859	9718	9559	19118	0.1	181626	10.53	18887	0.0219	1	0.012	15	0.0592
			0.5	2	4700	9400											
	5-3	2	0.5	1	4664	9328	9249	18498	0.1	181626	10.18	18267	0.0211	1	0.012	15	0.0572
			0.5	2	4585	9170											
	6-1	2	0.5	1	7205	14410	14321	28642	0.1	181626	15.77	28411	0.0329	1	0.012	15	0.0890
			0.5	2	7116	14232											
	6-2	2	0.5	1	7332	14664	14374	28748	0.1	181626	15.83	28517	0.0330	1	0.012	15	0.0894
			0.5	2	7042	14084											
	6-3	2	0.5	1	6905	13810	13850	27700	0.1	181626	15.25	27469	0.0318	1	0.012	15	0.0861
			0.5	2	6945	13890											
	7-1	2	0.5	1	8265	16530	16422	32844	0.1	181626	18.08	32613	0.0377	1	0.012	15	0.1022
			0.5	2	8157	16314											
	7-2	2	0.5	1	8095	16190	16069	32138	0.1	181626	17.69	31907	0.0369	1	0.012	15	0.1000
			0.5	2	7974	15948											

Table C-8. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 2 (Con't)

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
11343-29a Chemical 3 (con't)	7-3	2	0.5	1	7739	15478	15439	30878	0.1	181626	17.00	30647	0.0355	1	0.012	15	0.0960
			0.5	2	7700	15400											
	8-1	2	0.5	1	8334	16668	16451	32902	0.1	181626	18.12	32671	0.0378	1	0.012	15	0.1024
			0.5	2	8117	16234											
	8-2	2	0.5	1	8335	16670	16690	33380	0.1	181626	18.38	33149	0.0384	1	0.012	15	0.1039
			0.5	2	8355	16710											
	8-3	2	0.5	1	7557	15114	15039	30078	0.1	181626	16.56	29847	0.0345	1	0.012	15	0.0935
			0.5	2	7482	14964											

Table C-9. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 3

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	9614	19228	19026	38052	0.1	185009	20.57	37931	0.0442	1	0.014	15	0.1063
			0.5	2	9412	18824			0.1					1		15	
	2	2	0.5	1	9057	18114	18389	36778	0.1	185009	19.88	36657	0.0427	1	0.014	15	0.1027
			0.5	2	9332	18664			0.1					1		15	
	3	2	0.5	1	8803	17606	17527	35054	0.1	185009	18.95	34933	0.0407	1	0.014	15	0.0979
			0.5	2	8724	17448			0.1					1		15	
	4	2	0.5	1	8769	17538	17501	35002	0.1	185009	18.92	34881	0.0406	1	0.014	15	0.0978
			0.5	2	8732	17464			0.1					1		15	
Background control	1	2	0.5	1	31	62	59	118	0.1	185009	0.06	-4	0.0000	1	0.014	15	0.0000
			0.5	2	28	56			0.1					1		15	
	2	2	0.5	1	34	68	62	124	0.1	185009	0.07	3	0.0000	1	0.014	15	0.0000
			0.5	2	28	56			0.1					1		15	
	3	2	0.5	1	28	56	64	128	0.1	185009	0.07	7	0.0000	1	0.014	15	0.0000
			0.5	2	36	72			0.1					1		15	
	4	2	0.5	1	31	62	58	116	0.1	185009	0.06	-6	0.0000	1	0.014	15	0.0000
			0.5	2	27	54			0.1					1		15	
Positive control	1	2	0.5	1	4618	9236	9083	18166	0.1	185009	9.82	18045	0.0210	1	0.014	15	0.0506
			0.5	2	4465	8930			0.1					1		15	
	2	2	0.5	1	4236	8472	8512	17024	0.1	185009	9.20	16903	0.0197	1	0.014	15	0.0474
			0.5	2	4276	8552			0.1					1		15	
	3	2	0.5	1	3916	7832	7829	15658	0.1	185009	8.46	15537	0.0181	1	0.014	15	0.0435
			0.5	2	3913	7826			0.1					1		15	

Table C-9. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 3 (Con't)

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
Positive Control (con't)	4	2	0.5	1	4177	8354	8232	16464	0.1	185009	8.90	16343	0.0190	1	0.014	15	0.0458
			0.5	2	4055	8110			0.1					1		15	
Negative Control	1	2	0.5	1	9265	18530	18302	36604	0.1	185009	19.78	36483	0.0425	1	0.014	15	0.1023
			0.5	2	9037	18074			0.1					1		15	
	2	2	0.5	1	9235	18470	18362	36724	0.1	185009	19.85	36603	0.0426	1	0.014	15	0.1026
			0.5	2	9127	18254			0.1					1		15	
	3	2	0.5	1	8625	17250	17277	34554	0.1	185009	18.68	34433	0.0401	1	0.014	15	0.0965
			0.5	2	8652	17304			0.1					1		15	
	4	2	0.5	1	8256	16512	16487	32974	0.1	185009	17.82	32853	0.0383	1	0.014	15	0.0921
			0.5	2	8231	16462			0.1					1		15	
11343-29A Ref chem 3	1-1	2	0.5	1	557	1114	1128	2256	0.1	185009	1.22	2135	0.0025	1	0.014	15	0.0060
			0.5	2	571	1142			0.1					1		15	
	1-2	2	0.5	1	634	1268	1253	2506	0.1	185009	1.35	2385	0.0028	1	0.014	15	0.0067
			0.5	2	619	1238			0.1					1		15	
	1-3	2	0.5	1	648	1296	1244	2488	0.1	185009	1.34	2367	0.0028	1	0.014	15	0.0066
			0.5	2	596	1192			0.1					1		15	
	2-1	2	0.5	1	1217	2434	2421	4842	0.1	185009	2.62	4721	0.0055	1	0.014	15	0.0132
			0.5	2	1204	2408			0.1					1		15	
	2-2	2	0.5	1	1195	2390	2423	4846	0.1	185009	2.62	4725	0.0055	1	0.014	15	0.0132
			0.5	2	1228	2456			0.1					1		15	
	2-3	2	0.5	1	1193	2386	2321	4642	0.1	185009	2.51	4521	0.0053	1	0.014	15	0.0127
			0.5	2	1128	2256			0.1					1		15	

Table C-9. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 3 (Con't)

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
11343-29A Ref chem 3 (con't)	3-1	2	0.5	1	10100	20200	20259	40518	0.1	185009	21.90	40397	0.0471	1	0.014	15	0.1132
			0.5	2	10159	20318			0.1					1		15	
	3-2	2	0.5	1	9733	19466	19464	38928	0.1	185009	21.04	38807	0.0452	1	0.014	15	0.1088
			0.5	2	9731	19462			0.1					1		15	
	3-3	2	0.5	1	9704	19408	19410	38820	0.1	185009	20.98	38699	0.0451	1	0.014	15	0.1085
			0.5	2	9706	19412			0.1					1		15	
	4-1	2	0.5	1	1594	3188	3203	6406	0.1	185009	3.46	6285	0.0073	1	0.014	15	0.0176
			0.5	2	1609	3218			0.1					1		15	
	4-2	2	0.5	1	1650	3300	3227	6454	0.1	185009	3.49	6333	0.0074	1	0.014	15	0.0177
			0.5	2	1577	3154			0.1					1		15	
	4-3	2	0.5	1	1593	3186	3147	6294	0.1	185009	3.40	6173	0.0072	1	0.014	15	0.0173
			0.5	2	1554	3108			0.1					1		15	
	5-1	2	0.5	1	5462	10924	10795	21590	0.1	185009	11.67	21469	0.0250	1	0.014	15	0.0602
			0.5	2	5333	10666			0.1					1		15	
	5-2	2	0.5	1	5339	10678	10686	21372	0.1	185009	11.55	21251	0.0248	1	0.014	15	0.0596
			0.5	2	5347	10694			0.1					1		15	
	5-3	2	0.5	1	5290	10580	10381	20762	0.1	185009	11.22	20641	0.0240	1	0.014	15	0.0578
			0.5	2	5091	10182			0.1					1		15	
	6-1	2	0.5	1	8082	16164	16119	32238	0.1	185009	17.43	32117	0.0374	1	0.014	15	0.0900
			0.5	2	8037	16074			0.1					1		15	
	6-2	2	0.5	1	7675	15350	15446	30892	0.1	185009	16.70	30771	0.0358	1	0.014	15	0.0862
			0.5	2	7771	15542			0.1					1		15	
	6-3	2	0.5	1	7832	15664	15601	31202	0.1	185009	16.87	31081	0.0362	1	0.014	15	0.0871
			0.5	2	7769	15538			0.1					1		15	

Table C-9. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 3 (Con't)

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
11343-29A Ref chem 3 (con't)	7-1	2	0.5	1	9126	18252	18326	36652	0.1	185009	19.81	36531	0.0426	1	0.014	15	0.1024
			0.5	2	9200	18400			0.1					1		15	
	7-2	2	0.5	1	8805	17610	17555	35110	0.1	185009	18.98	34989	0.0408	1	0.014	15	0.0981
			0.5	2	8750	17500			0.1					1		15	
	7-3	2	0.5	1	8368	16736	16613	33226	0.1	185009	17.96	33105	0.0386	1	0.014	15	0.0928
			0.5	2	8245	16490			0.1					1		15	
	8-1	2	0.5	1	9159	18318	18262	36524	0.1	185009	19.74	36403	0.0424	1	0.014	15	0.1020
			0.5	2	9103	18206			0.1					1		15	
	8-2	2	0.5	1	8693	17386	17408	34816	0.1	185009	18.82	34695	0.0404	1	0.014	15	0.0972
			0.5	2	8715	17430			0.1					1		15	
	8-3	2	0.5	1	8006	16012	15981	31962	0.1	185009	17.28	31841	0.0371	1	0.014	15	0.0892
			0.5	2	7975	15950			0.1					1		15	

Table C-10. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 4

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	5228	10456	10481	20962	0.1	91894	22.81	20910	0.0458	1	0.012	15	0.1311
			0.5	2	5253	10506											
	2	2	0.5	1	5373	10746	10624	21248	0.1	91894	23.12	21196	0.0465	1	0.012	15	0.1329
			0.5	2	5251	10502											
	3	2	0.5	1	4906	9812	9720	19440	0.1	91894	21.15	19388	0.0425	1	0.012	15	0.1215
			0.5	2	4814	9628											
	4	2	0.5	1	4967	9934	9890	19780	0.1	91894	21.52	19728	0.0432	1	0.012	15	0.1237
			0.5	2	4923	9846											
Background control	1	2	0.5	1	9	18	16	32	0.1	91894	0.03	-21	0.0000	1	0.012	15	-0.0001
			0.5	2	7	14											
	2	2	0.5	1	18	36	31	62	0.1	91894	0.07	10	0.0000	1	0.012	15	0.0001
			0.5	2	13	26											
	3	2	0.5	1	9	18	19	38	0.1	91894	0.04	-15	0.0000	1	0.012	15	-0.0001
			0.5	2	10	20											
	4	2	0.5	1	18	36	39	78	0.1	91894	0.08	26	0.0001	1	0.012	15	0.0002
			0.5	2	21	42											
Positive control	1	2	0.5	1	2380	4760	4693	9386	0.1	91894	10.21	9334	0.0205	1	0.012	15	0.0585
			0.5	2	2313	4626											
	2	2	0.5	1	2224	4448	4551	9102	0.1	91894	9.90	9050	0.0198	1	0.012	15	0.0567
			0.5	2	2327	4654											
	3	2	0.5	1	2120	4240	4249	8498	0.1	91894	9.25	8446	0.0185	1	0.012	15	0.0529
			0.5	2	2129	4258											
	4	2	0.5	1	2157	4314	4327	8654	0.1	91894	9.42	8602	0.0189	1	0.012	15	0.0539

Table C-10. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 4 (Con't)

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
Positive control (con't)			0.5	2	2170	4340											
Negative Control	1	2	0.5	1	5022	10044	10191	20382	0.1	91894	22.18	20330	0.0446	1	0.012	15	0.1274
			0.5	2	5169	10338											
	2	2	0.5	1	5193	10386	10256	20512	0.1	91894	22.32	20460	0.0448	1	0.012	15	0.1283
			0.5	2	5063	10126											
	3	2	0.5	1	4922	9844	9779	19558	0.1	91894	21.28	19506	0.0428	1	0.012	15	0.1223
			0.5	2	4857	9714											
	4	2	0.5	1	4740	9480	9339	18678	0.1	91894	20.33	18626	0.0408	1	0.012	15	0.1168
			0.5	2	4599	9198											
Ref Chem 3 11343-29A	1-1	2	0.5	1	334	668	659	1318	0.1	91894	1.43	1266	0.0028	1	0.012	15	0.0079
			0.5	2	325	650											
	1-2	2	0.5	1	330	660	643	1286	0.1	91894	1.40	1234	0.0027	1	0.012	15	0.0077
			0.5	2	313	626											
	1-3	2	0.5	1	308	616	640	1280	0.1	91894	1.39	1228	0.0027	1	0.012	15	0.0077
			0.5	2	332	664											
	2-1	2	0.5	1	723	1446	1458	2916	0.1	91894	3.17	2864	0.0063	1	0.012	15	0.0180
			0.5	2	735	1470											
	2-2	2	0.5	1	705	1410	1357	2714	0.1	91894	2.95	2662	0.0058	1	0.012	15	0.0167
			0.5	2	652	1304											
	2-3	2	0.5	1	709	1418	1419	2838	0.1	91894	3.09	2786	0.0061	1	0.012	15	0.0175
			0.5	2	710	1420											
	3-1	2	0.5	1	1179	2358	2403	4806	0.1	91894	5.23	4754	0.0104	1	0.012	15	0.0298
			0.5	2	1224	2448											

Table C-10. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 4 (Con't)

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
Ref Chem 3 11343-29A (con't)	3-2	2	0.5	1	1206	2412	2384	4768	0.1	91894	5.19	4716	0.0103	1	0.012	15	0.0296
			0.5	2	1178	2356											
	3-3	2	0.5	1	1133	2266	2306	4612	0.1	91894	5.02	4560	0.0100	1	0.012	15	0.0286
			0.5	2	1173	2346											
	4-1	2	0.5	1	2248	4496	4425	8850	0.1	91894	9.63	8798	0.0193	1	0.012	15	0.0551
			0.5	2	2177	4354											
	4-2	2	0.5	1	2220	4440	4399	8798	0.1	91894	9.57	8746	0.0192	1	0.012	15	0.0548
			0.5	2	2179	4358											
	4-3	2	0.5	1	2076	4152	4146	8292	0.1	91894	9.02	8240	0.0181	1	0.012	15	0.0517
			0.5	2	2070	4140											
	5-1	2	0.5	1	3069	6138	6134	12268	0.1	91894	13.35	12216	0.0268	1	0.012	15	0.0766
			0.5	2	3065	6130											
	5-2	2	0.5	1	2938	5876	5910	11820	0.1	91894	12.86	11768	0.0258	1	0.012	15	0.0738
			0.5	2	2972	5944											
	5-3	2	0.5	1	2969	5938	5919	11838	0.1	91894	12.88	11786	0.0258	1	0.012	15	0.0739
			0.5	2	2950	5900											
	6-1	2	0.5	1	4370	8740	8873	17746	0.1	91894	19.31	17694	0.0388	1	0.012	15	0.1109
			0.5	2	4503	9006											
	6-2	2	0.5	1	4377	8754	8767	17534	0.1	91894	19.08	17482	0.0383	1	0.012	15	0.1096
			0.5	2	4390	8780											
	6-3	2	0.5	1	4295	8590	8552	17104	0.1	91894	18.61	17052	0.0374	1	0.012	15	0.1069
			0.5	2	4257	8514											
	7-1	2	0.5	1	5081	10162	10197	20394	0.1	91894	22.19	20342	0.0446	1	0.012	15	0.1275
			0.5	2	5116	10232											

Table C-10. Data for the Calculation of Aromatase Activity: Ketoconazole, Replicate 4 (Con't)

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
Ref Chem 3 11343-29A (con't)	7-2	2	0.5	1	5031	10062	10026	20052	0.1	91894	21.82	20000	0.0438	1	0.012	15	0.1254
			0.5	2	4995	9990											
	7-3	2	0.5	1	4911	9822	9650	19300	0.1	91894	21.00	19248	0.0422	1	0.012	15	0.1207
			0.5	2	4739	9478											
	8-1	2	0.5	1	5107	10214	10254	20508	0.1	91894	22.32	20456	0.0448	1	0.012	15	0.1282
			0.5	2	5147	10294											
	8-2	2	0.5	1	5082	10164	10191	20382	0.1	91894	22.18	20330	0.0446	1	0.012	15	0.1274
			0.5	2	5109	10218											
	8-3	2	0.5	1	4854	9708	9667	19334	0.1	91894	21.04	19282	0.0423	1	0.012	15	0.1209
			0.5	2	4813	9626											

Table C-11. Data for the Calculation of Aromatase Activity: Econazole, Replicate 1

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	5925	11850	11740	23480	0.1	202182	11.61	23365	0.0236	1	0.010	15	0.0753
			0.5	2	5815	11630			0.1					1		15	
	2	2	0.5	1	6140	12280	12081	24162	0.1	202182	11.95	24047	0.0243	1	0.010	15	0.0775
			0.5	2	5941	11882			0.1					1		15	
	3	2	0.5	1	5364	10728	10611	21222	0.1	202182	10.50	21107	0.0213	1	0.010	15	0.0680
			0.5	2	5247	10494			0.1					1		15	
	4	2	0.5	1	4077	8154	8301	16602	0.1	202182	8.21	16487	0.0166	1	0.010	15	0.0532
			0.5	2	4224	8448			0.1					1		15	
Background control	1	2	0.5	1	24	48	53	106	0.1	202182	0.05	-9	0.0000	1	0.010	15	0.0000
			0.5	2	29	58			0.1					1		15	
	2	2	0.5	1	23	46	57	114	0.1	202182	0.06	-1	0.0000	1	0.010	15	0.0000
			0.5	2	34	68			0.1					1		15	
	3	2	0.5	1	29	58	51	102	0.1	202182	0.05	-13	0.0000	1	0.010	15	0.0000
			0.5	2	22	44			0.1					1		15	
	4	2	0.5	1	28	56	69	138	0.1	202182	0.07	23	0.0000	1	0.010	15	0.0001
			0.5	2	41	82			0.1					1		15	
Positive control	1	2	0.5	1	2807	5614	5622	11244	0.1	202182	5.56	11129	0.0112	1	0.010	15	0.0359
			0.5	2	2815	5630			0.1					1		15	
	2	2	0.5	1	2707	5414	5461	10922	0.1	202182	5.40	10807	0.0109	1	0.010	15	0.0348
			0.5	2	2754	5508			0.1					1		15	
	3	2	0.5	1	2480	4960	4799	9598	0.1	202182	4.75	9483	0.0096	1	0.010	15	0.0306
			0.5	2	2319	4638			0.1					1		15	
	4	2	0.5	1	2475	4950	4842	9684	0.1	202182	4.79	9569	0.0097	1	0.010	15	0.0308
			0.5	2	2367	4734			0.1					1		15	

Table C-11. Data for the Calculation of Aromatase Activity: Econazole, Replicate 1 (Con't)

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
Negative Control	1	2	0.5	1	5776	11552	11716	23432	0.1	202182	11.59	23317	0.0235	1	0.010	15	0.0752
			0.5	2	5940	11880			0.1					1		15	
	2	2	0.5	1	6071	12142	11855	23710	0.1	202182	11.73	23595	0.0238	1	0.010	15	0.0761
			0.5	2	5784	11568			0.1					1		15	
	3	2	0.5	1	4462	8924	8932	17864	0.1	202182	8.84	17749	0.0179	1	0.010	15	0.0572
			0.5	2	4470	8940			0.1					1		15	
	4	2	0.5	1	4765	9530	9626	19252	0.1	202182	9.52	19137	0.0193	1	0.010	15	0.0617
			0.5	2	4861	9722			0.1					1		15	
11343-29B	1-1	2	0.5	1	19	38	47	94	0.1	202182	0.05	-21	0.0000	1	0.010	15	-0.0001
			0.5	2	28	56			0.1					1		15	
	1-2	2	0.5	1	29	58	50	100	0.1	202182	0.05	-15	0.0000	1	0.010	15	0.0000
			0.5	2	21	42			0.1					1		15	
	1-3	2	0.5	1	29	58	55	110	0.1	202182	0.05	-5	0.0000	1	0.010	15	0.0000
			0.5	2	26	52			0.1					1		15	
	2-1	2	0.5	1	45	90	74	148	0.1	202182	0.07	33	0.0000	1	0.010	15	0.0001
			0.5	2	29	58			0.1					1		15	
	2-2	2	0.5	1	31	62	57	114	0.1	202182	0.06	-1	0.0000	1	0.010	15	0.0000
			0.5	2	26	52			0.1					1		15	
	2-3	2	0.5	1	26	52	49	98	0.1	202182	0.05	-17	0.0000	1	0.010	15	-0.0001
			0.5	2	23	46			0.1					1		15	

Table C-11. Data for the Calculation of Aromatase Activity: Econzole, Replicate 1 (Con't)

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
11343-29B (cont't)	3-1	2	0.5	1	30	60	53	106	0.1	202182	0.05	-9	0.0000	1	0.010	15	0.0000
			0.5	2	23	46			0.1					1		15	
	3-2	2	0.5	1	30	60	62	124	0.1	202182	0.06	9	0.0000	1	0.010	15	0.0000
			0.5	2	32	64			0.1					1		15	
	3-3	2	0.5	1	23	46	60	120	0.1	202182	0.06	5	0.0000	1	0.010	15	0.0000
			0.5	2	37	74			0.1					1		15	
	4-1	2	0.5	1	44	88	86	172	0.1	202182	0.09	57	0.0001	1	0.010	15	0.0002
			0.5	2	42	84			0.1					1		15	
	4-2	2	0.5	1	42	84	81	162	0.1	202182	0.08	47	0.0000	1	0.010	15	0.0002
			0.5	2	39	78			0.1					1		15	
	4-3	2	0.5	1	53	106	89	178	0.1	202182	0.09	63	0.0001	1	0.010	15	0.0002
			0.5	2	36	72			0.1					1		15	
	5-1	2	0.5	1	112	224	225	450	0.1	202182	0.22	335	0.0003	1	0.010	15	0.0011
			0.5	2	113	226			0.1					1		15	
	5-2	2	0.5	1	95	190	191	382	0.1	202182	0.19	267	0.0003	1	0.010	15	0.0009
			0.5	2	96	192			0.1					1		15	
	5-3	2	0.5	1	108	216	202	404	0.1	202182	0.20	289	0.0003	1	0.010	15	0.0009
			0.5	2	94	188			0.1					1		15	
	6-1	2	0.5	1	207	414	430	860	0.1	202182	0.43	745	0.0008	1	0.010	15	0.0024
			0.5	2	223	446			0.1					1		15	
	6-2	2	0.5	1	114	228	236	472	0.1	202182	0.23	357	0.0004	1	0.010	15	0.0012
			0.5	2	122	244			0.1					1		15	
	6-3	2	0.5	1	259	518	506	1012	0.1	202182	0.50	897	0.0009	1	0.010	15	0.0029
			0.5	2	247	494			0.1					1		15	

Table C-11. Data for the Calculation of Aromatase Activity: Econzole, Replicate 1 (Con't)

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
11343-29B (cont't)	7-1	2	0.5	1	703	1406	1401	2802	0.1	202182	1.39	2687	0.0027	1	0.010	15	0.0087
			0.5	2	698	1396			0.1					1		15	
	7-2	2	0.5	1	562	1124	1104	2208	0.1	202182	1.09	2093	0.0021	1	0.010	15	0.0067
			0.5	2	542	1084			0.1					1		15	
	7-3	2	0.5	1	1136	2272	2241	4482	0.1	202182	2.22	4367	0.0044	1	0.010	15	0.0141
			0.5	2	1105	2210			0.1					1		15	
	8-1	2	0.5	1	1251	2502	2474	4948	0.1	202182	2.45	4833	0.0049	1	0.010	15	0.0156
			0.5	2	1223	2446			0.1					1		15	
	8-2	2	0.5	1	1330	2660	2667	5334	0.1	202182	2.64	5219	0.0053	1	0.010	15	0.0168
			0.5	2	1337	2674			0.1					1		15	
	8-3	2	0.5	1	1525	3050	2999	5998	0.1	202182	2.97	5883	0.0059	1	0.010	15	0.0190
			0.5	2	1474	2948			0.1					1		15	

Table C-12. Data for the Calculation of Aromatase Activity: Econazole, Replicate 2

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	7521	15042	14572	29144	0.1	176615	16.50	29008	0.0334	1	0.017	15	0.0654
			0.5	2	7051	14102			0.1					1		15	
	2	2	0.5	1	7722	15444	15163	30326	0.1	176615	17.17	30190	0.0347	1	0.017	15	0.0681
			0.5	2	7441	14882			0.1					1		15	
	3	2	0.5	1	6624	13248	13286	26572	0.1	176615	15.05	26436	0.0304	1	0.017	15	0.0596
			0.5	2	6662	13324			0.1					1		15	
	4	2	0.5	1	6895	13790	13683	27366	0.1	176615	15.49	27230	0.0313	1	0.017	15	0.0614
			0.5	2	6788	13576			0.1					1		15	
Background control	1	2	0.5	1	19	38	38	76	0.1	176615	0.04	-61	-0.0001	1	0.017	15	-0.0001
			0.5	2	19	38			0.1					1		15	
	2	2	0.5	1	44	88	78	156	0.1	176615	0.09	20	0.0000	1	0.017	15	0.0000
			0.5	2	34	68			0.1					1		15	
	3	2	0.5	1	35	70	73	146	0.1	176615	0.08	10	0.0000	1	0.017	15	0.0000
			0.5	2	38	76			0.1					1		15	
	4	2	0.5	1	45	90	84	168	0.1	176615	0.10	32	0.0000	1	0.017	15	0.0001
			0.5	2	39	78			0.1					1		15	
Positive control	1	2	0.5	1	3643	7286	7278	14556	0.1	176615	8.24	14420	0.0166	1	0.017	15	0.0325
			0.5	2	3635	7270			0.1					1		15	
	2	2	0.5	1	3501	7002	6929	13858	0.1	176615	7.85	13722	0.0158	1	0.017	15	0.0309
			0.5	2	3428	6856			0.1					1		15	
	3	2	0.5	1	3277	6554	6536	13072	0.1	176615	7.40	12936	0.0149	1	0.017	15	0.0292
			0.5	2	3259	6518			0.1					1		15	
	4	2	0.5	1	3160	6320	6342	12684	0.1	176615	7.18	12548	0.0144	1	0.017	15	0.0283
			0.5	2	3182	6364			0.1					1		15	

Table C-12. Data for the Calculation of Aromatase Activity: Econzole, Replicate 2 (Con't)

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
Negative Control	1	2	0.5	1	7095	14190	14241	28482	0.1	176615	16.13	28346	0.0326	1	0.017	15	0.0639
			0.5	2	7146	14292			0.1					1		15	
	2	2	0.5	1	7354	14708	14639	29278	0.1	176615	16.58	29142	0.0335	1	0.017	15	0.0657
			0.5	2	7285	14570			0.1					1		15	
	3	2	0.5	1	7093	14186	14125	28250	0.1	176615	16.00	28114	0.0323	1	0.017	15	0.0634
			0.5	2	7032	14064			0.1					1		15	
	4	2	0.5	1	6955	13910	13846	27692	0.1	176615	15.68	27556	0.0317	1	0.017	15	0.0621
			0.5	2	6891	13782			0.1					1		15	
11343-29B	1-1	2	0.5	1	168	336	333	666	0.1	176615	0.38	530	0.0006	1	0.017	15	0.0012
			0.5	2	165	330			0.1					1		15	
	1-2	2	0.5	1	159	318	336	672	0.1	176615	0.38	536	0.0006	1	0.017	15	0.0012
			0.5	2	177	354			0.1					1		15	
	1-3	2	0.5	1	175	350	339	678	0.1	176615	0.38	542	0.0006	1	0.017	15	0.0012
			0.5	2	164	328			0.1					1		15	
	2-1	2	0.5	1	1170	2340	2326	4652	0.1	176615	2.63	4516	0.0052	1	0.017	15	0.0102
			0.5	2	1156	2312			0.1					1		15	
	2-2	2	0.5	1	1166	2332	2285	4570	0.1	176615	2.59	4434	0.0051	1	0.017	15	0.0100
			0.5	2	1119	2238			0.1					1		15	
	2-3	2	0.5	1	1271	2542	2495	4990	0.1	176615	2.83	4854	0.0056	1	0.017	15	0.0109
			0.5	2	1224	2448			0.1					1		15	
	3-1	2	0.5	1	2181	4362	4258	8516	0.1	176615	4.82	8380	0.0096	1	0.017	15	0.0189
			0.5	2	2077	4154			0.1					1		15	
	3-2	2	0.5	1	2291	4582	4287	8574	0.1	176615	4.85	8438	0.0097	1	0.017	15	0.0190
			0.5	2	1996	3992			0.1					1		15	

Table C-12. Data for the Calculation of Aromatase Activity: Econzole, Replicate 2 (Con't)

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
11343-29B (con't)	3-3	2	0.5	1	2034	4068	4004	8008	0.1	176615	4.53	7872	0.0091	1	0.017	15	0.0177
			0.5	2	1970	3940			0.1					1		15	
	4-1	2	0.5	1	3195	6390	6409	12818	0.1	176615	7.26	12682	0.0146	1	0.017	15	0.0286
			0.5	2	3214	6428			0.1					1		15	
	4-2	2	0.5	1	3179	6358	6419	12838	0.1	176615	7.27	12702	0.0146	1	0.017	15	0.0286
			0.5	2	3240	6480			0.1					1		15	
	4-3	2	0.5	1	3197	6394	6415	12830	0.1	176615	7.26	12694	0.0146	1	0.017	15	0.0286
			0.5	2	3218	6436			0.1					1		15	
	5-1	2	0.5	1	5044	10088	9972	19944	0.1	176615	11.29	19808	0.0228	1	0.017	15	0.0447
			0.5	2	4928	9856			0.1					1		15	
	5-2	2	0.5	1	4991	9982	9985	19970	0.1	176615	11.31	19834	0.0228	1	0.017	15	0.0447
			0.5	2	4994	9988			0.1					1		15	
	5-3	2	0.5	1	4717	9434	9570	19140	0.1	176615	10.84	19004	0.0219	1	0.017	15	0.0429
			0.5	2	4853	9706			0.1					1		15	
	6-1	2	0.5	1	5994	11988	12032	24064	0.1	176615	13.63	23928	0.0275	1	0.017	15	0.0540
			0.5	2	6038	12076			0.1					1		15	
	6-2	2	0.5	1	5890	11780	11841	23682	0.1	176615	13.41	23546	0.0271	1	0.017	15	0.0531
			0.5	2	5951	11902			0.1					1		15	
	6-3	2	0.5	1	5954	11908	11844	23688	0.1	176615	13.41	23552	0.0271	1	0.017	15	0.0531
			0.5	2	5890	11780			0.1					1		15	
	7-1	2	0.5	1	6419	12838	12829	25658	0.1	176615	14.53	25522	0.0294	1	0.017	15	0.0575
			0.5	2	6410	12820			0.1					1		15	
	7-2	2	0.5	1	6186	12372	12325	24650	0.1	176615	13.96	24514	0.0282	1	0.017	15	0.0553
			0.5	2	6139	12278			0.1					1		15	

Table C-12. Data for the Calculation of Aromatase Activity: Econzole, Replicate 2 (Con't)

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
	7-3	2	0.5	1	6062	12124	12074	24148	0.1	176615	13.67	24012	0.0276	1	0.017	15	0.0541
			0.5	2	6012	12024			0.1					1		15	
	8-1	2	0.5	1	6702	13404	13284	26568	0.1	176615	15.04	26432	0.0304	1	0.017	15	0.0596
			0.5	2	6582	13164			0.1					1		15	
	8-2	2	0.5	1	6740	13480	13507	27014	0.1	176615	15.30	26878	0.0309	1	0.017	15	0.0606
			0.5	2	6767	13534			0.1					1		15	
	8-3	2	0.5	1	6753	13506	13337	26674	0.1	176615	15.10	26538	0.0305	1	0.017	15	0.0598
			0.5	2	6584	13168			0.1					1		15	

Table C-13. Data for the Calculation of Aromatase Activity: Econazole, Replicate 3

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	7874	15748	15853	31706	0.1	196685	16.12	31573	0.0327	1	0.012	15	0.0930
			0.5	2	7979	15958			0.1					1		15	
	2	2	0.5	1	8043	16086	16073	32146	0.1	196685	16.34	32013	0.0332	1	0.012	15	0.0943
			0.5	2	8030	16060			0.1					1		15	
	3	2	0.5	1	7640	15280	15243	30486	0.1	196685	15.50	30353	0.0315	1	0.012	15	0.0894
			0.5	2	7603	15206			0.1					1		15	
	4	2	0.5	1	7429	14858	14837	29674	0.1	196685	15.09	29541	0.0306	1	0.012	15	0.0870
			0.5	2	7408	14816			0.1					1		15	
Background control	1	2	0.5	1	30	60	62	124	0.1	196685	0.06	-10	0.0000	1	0.012	15	0.0000
			0.5	2	32	64			0.1					1		15	
	2	2	0.5	1	31	62	68	136	0.1	196685	0.07	3	0.0000	1	0.012	15	0.0000
			0.5	2	37	74			0.1					1		15	
	3	2	0.5	1	38	76	77	154	0.1	196685	0.08	21	0.0000	1	0.012	15	0.0001
			0.5	2	39	78			0.1					1		15	
	4	2	0.5	1	28	56	60	120	0.1	196685	0.06	-14	0.0000	1	0.012	15	0.0000
			0.5	2	32	64			0.1					1		15	
Positive control	1	2	0.5	1	3610	7220	7094	14188	0.1	196685	7.21	14055	0.0146	1	0.012	15	0.0414
			0.5	2	3484	6968			0.1					1		15	
	2	2	0.5	1	4264	8528	8510	17020	0.1	196685	8.65	16887	0.0175	1	0.012	15	0.0497
			0.5	2	4246	8492			0.1					1		15	
	3	2	0.5	1	3582	7164	6985	13970	0.1	196685	7.10	13837	0.0143	1	0.012	15	0.0407
			0.5	2	3403	6806			0.1					1		15	
	4	2	0.5	1	3699	7398	7401	14802	0.1	196685	7.53	14669	0.0152	1	0.012	15	0.0432
			0.5	2	3702	7404			0.1					1		15	

Table C-13. Data for the Calculation of Aromatase Activity: Econzole, Replicate 3 (Con't)

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
Negative Control	1	2	0.5	1	7833	15666	15632	31264	0.1	196685	15.90	31131	0.0323	1	0.012	15	0.0917
			0.5	2	7799	15598			0.1					1		15	
	2	2	0.5	1	7854	15708	15606	31212	0.1	196685	15.87	31079	0.0322	1	0.012	15	0.0915
			0.5	2	7752	15504			0.1					1		15	
	3	2	0.5	1	7459	14918	14785	29570	0.1	196685	15.03	29437	0.0305	1	0.012	15	0.0867
			0.5	2	7326	14652			0.1					1		15	
	4	2	0.5	1	7313	14626	14699	29398	0.1	196685	14.95	29265	0.0303	1	0.012	15	0.0862
			0.5	2	7386	14772			0.1					1		15	
11343-29B	1-1	2	0.5	1	179	358	347	694	0.1	196685	0.35	561	0.0006	1	0.012	15	0.0017
			0.5	2	168	336			0.1					1		15	
	1-2	2	0.5	1	168	336	332	664	0.1	196685	0.34	531	0.0006	1	0.012	15	0.0016
			0.5	2	164	328			0.1					1		15	
	1-3	2	0.5	1	154	308	321	642	0.1	196685	0.33	509	0.0005	1	0.012	15	0.0015
			0.5	2	167	334			0.1					1		15	
	2-1	2	0.5	1	1229	2458	2394	4788	0.1	196685	2.43	4655	0.0048	1	0.012	15	0.0137
			0.5	2	1165	2330			0.1					1		15	
	2-2	2	0.5	1	1228	2456	2411	4822	0.1	196685	2.45	4689	0.0049	1	0.012	15	0.0138
			0.5	2	1183	2366			0.1					1		15	
	2-3	2	0.5	1	1169	2338	2383	4766	0.1	196685	2.42	4633	0.0048	1	0.012	15	0.0136
			0.5	2	1214	2428			0.1					1		15	
	3-1	2	0.5	1	2111	4222	4156	8312	0.1	196685	4.23	8179	0.0085	1	0.012	15	0.0241
			0.5	2	2045	4090			0.1					1		15	
	3-2	2	0.5	1	1981	3962	4054	8108	0.1	196685	4.12	7975	0.0083	1	0.012	15	0.0235
			0.5	2	2073	4146			0.1					1		15	
	3-3	2	0.5	1	2072	4144	4130	8260	0.1	196685	4.20	8127	0.0084	1	0.012	15	0.0239
			0.5	2	2058	4116			0.1					1		15	

Table C-13. Data for the Calculation of Aromatase Activity: Econzole, Replicate 3 (Con't)

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
11343-29B (con't)	4-1	2	0.5	1	3385	6770	6656	13312	0.1	196685	6.77	13179	0.0137	1	0.012	15	0.0388
			0.5	2	3271	6542			0.1					1		15	
	4-2	2	0.5	1	3274	6548	6582	13164	0.1	196685	6.69	13031	0.0135	1	0.012	15	0.0384
			0.5	2	3308	6616			0.1					1		15	
	4-3	2	0.5	1	3249	6498	6442	12884	0.1	196685	6.55	12751	0.0132	1	0.012	15	0.0375
			0.5	2	3193	6386			0.1					1		15	
	5-1	2	0.5	1	5420	10840	10703	21406	0.1	196685	10.88	21273	0.0221	1	0.012	15	0.0626
			0.5	2	5283	10566			0.1					1		15	
	5-2	2	0.5	1	5175	10350	10348	20696	0.1	196685	10.52	20563	0.0213	1	0.012	15	0.0606
			0.5	2	5173	10346			0.1					1		15	
	5-3	2	0.5	1	5147	10294	10144	20288	0.1	196685	10.31	20155	0.0209	1	0.012	15	0.0593
			0.5	2	4997	9994			0.1					1		15	
	6-1	2	0.5	1	6137	12274	12190	24380	0.1	196685	12.40	24247	0.0251	1	0.012	15	0.0714
			0.5	2	6053	12106			0.1					1		15	
	6-2	2	0.5	1	5993	11986	11964	23928	0.1	196685	12.17	23795	0.0247	1	0.012	15	0.0701
			0.5	2	5971	11942			0.1					1		15	
	6-3	2	0.5	1	6115	12230	12294	24588	0.1	196685	12.50	24455	0.0254	1	0.012	15	0.0720
			0.5	2	6179	12358			0.1					1		15	
	7-1	2	0.5	1	7293	14586	14084	28168	0.1	196685	14.32	28035	0.0291	1	0.012	15	0.0826
			0.5	2	6791	13582			0.1					1		15	
	7-2	2	0.5	1	6725	13450	13519	27038	0.1	196685	13.75	26905	0.0279	1	0.012	15	0.0792
			0.5	2	6794	13588			0.1					1		15	
	7-3	2	0.5	1	6950	13900	13964	27928	0.1	196685	14.20	27795	0.0288	1	0.012	15	0.0818
			0.5	2	7014	14028			0.1					1		15	

Table C-13. Data for the Calculation of Aromatase Activity: Econzole, Replicate 3 (Con't)

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
11343-29B (con't)	8-1	2	0.5	1	7132	14264	14100	28200	0.1	196685	14.34	28067	0.0291	1	0.012	15	0.0826
			0.5	2	6968	13936			0.1					1		15	
	8-2	2	0.5	1	7041	14082	14214	28428	0.1	196685	14.45	28295	0.0293	1	0.012	15	0.0833
			0.5	2	7173	14346			0.1					1		15	
	8-3	2	0.5	1	7450	14900	14808	29616	0.1	196685	15.06	29483	0.0306	1	0.012	15	0.0868
			0.5	2	7358	14716			0.1					1		15	

Table C-14. Data for the Calculation of Aromatase Activity: Econazole, Replicate 4

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	7414	14828	14838	29676	0.1	190328	15.59	29440	0.0314	1	0.010	15	0.1016
			0.5	2	7424	14848			0.1					1		15	
	2	2	0.5	1	8181	16362	16101	32202	0.1	190328	16.92	31966	0.0341	1	0.010	15	0.1103
			0.5	2	7920	15840			0.1					1		15	
	3	2	0.5	1	8293	16586	16290	32580	0.1	190328	17.12	32344	0.0345	1	0.010	15	0.1116
			0.5	2	7997	15994			0.1					1		15	
	4	2	0.5	1	1623	3246	3269	6538	0.1	190328	3.44	6302	0.0067	1	0.010	15	0.0217
			0.5	2	1646	3292			0.1					1		15	
Background control	1	2	0.5	1	31	62	59	118	0.1	190328	0.06	-118	-0.0001	1	0.010	15	-0.0004
			0.5	2	28	56			0.1					1		15	
	2	2	0.5	1	143	286	288	576	0.1	190328	0.30	340	0.0004	1	0.010	15	0.0012
			0.5	2	145	290			0.1					1		15	
	3	2	0.5	1	36	72	74	148	0.1	190328	0.08	-88	-0.0001	1	0.010	15	-0.0003
			0.5	2	38	76			0.1					1		15	
	4	2	0.5	1	24	48	51	102	0.1	190328	0.05	-134	-0.0001	1	0.010	15	-0.0005
			0.5	2	27	54			0.1					1		15	
Positive control	1	2	0.5	1	3786	7572	7601	15202	0.1	190328	7.99	14966	0.0160	1	0.010	15	0.0516
			0.5	2	3815	7630			0.1					1		15	
	2	2	0.5	1	4585	9170	9266	18532	0.1	190328	9.74	18296	0.0195	1	0.010	15	0.0631
			0.5	2	4681	9362			0.1					1		15	
	3	2	0.5	1	3791	7582	7510	15020	0.1	190328	7.89	14784	0.0158	1	0.010	15	0.0510
			0.5	2	3719	7438			0.1					1		15	
	4	2	0.5	1	3935	7870	7834	15668	0.1	190328	8.23	15432	0.0165	1	0.010	15	0.0532
			0.5	2	3899	7798			0.1					1		15	

Table C-14. Data for the Calculation of Aromatase Activity: Econzole, Replicate 4 (Con't)

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
Negative Control	1	2	0.5	1	3308	6616	6467	12934	0.1	190328	6.80	12698	0.0135	1	0.010	15	0.0438
			0.5	2	3159	6318			0.1					1		15	
	2	2	0.5	1	3397	6794	6681	13362	0.1	190328	7.02	13126	0.0140	1	0.010	15	0.0453
			0.5	2	3284	6568			0.1					1		15	
	3	2	0.5	1	3002	6004	5889	11778	0.1	190328	6.19	11542	0.0123	1	0.010	15	0.0398
			0.5	2	2887	5774			0.1					1		15	
	4	2	0.5	1	2970	5940	5867	11734	0.1	190328	6.17	11498	0.0123	1	0.010	15	0.0397
			0.5	2	2897	5794			0.1					1		15	
11343-29B	1-1	2	0.5	1	213	426	422	844	0.1	190328	0.44	608	0.0006	1	0.010	15	0.0021
			0.5	2	209	418			0.1					1		15	
	1-2	2	0.5	1	183	366	377	754	0.1	190328	0.40	518	0.0006	1	0.010	15	0.0018
			0.5	2	194	388			0.1					1		15	
	1-3	2	0.5	1	192	384	415	830	0.1	190328	0.44	594	0.0006	1	0.010	15	0.0020
			0.5	2	223	446			0.1					1		15	
	2-1	2	0.5	1	1531	3062	3096	6192	0.1	190328	3.25	5956	0.0064	1	0.010	15	0.0205
			0.5	2	1565	3130			0.1					1		15	
	2-2	2	0.5	1	1540	3080	2955	5910	0.1	190328	3.11	5674	0.0060	1	0.010	15	0.0196
			0.5	2	1415	2830			0.1					1		15	
	2-3	2	0.5	1	1523	3046	3059	6118	0.1	190328	3.21	5882	0.0063	1	0.010	15	0.0203
			0.5	2	1536	3072			0.1					1		15	
	3-1	2	0.5	1	2733	5466	5373	10746	0.1	190328	5.65	10510	0.0112	1	0.010	15	0.0363
			0.5	2	2640	5280			0.1					1		15	
	3-2	2	0.5	1	1415	2830	2814	5628	0.1	190328	2.96	5392	0.0057	1	0.010	15	0.0186
			0.5	2	1399	2798			0.1					1		15	
	3-3	2	0.5	1	1771	3542	3515	7030	0.1	190328	3.69	6794	0.0072	1	0.010	15	0.0234
			0.5	2	1744	3488			0.1					1		15	

Table C-14. Data for the Calculation of Aromatase Activity: Econzole, Replicate 4 (Con't)

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
	4-1	2	0.5	1	4065	8130	8092	16184	0.1	190328	8.50	15948	0.0170	1	0.010	15	0.0550
			0.5	2	4027	8054			0.1					1		15	
	4-2	2	0.5	1	3943	7886	7889	15778	0.1	190328	8.29	15542	0.0166	1	0.010	15	0.0536
			0.5	2	3946	7892			0.1					1		15	
	4-3	2	0.5	1	2163	4326	4367	8734	0.1	190328	4.59	8498	0.0091	1	0.010	15	0.0293
			0.5	2	2204	4408			0.1					1		15	
	5-1	2	0.5	1	5699	11398	11367	22734	0.1	190328	11.94	22498	0.0240	1	0.010	15	0.0776
			0.5	2	5668	11336			0.1					1		15	
	5-2	2	0.5	1	5508	11016	10972	21944	0.1	190328	11.53	21708	0.0231	1	0.010	15	0.0749
			0.5	2	5464	10928			0.1					1		15	
	5-3	2	0.5	1	3756	7512	7486	14972	0.1	190328	7.87	14736	0.0157	1	0.010	15	0.0508
			0.5	2	3730	7460			0.1					1		15	
	6-1	2	0.5	1	6706	13412	13406	26812	0.1	190328	14.09	26576	0.0283	1	0.010	15	0.0917
			0.5	2	6700	13400			0.1					1		15	
	6-2	2	0.5	1	6453	12906	12931	25862	0.1	190328	13.59	25626	0.0273	1	0.010	15	0.0884
			0.5	2	6478	12956			0.1					1		15	
	6-3	2	0.5	1	6743	13486	13344	26688	0.1	190328	14.02	26452	0.0282	1	0.010	15	0.0913
			0.5	2	6601	13202			0.1					1		15	
	7-1	2	0.5	1	7286	14572	14560	29120	0.1	190328	15.30	28884	0.0308	1	0.010	15	0.0996
			0.5	2	7274	14548			0.1					1		15	
	7-2	2	0.5	1	6614	13228	13159	26318	0.1	190328	13.83	26082	0.0278	1	0.010	15	0.0900
			0.5	2	6545	13090			0.1					1		15	
	7-3	2	0.5	1	4359	8718	8732	17464	0.1	190328	9.18	17228	0.0184	1	0.010	15	0.0594
			0.5	2	4373	8746			0.1					1		15	

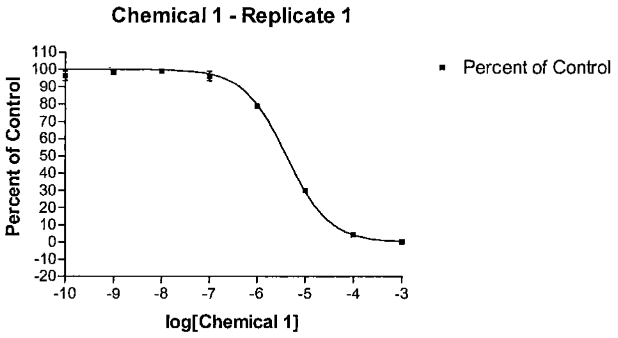
Table C-14. Data for the Calculation of Aromatase Activity: Econzole, Replicate 4 (Con't)

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
	8-1	2	0.5	1	6828	13656	13699	27398	0.1	190328	14.40	27162	0.0290	1	0.010	15	0.0937
			0.5	2	6871	13742			0.1					1		15	
	8-2	2	0.5	1	7925	15850	15798	31596	0.1	190328	16.60	31360	0.0334	1	0.010	15	0.1082
			0.5	2	7873	15746			0.1					1		15	
	8-3	2	0.5	1	7586	15172	15075	30150	0.1	190328	15.84	29914	0.0319	1	0.010	15	0.1032
			0.5	2	7489	14978			0.1					1		15	

Appendix D

Prism Outputs

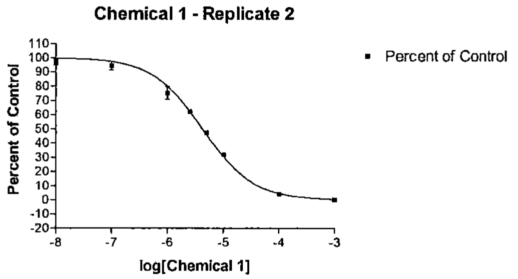
Aminoglutethimide
Replicate 1



log[Ref chem 1]	Percent of Control		
	Y1	Y2	Y3
-3.0	0.4	0.3	0.4
-4.0	4.4	4.3	4.3
-5.0	31.0	29.6	29.6
-6.0	79.3	79.7	77.3
-7.0	99.0	95.6	93.4
-8.0	99.5	99.0	98.6
-9.0	98.7	98.0	98.3
-10.0	99.5	94.6	94.6

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.389
HILLSLOPE	-0.9702
EC50	4.0860e-006
Std. Error	
LOGEC50	0.01560
HILLSLOPE	0.01865
95% Confidence Intervals	
LOGEC50	-5.421 to -5.356
HILLSLOPE	-1.009 to -0.9315
EC50	3.7930e-006 to 4.4020e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9976
Weighted Sum of Squares (1/Y)	1.206
Absolute Sum of Squares	96.52
Sy.x	2.095
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

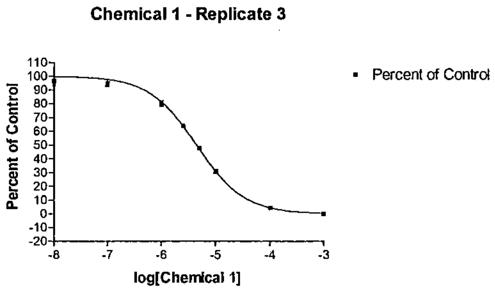
Aminoglutethimide
Replicate 2



log[Ref chem 1]	Percent of Control		
	Y1	Y2	Y3
-3.0	0.4	0.4	0.3
-4.0	4.5	4.1	4.2
-5.0	32.5	31.7	31.5
-5.3	47.6	47.1	47.7
-5.6	63.1	61.6	62.2
-6.0	70.1	78.5	77.3
-7.0	97.8	93.6	91.7
-8.0	99.5	95.3	96.4

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.374
HILLSLOPE	-0.9578
EC50	4.2250e-006
Std. Error	
LOGEC50	0.01590
HILLSLOPE	0.02456
95% Confidence Intervals	
LOGEC50	-5.407 to -5.341
HILLSLOPE	-1.009 to -0.9069
EC50	3.9160e-006 to 4.5580e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9934
Weighted Sum of Squares (1/Y)	3.153
Absolute Sum of Squares	196.4
Sy.x	2.988
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Aminoglutethimide
Replicate 3

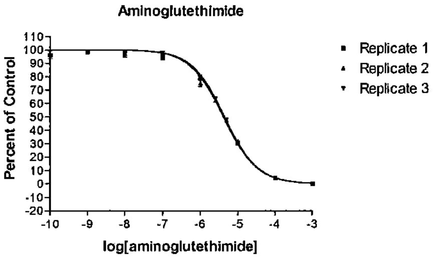


Chemical 1 - Replicate 3

log[Ref chem 1]	Percent of Control		
	Y1	Y2	Y3
-3.0	0.3	0.3	0.4
-4.0	4.6	4.6	4.4
-5.0	32.4	30.6	29.4
-5.3	47.0	48.7	48.4
-5.6	65.2	64.1	63.0
-6.0	82.0	80.4	78.1
-7.0	98.1	93.7	93.6
-8.0	99.2	97.4	92.9

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.351
HILLSLOPE	-0.9904
EC50	4.4600e-006
Std. Error	
LOGEC50	0.01073
HILLSLOPE	0.01854
95% Confidence Intervals	
LOGEC50	-5.373 to -5.328
HILLSLOPE	-1.029 to -0.9520
EC50	4.2370e-006 to 4.6940e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9964
Weighted Sum of Squares (1/Y)	1.516
Absolute Sum of Squares	110.7
Sy.x	2.243
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

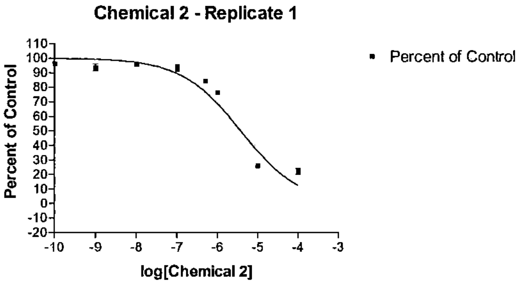
Aminoglutethimide
All Replicates



log[Ref chem 1]	Replicate 1			Replicate 2			Replicate 3		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
-3.0	0.4	0.3	0.4	0.4	0.4	0.3	0.3	0.3	0.4
-4.0	4.4	4.3	4.3	4.5	4.1	4.2	4.6	4.6	4.4
-5.0	31.0	29.6	29.6	32.5	31.7	31.5	32.4	30.6	29.4
-5.3				47.6	47.1	47.7	47.0	48.7	48.4
-5.6				63.1	61.6	62.2	65.2	64.1	63.0
-6.0	79.3	79.7	77.3	70.1	78.5	77.3	82.0	80.4	78.1
-7.0	99.0	95.6	93.4	97.8	93.6	91.7	98.1	93.7	93.6
-8.0	99.5	99.0	98.6	99.5	95.3	96.4	99.2	97.4	92.9
-9.0	98.7	98.0	98.3						
-10.0	99.5	94.6	94.6						

	Replicate 1	Replicate 2	Replicate 3
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-5.389	-5.374	-5.351
HILLSLOPE	-0.9702	-0.9578	-0.9904
EC50	4.0860e-006	4.2250e-006	4.4600e-006
Std. Error			
LOGEC50	0.01560	0.01590	0.01073
HILLSLOPE	0.01865	0.02456	0.01854
95% Confidence Intervals			
LOGEC50	-5.421 to -5.356	-5.407 to -5.341	-5.373 to -5.328
HILLSLOPE	-1.009 to -0.9315	-1.009 to -0.9069	-1.029 to -0.9520
EC50	3.7930e-006 to 4.4020e-006	3.9160e-006 to 4.5580e-006	4.2370e-006 to 4.6940e-006
Goodness of Fit			
Degrees of Freedom	22	22	22
R² (unweighted)	0.9976	0.9934	0.9964
Weighted Sum of Squares (1/Y)	1.206	3.153	1.516
Absolute Sum of Squares	96.52	196.4	110.7
Sy.x	2.095	2.988	2.243
Data			
Number of X values	10	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	6	0	0

Chrysin
Replicate 1

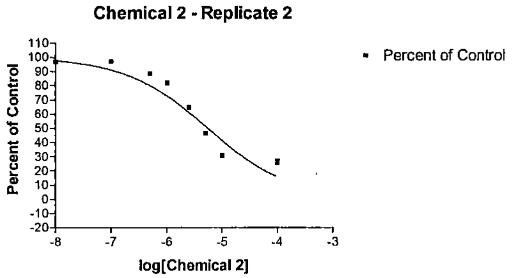


Chemical 2 - Replicate 1

log[Ref chem 2]	Percent of Control		
	Y1	Y2	Y3
-4.0	19.8	23.4	23.0
-5.0	26.8	25.1	26.3
-6.0	74.9	77.2	76.2
-6.3	84.7	84.2	83.8
-7.0	95.7	92.6	91.0
-8.0	96.1	96.2	95.7
-9.0	96.2	93.2	91.4
-10.0	94.9	97.0	97.0

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.427
HILLSLOPE	-0.5892
EC50	3.7450e-006
Std. Error	
LOGEC50	0.08528
HILLSLOPE	0.06366
95% Confidence Intervals	
LOGEC50	-5.603 to -5.250
HILLSLOPE	-0.7212 to -0.4571
EC50	2.4920e-006 to 5.6270e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9454
Weighted Sum of Squares (1/Y)	30.11
Absolute Sum of Squares	1121
Sy.x	7.138
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

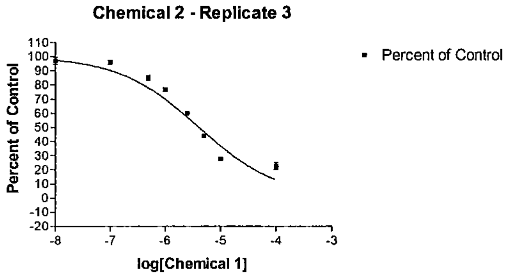
Chrysin
Replicate 2



log[Ref chem 2]	Percent of Control		
	Y1	Y2	Y3
-4.0	26.0	28.0	24.6
-5.0	30.4	32.1	30.3
-5.3	46.0	46.0	47.3
-5.6	64.9	62.9	66.3
-6.0	82.8	82.1	81.2
-6.3	89.6	89.0	87.6
-7.0	97.0	98.1	96.1
-8.0	97.9	96.5	96.5

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.260
HILLSLOPE	-0.5774
EC50	5.4980e-006
Std. Error	
LOGEC50	0.07054
HILLSLOPE	0.06615
95% Confidence Intervals	
LOGEC50	-5.406 to -5.114
HILLSLOPE	-0.7146 to -0.4402
EC50	3.9250e-006 to 7.7000e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9213
Weighted Sum of Squares (1/Y)	32.62
Absolute Sum of Squares	1392
Sy.x	7.953
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

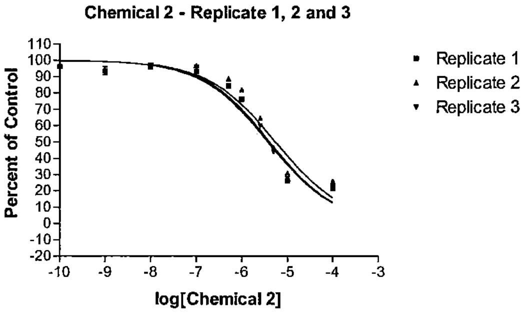
Chrysin
Replicate 3



log[Ref chem 2]	Percent of Control		
	Y1	Y2	Y3
-4.0	19.9	24.0	24.1
-5.0	28.0	26.9	28.0
-5.3	43.5	44.1	44.4
-5.6	59.6	60.0	59.9
-6.0	76.6	75.0	77.8
-6.3	83.5	86.8	83.8
-7.0	97.4	94.4	95.9
-8.0	94.5	98.0	98.7

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.388
HILLSLOPE	-0.5976
EC50	4.0890e-006
Std. Error	
LOGEC50	0.06225
HILLSLOPE	0.06115
95% Confidence Intervals	
LOGEC50	-5.517 to -5.259
HILLSLOPE	-0.7244 to -0.4708
EC50	3.0370e-006 to 5.5040e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9456
Weighted Sum of Squares (1/Y)	27.98
Absolute Sum of Squares	1005
Sy.x	6.759
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Chrysin
All Replicates

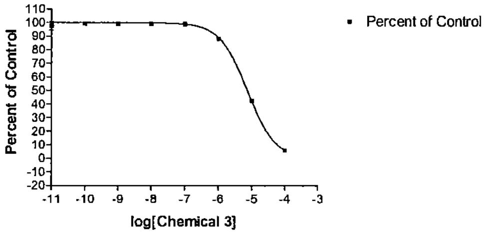


log[Ref chem 2]	Replicate 1			Replicate 2			Replicate 3		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
-4.0	19.8	23.4	23.0	26.0	28.0	24.6	19.9	24.0	24.1
-5.0	26.8	25.1	26.3	30.4	32.1	30.3	28.0	26.9	28.0
-5.3				46.0	46.0	47.3	43.5	44.1	44.4
-5.6				64.9	62.9	66.3	59.6	60.0	59.9
-6.0	74.9	77.2	76.2	82.8	82.1	81.2	76.6	75.0	77.8
-6.3	84.7	84.2	83.8	89.6	89.0	87.6	83.5	86.8	83.8
-7.0	95.7	92.6	91.0	97.0	98.1	96.1	97.4	94.4	95.9
-8.0	96.1	96.2	95.7	97.9	96.5	96.5	94.5	98.0	98.7
-9.0	96.2	93.2	91.4						
-10.0	94.9	97.0	97.0						

	Replicate 1	Replicate 2	Replicate 3
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-5.427	-5.260	-5.388
HILLSLOPE	-0.5892	-0.5774	-0.5976
EC50	3.7450e-006	5.4980e-006	4.0890e-006
Std. Error			
LOGEC50	0.08528	0.07054	0.06225
HILLSLOPE	0.06366	0.06615	0.06115
95% Confidence Intervals			
LOGEC50	-5.603 to -5.250	-5.406 to -5.114	-5.517 to -5.259
HILLSLOPE	-0.7212 to -0.4571	-0.7146 to -0.4402	-0.7244 to -0.4708
EC50	2.4920e-006 to 5.6270e-006	3.9250e-006 to 7.7000e-006	3.0370e-006 to 5.5040e-006
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9454	0.9213	0.9456
Weighted Sum of Squares (1/Y)	30.11	32.62	27.98
Absolute Sum of Squares	1121	1392	1005
Sy.x	7.138	7.953	6.759
Data			
Number of X values	10	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	6	0	0

Ketoconazole
Replicate 1

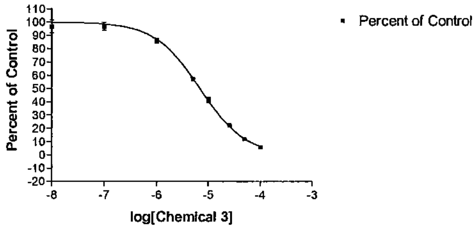
Chemical 3 - Replicate 1



log[Ref chem 1]	Percent of Control		
	Y1	Y2	Y3
-4.0	6.1	6.3	5.9
-5.0	42.1	43.0	43.0
-6.0	87.7	89.3	87.4
-7.0	99.5	99.5	98.4
-8.0	99.5	99.5	98.7
-9.0	99.5	98.6	99.7
-10.0	99.5	99.5	99.4
-11.0	99.5	99.5	93.8

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.128
HILLSLOPE	-1.047
EC50	7.4400e-006
Std. Error	
LOGEC50	0.01013
HILLSLOPE	0.01686
95% Confidence Intervals	
LOGEC50	-5.149 to -5.107
HILLSLOPE	-1.082 to -1.012
EC50	7.0890e-006 to 7.8090e-006
Goodness of Fit	
Degrees of Freedom	22
R² (unweighted)	0.9981
Weighted Sum of Squares (1/Y)	0.5664
Absolute Sum of Squares	50.44
Sy.x	1.514
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

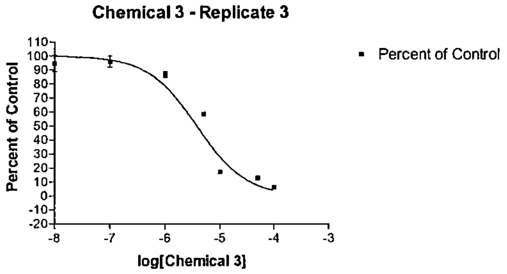
Ketoconazole
Replicate 2


Chemical 3 - Replicate 2

log[Ref chem 1]	Percent of Control		
	Y1	Y2	Y3
-4.0	6.2	6.0	5.7
-4.3	12.4	12.4	11.7
-4.6	23.1	22.5	22.0
-5.0	43.3	42.1	38.9
-5.3	58.0	57.6	55.7
-6.0	86.7	87.0	83.8
-7.0	99.5	97.3	93.5
-8.0	99.7	99.5	91.1

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.164
HILLSLOPE	-0.9929
EC50	6.8530e-006
Std. Error	
LOGEC50	0.01360
HILLSLOPE	0.02162
95% Confidence Intervals	
LOGEC50	-5.192 to -5.136
HILLSLOPE	-1.038 to -0.9481
EC50	6.4230e-006 to 7.3130e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9954
Weighted Sum of Squares (1/Y)	1.988
Absolute Sum of Squares	136.8
Sy.x	2.494
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

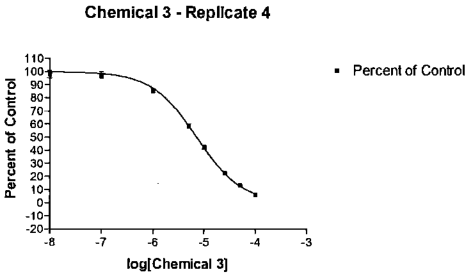
Ketoconazole
Replicate 3



log[Ref chem 3]	Percent of Control		
	Y1	Y2	Y3
-4.0	5.9	6.6	6.6
-4.3	13.1	13.1	12.5
-4.6	~~99.5~~	~~99.5~~	~~99.5~~
-5.0	17.4	17.5	17.1
-5.3	59.5	58.9	57.2
-6.0	89.0	85.2	86.1
-7.0	99.5	96.9	91.7
-8.0	99.5	96.1	88.2

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.424
HILLSLOPE	-0.9693
EC50	3.7700e-006
Std. Error	
LOGEC50	0.08047
HILLSLOPE	0.1316
95% Confidence Intervals	
LOGEC50	-5.592 to -5.255
HILLSLOPE	-1.245 to -0.6939
EC50	2.5580e-006 to 5.5560e-006
Goodness of Fit	
Degrees of Freedom	19
R^2 (unweighted)	0.9474
Weighted Sum of Squares (1/Y)	45.55
Absolute Sum of Squares	1554
Sy.x	9.042
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	21
Number of missing values	3

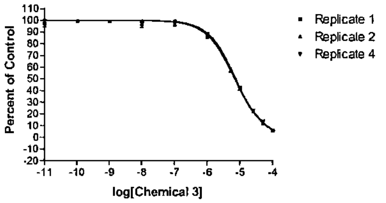
Ketoconazole
Replicate 4



log[Ref chem 3]	Percent of Control		
	Y1	Y2	Y3
-4.0	6.2	6.1	6.1
-4.3	14.1	13.1	13.7
-4.6	23.4	23.2	22.5
-5.0	43.3	43.1	40.6
-5.3	60.2	58.0	58.0
-6.0	87.1	86.1	84.0
-7.0	99.5	98.5	94.8
-8.0	99.5	99.5	95.0

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-5.143
HILLSLOPE	-0.9865
EC50	7.1990e-006
Std. Error	
LOGEC50	0.01103
HILLSLOPE	0.01757
95% Confidence Intervals	
LOGEC50	-5.166 to -5.120
HILLSLOPE	-1.023 to -0.9501
EC50	6.8290e-006 to 7.5880e-006
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9977
Weighted Sum of Squares (1/Y)	1.316
Absolute Sum of Squares	67.62
Sy.x	1.753
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

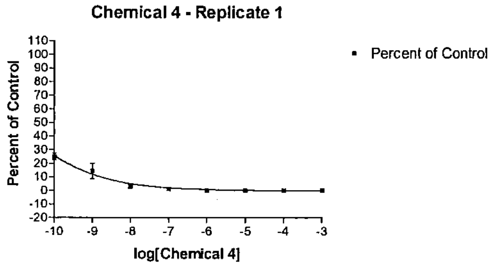
Ketoconazole
Replicates 1, 2, 4


Chemical 3 - Replicate 1, 2 and 4

log[Ref chem 3]	Replicate 1			Replicate 2			Replicate 4		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
-4.0	6.1	6.3	5.9	6.2	6.0	5.7	6.2	6.1	6.1
-4.3				12.4	12.4	11.7	14.1	13.1	13.7
-4.6				23.1	22.5	22.0	23.4	23.2	22.5
-5.0	42.1	43.0	43.0	43.3	42.1	38.9	43.3	43.1	40.6
-5.3				58.0	57.6	55.7	60.2	58.0	58.0
-6.0	87.7	89.3	87.4	86.7	87.0	83.8	87.1	86.1	84.0
-7.0	99.5	99.5	98.4	99.5	97.3	93.5	99.5	98.5	94.8
-8.0	99.5	99.5	98.7	99.7	99.5	91.1	99.5	99.5	95.0
-9.0	99.5	98.6	99.7						
-10.0	99.5	99.5	99.4						
-11.0	99.5	99.5	93.8						

	Replicate 1	Replicate 2	Replicate 4
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-5.128	-5.164	-5.143
HILLSLOPE	-1.047	-0.9929	-0.9865
EC50	7.4400e-006	6.8530e-006	7.1990e-006
Std. Error			
LOGEC50	0.01013	0.01360	0.01103
HILLSLOPE	0.01686	0.02162	0.01757
95% Confidence Intervals			
LOGEC50	-5.149 to -5.107	-5.192 to -5.136	-5.166 to -5.120
HILLSLOPE	-1.082 to -1.012	-1.038 to -0.9481	-1.023 to -0.9501
EC50	7.0890e-006 to 7.8090e-006	6.4230e-006 to 7.3130e-006	6.8290e-006 to 7.5880e-006
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9981	0.9954	0.9977
Weighted Sum of Squares (1/Y)	0.5664	1.988	1.316
Absolute Sum of Squares	50.44	136.8	67.62
Sy.x	1.514	2.494	1.753
Data			
Number of X values	11	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	9	0	0

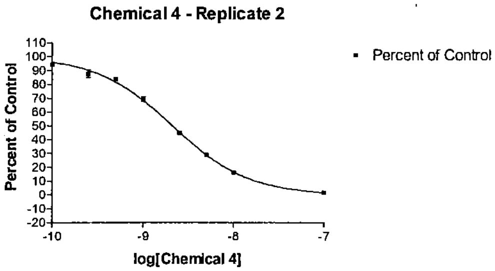
Econazole
Replicate 1



log[Ref chem 4]	Percent of Control		
	Y1	Y2	Y3
-3.0	0.5	0.5	0.5
-4.0	0.2	0.0	0.5
-5.0	0.5	4.0e-002	2.0e-002
-6.0	0.3	0.2	0.3
-7.0	1.6	1.3	1.4
-8.0	3.5	1.7	4.2
-9.0	12.6	9.9	20.5
-10.0	22.7	24.6	27.7

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-11.14
HILLSLOPE	-0.4046
EC50	7.2560e-012
Std. Error	
LOGEC50	0.1596
HILLSLOPE	0.04013
95% Confidence Intervals	
LOGEC50	-11.47 to -10.81
HILLSLOPE	-0.4878 to -0.3214
EC50	3.3860e-012 to 1.5550e-011
Goodness of Fit	
Degrees of Freedom	22
R² (unweighted)	0.9401
Weighted Sum of Squares (1/Y)	Impossible
Absolute Sum of Squares	110.5
Sy.x	2.241
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

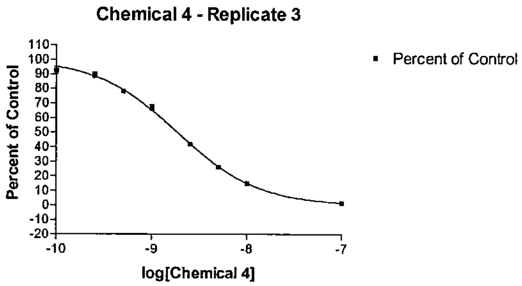
Econazole
Replicate 2



log[Ref chem 4]	Percent of Control		
	Y1	Y2	Y3
-7.0	1.9	1.9	1.9
-8.0	16.0	15.7	17.2
-8.3	29.7	29.9	27.9
-8.6	45.0	45.0	45.0
-9.0	70.2	70.3	67.3
-9.3	84.8	83.4	83.5
-9.6	90.4	86.9	85.1
-10.0	93.7	95.3	94.1

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-8.676
HILLSLOPE	-1.037
EC50	2.1090e-009
Std. Error	
LOGEC50	0.008105
HILLSLOPE	0.01570
95% Confidence Intervals	
LOGEC50	-8.693 to -8.659
HILLSLOPE	-1.069 to -1.004
EC50	2.0290e-009 to 2.1920e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9972
Weighted Sum of Squares (1/Y)	1.038
Absolute Sum of Squares	73.93
Sy.x	1.833
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

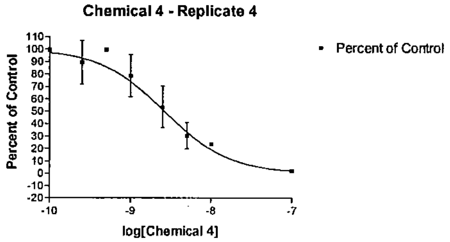
Econazole
Replicate 3



log[Ref chem 4]	Percent of Control		
	Y1	Y2	Y3
-7.0	1.8	1.7	1.6
-8.0	15.1	15.2	15.0
-8.3	26.5	25.8	26.3
-8.6	42.7	42.2	41.3
-9.0	68.9	66.6	65.3
-9.3	78.6	77.1	79.2
-9.6	90.8	87.2	90.0
-10.0	90.9	91.7	95.5

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-8.732
HILLSLOPE	-1.023
EC50	1.8520e-009
Std. Error	
LOGEC50	0.007136
HILLSLOPE	0.01321
95% Confidence Intervals	
LOGEC50	-8.747 to -8.718
HILLSLOPE	-1.050 to -0.9951
EC50	1.7900e-009 to 1.9160e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9976
Weighted Sum of Squares (1/Y)	0.7921
Absolute Sum of Squares	62.03
Sy.x	1.679
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

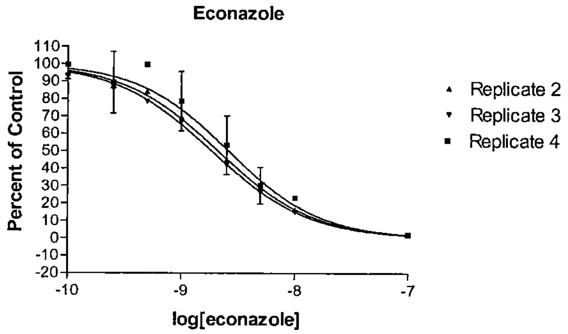
Econazole
Replicate 4



log[Ref chem 4]	Percent of Control		
	Y1	Y2	Y3
-7.0	2.4	2.1	2.4
-8.0	23.8	22.7	23.5
-8.3	42.0	21.6	27.2
-8.6	63.8	62.1	34.0
-9.0	89.9	86.8	58.9
-9.3	99.5	99.5	99.5
-9.6	99.5	99.5	68.9
-10.0	99.5	99.5	99.5

	Percent of Control
Equation 1	
Best-fit values	
BOTTOM (Constant)	0.0
TOP (Constant)	100.0
LOGEC50	-8.584
HILLSLOPE	-1.081
EC50	2.6090e-009
Std. Error	
LOGEC50	0.05389
HILLSLOPE	0.1224
95% Confidence Intervals	
LOGEC50	-8.695 to -8.472
HILLSLOPE	-1.334 to -0.8267
EC50	2.0170e-009 to 3.3740e-009
Goodness of Fit	
Degrees of Freedom	22
R^2 (unweighted)	0.9123
Weighted Sum of Squares (1/Y)	48.83
Absolute Sum of Squares	2788
Sy.x	11.26
Data	
Number of X values	8
Number of Y replicates	3
Total number of values	24
Number of missing values	0

Econazole
Replicates 2, 3, 4



log[Ref chem 4]	Replicate 4			Replicate 2			Replicate 3		
	Y1	Y2	Y3	Y1	Y2	Y3	Y1	Y2	Y3
-7.0	2.4	2.1	2.4	1.9	1.9	1.9	1.8	1.7	1.6
-8.0	23.8	22.7	23.5	16.0	15.7	17.2	15.1	15.2	15.0
-8.3	42.0	21.6	27.2	29.7	29.9	27.9	26.5	25.8	26.3
-8.6	63.8	62.1	34.0	45.0	45.0	45.0	42.7	42.2	41.3
-9.0	89.9	86.8	58.9	70.2	70.3	67.3	68.9	66.6	65.3
-9.3	99.5	99.5	99.5	84.8	83.4	83.5	78.6	77.1	79.2
-9.6	99.5	99.5	68.9	90.4	86.9	85.1	90.8	87.2	90.0
-10.0	99.5	99.5	99.5	93.7	95.3	94.1	90.9	91.7	95.5

	Replicate 4	Replicate 2	Replicate 3
Equation 1			
Best-fit values			
BOTTOM (Constant)	0.0	0.0	0.0
TOP (Constant)	100.0	100.0	100.0
LOGEC50	-8.584	-8.676	-8.732
HILLSLOPE	-1.081	-1.037	-1.023
EC50	2.6090e-009	2.1090e-009	1.8520e-009
Std. Error			
LOGEC50	0.05389	0.008105	0.007136
HILLSLOPE	0.1224	0.01570	0.01321
95% Confidence Intervals			
LOGEC50	-8.695 to -8.472	-8.693 to -8.659	-8.747 to -8.718
HILLSLOPE	-1.334 to -0.8267	-1.069 to -1.004	-1.050 to -0.9951
EC50	2.0170e-009 to 3.3740e-009	2.0290e-009 to 2.1920e-009	1.7900e-009 to 1.9160e-009
Goodness of Fit			
Degrees of Freedom	22	22	22
R^2 (unweighted)	0.9123	0.9972	0.9976
Weighted Sum of Squares (1/Y)	48.83	1.038	0.7921
Absolute Sum of Squares	2788	73.93	62.03
Sy.x	11.26	1.833	1.679
Data			
Number of X values	8	8	8
Number of Y replicates	3	3	3
Total number of values	24	24	24
Number of missing values	0	0	0

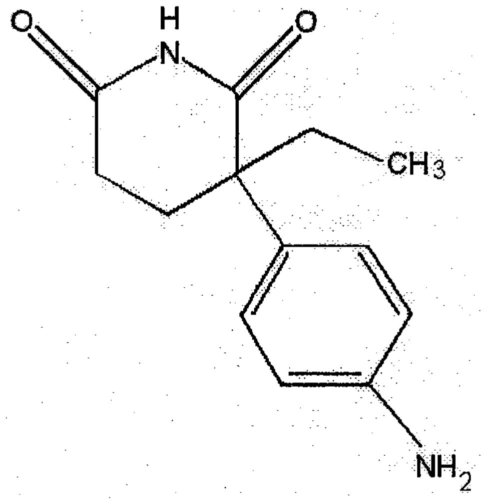
Appendix E

Chemistry Reports

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

AMINOGLUTETHIMIDE

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

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

Prepared By:

Denise A. Contos

Denise A. Contos, M.S.

Approved By:

10-18-05

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

QUALITY ASSURANCE STATEMENT

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

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

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

Quality Assurance Unit 10-14-05
Date

EXECUTIVE SUMMARY

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

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

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

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

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

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 aminoglutethimide on EPA Work Assignment 4-16/17, and consisted of:

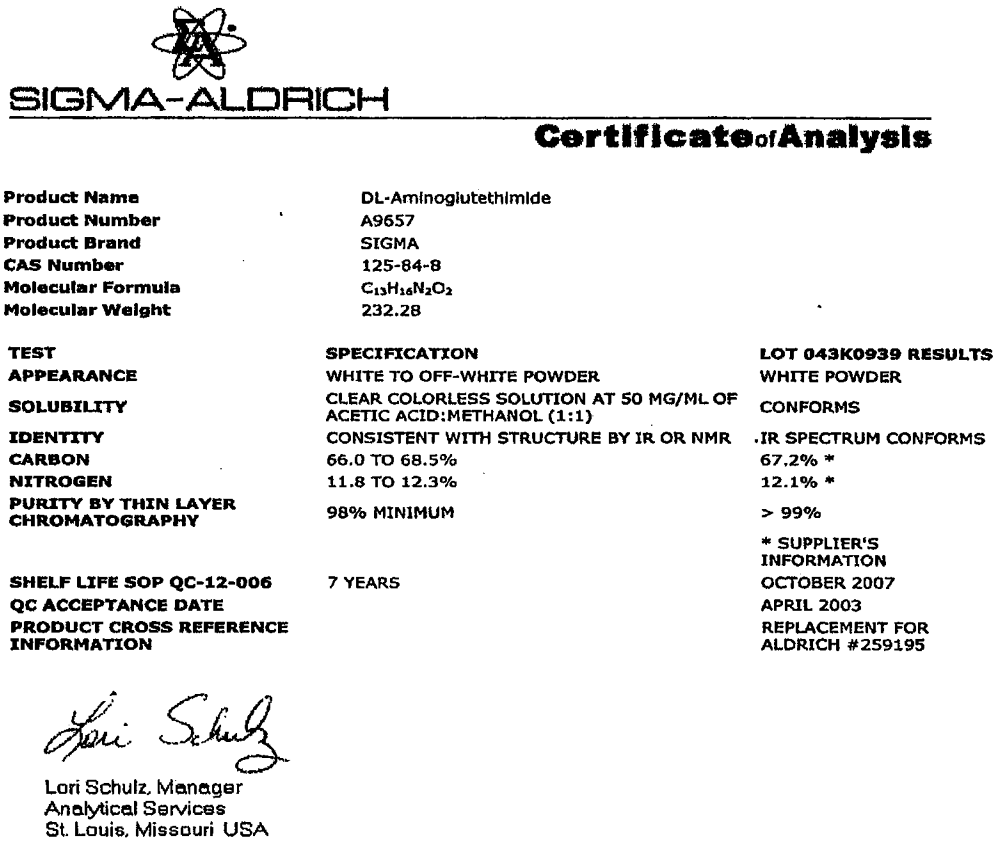
- Determining solubility in dimethylsulfoxide (DMSO)
- Preparing and analyzing a stock formulation and a formulation stability sample.

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

2 CHEMICAL RECEIPT AND STORAGE

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

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



SIGMA-ALDRICH

Certificate of Analysis

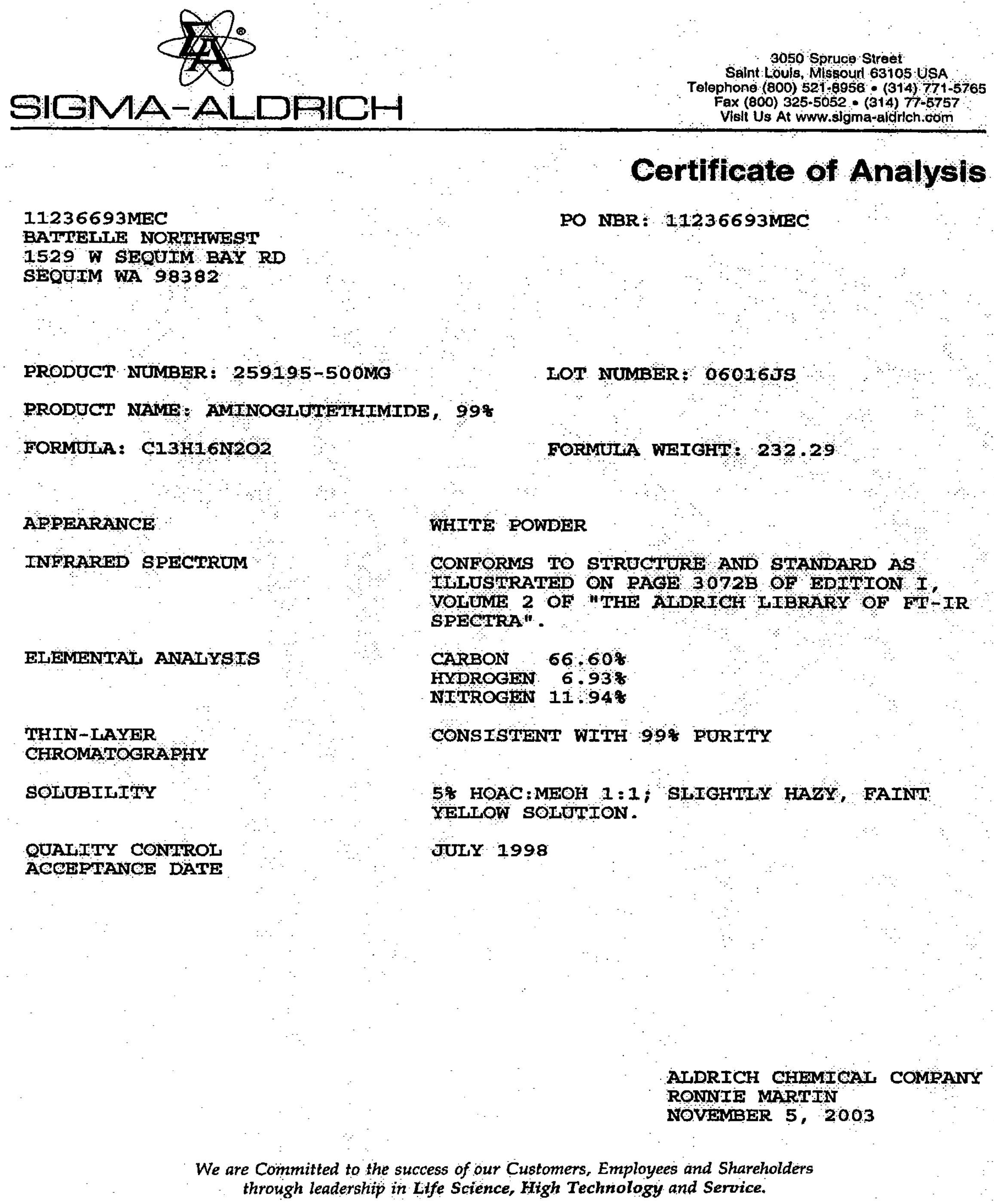
Product Name	DL-Aminoglutethimide
Product Number	A9657
Product Brand	SIGMA
CAS Number	125-84-8
Molecular Formula	$C_{13}H_{16}N_2O_2$
Molecular Weight	232.28

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

Lori Schulz

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

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



SIGMA-ALDRICH

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

Certificate of Analysis

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

PO NBR: 11236693MEC

PRODUCT NUMBER: 259195-500MG

LOT NUMBER: 06016JS

PRODUCT NAME: AMINOGLUTETHIMIDE, 99%

FORMULA: C13H16N2O2

FORMULA WEIGHT: 232.29

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

ALDRICH CHEMICAL COMPANY
RONNIE MARTIN
NOVEMBER 5, 2003

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

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

3 SOLUBILITY STUDY

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

4 FORMULATION PREPARATION AND ANALYSIS

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

4.1 Preparation of Formulation

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

4.2 Preparation of Standards and Blanks

4.2.1 Internal Standard (IS)

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

4.2.2 Stock Standards

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

4.2.3 Vehicle/Calibration Standards

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

Table 1 – Preparation of Vehicle/Calibration Standards

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

4.2.4 Blanks

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

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

4.3 Preparation of Formulation and Formulation Stability Samples

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

4.4 Analysis

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

Table 2 – GC System

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

4.5 Calculations

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

4.6 Results

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

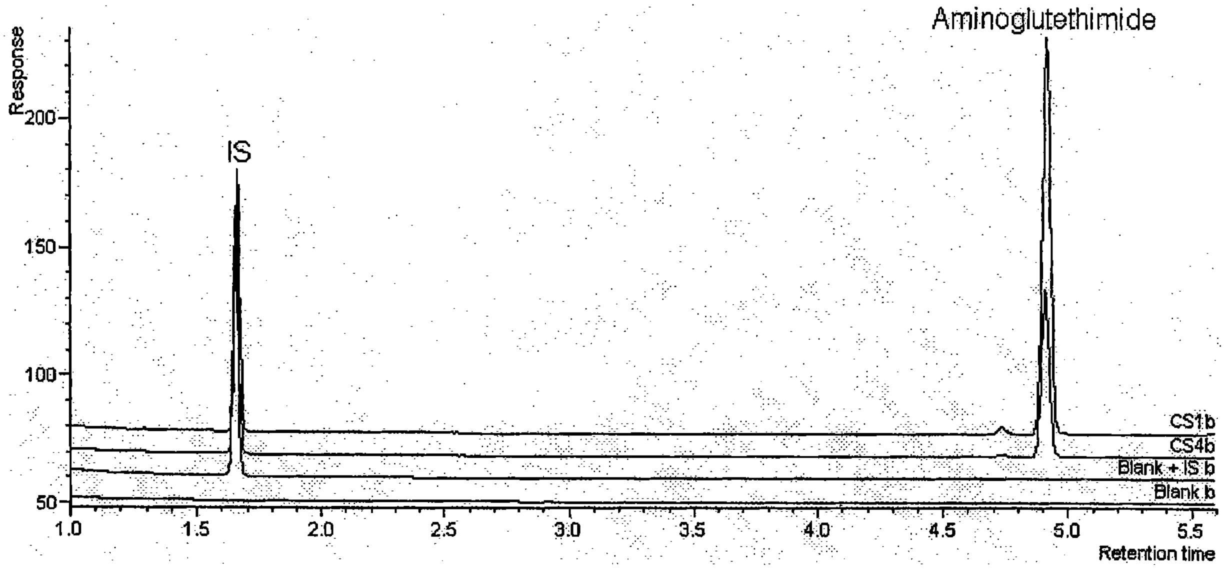


Figure 3 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from 1/24/2005 Formulation Analysis (Shown Top to Bottom)

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

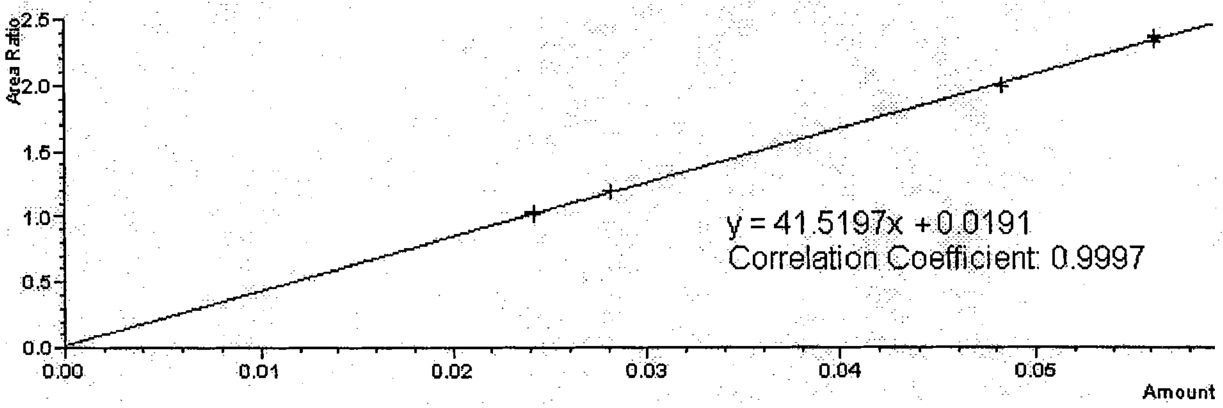


Figure 4 – Vehicle/Calibration Standard Curve for 1/24/2005 Analysis

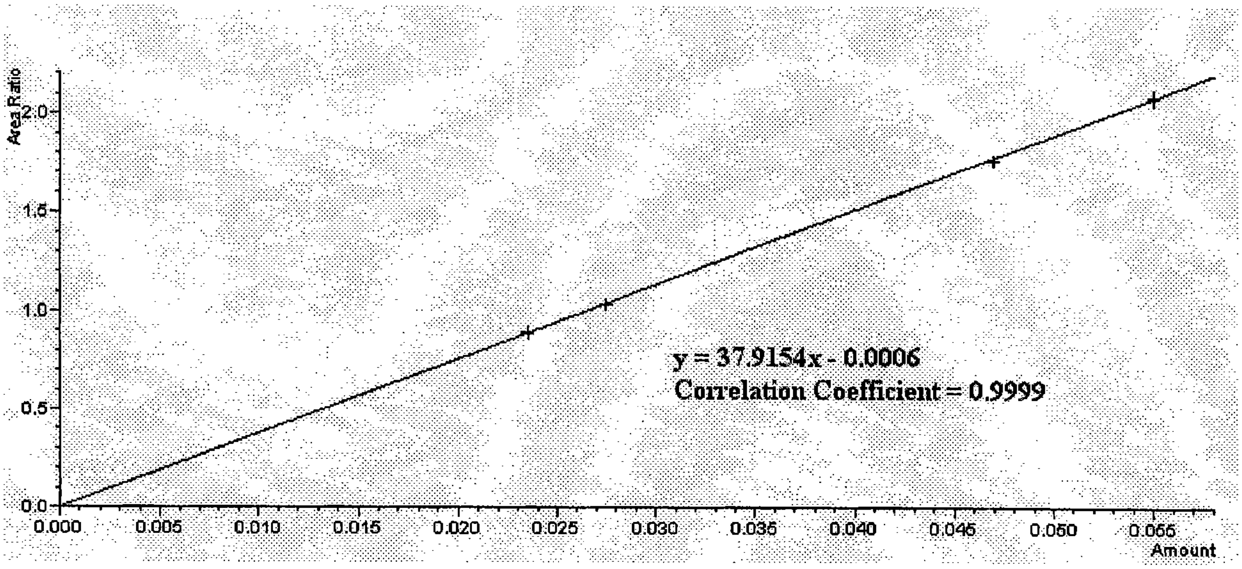


Figure 5 – Vehicle/Calibration Standard Curve for 6/30/2005 Analysis

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

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

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

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

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

Table 4 – Formulation Analysis Results

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

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

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

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

4.7 Conclusions

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

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

5 ACKNOWLEDGMENTS

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

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

CHRYSIN

CAS No.: 480-40-0

Lot No.: 10101DC (Sigma Aldrich)

Receipt Date: 10/26/04

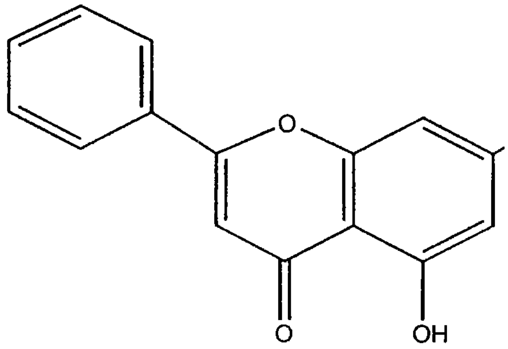
Amount Received: 25 g

Appearance: Solid

Vendor Purity: 98.20% by HPLC

Storage Conditions (@ Battelle): Room temperature (~25°C)

STRUCTURE:



Mol. Wt.: 254.24 g/mol

Mol. Formula: $C_{15}H_{10}O_4$

Prepared By:

Denise A. Contos 12/22/05

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

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

QUALITY ASSURANCE STATEMENT

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

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

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

12-22-05

Quality Assurance Unit — Date

Battelle

EXECUTIVE SUMMARY

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

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

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

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

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

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1 INTRODUCTION

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

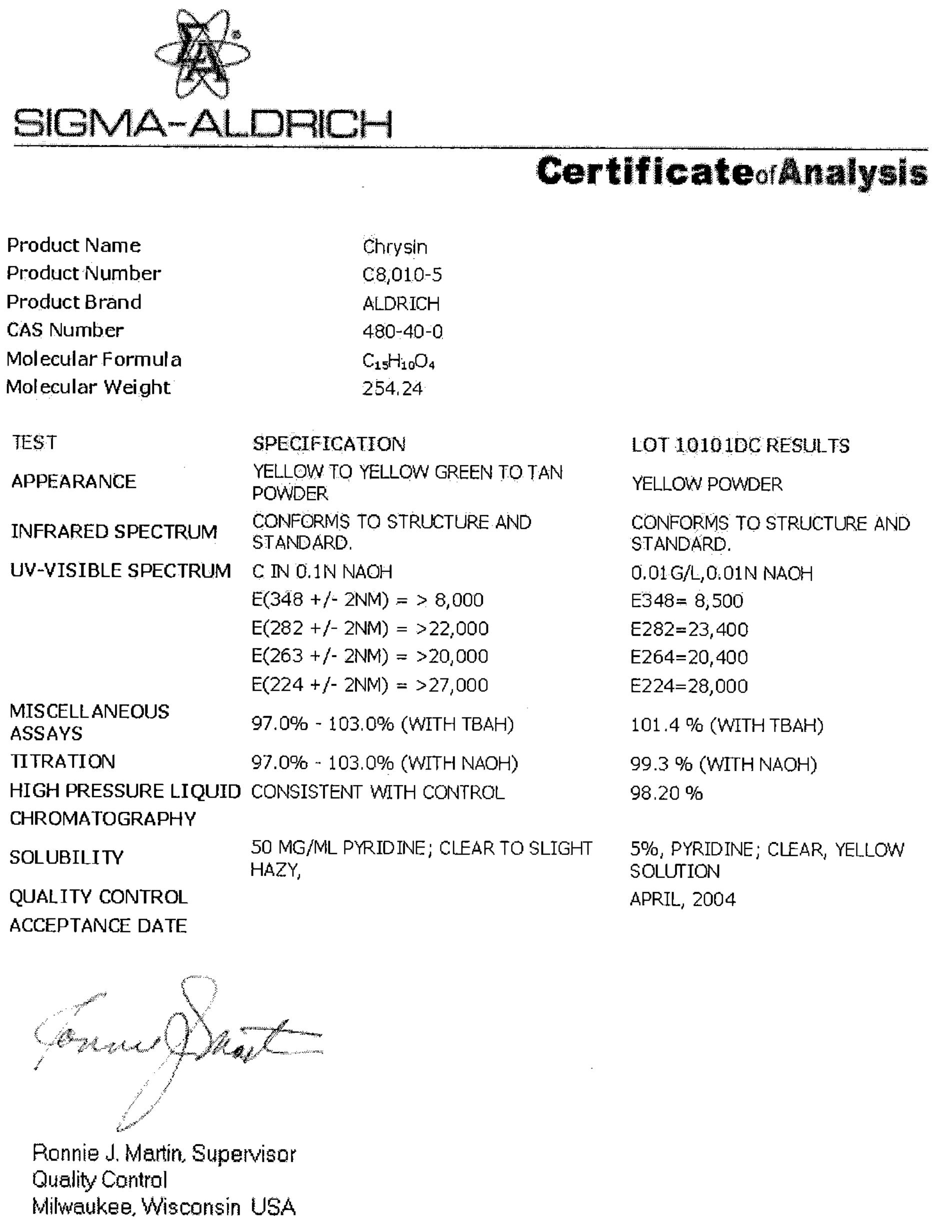
- Determining solubility in dimethylsulfoxide (DMSO)
- Developing and validating a formulation analysis method
- Conducting a storage stability study
- Preparing and analyzing a stock formulation.

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

2 CHEMICAL RECEIPT AND STORAGE

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

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



SIGMA-ALDRICH

Certificate of Analysis

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

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

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

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

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

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

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

4.1 Method Development

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

4.2 Method

The HPLC parameters for CHRY are presented in Table 1.

Table 1 – HPLC System

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

4.3 Method Validation

Validation was accomplished using a single experiment.

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

4.3.1 Preparation of Mobile Phase

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

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

4.3.2 Preparation of Standards and Blanks

4.3.2.1 Internal Standard (IS)

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

4.3.2.2 Stock Standards

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

4.3.2.3 Working Standards

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

Table 2 – Preparation of Working Standards

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

4.3.2.4 Vehicle/Calibration Standards

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

Table 3 – Preparation of Vehicle/Calibration Standards

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

4.3.2.5 Triplicate Blanks without and with IS

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

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

4.3.3 Analysis

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

4.3.4 Calculations

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

4.3.5 Results

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

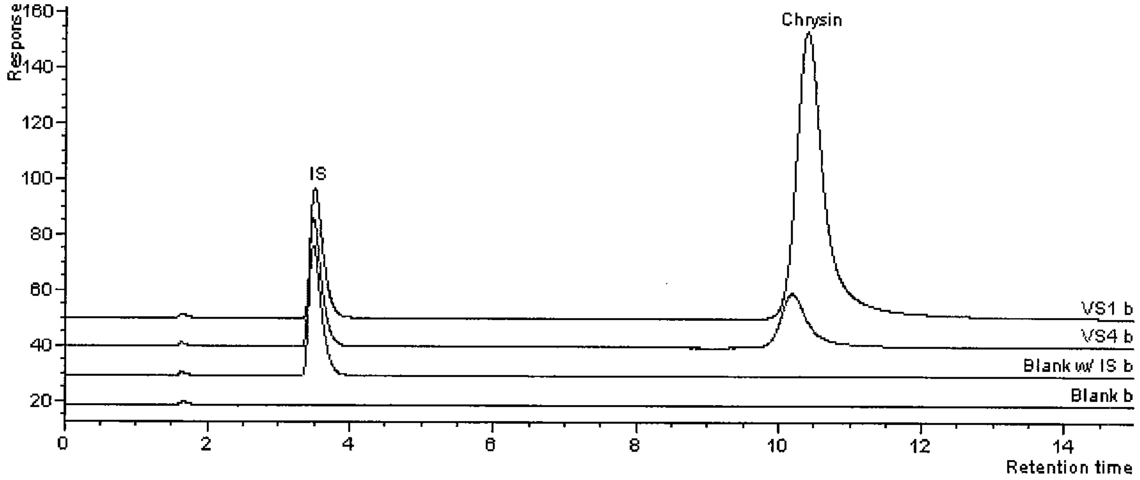


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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

Table 4 – Regression Analysis Results

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

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

Table 5 – Vehicle/Calibration Standard Validation Results

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

For the method validation the LOD is 0.18 µg/mL and is defined as three times the standard deviation of the lowest vehicle/calibration standard. This is equivalent to a formulation concentration of 18 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ is 0.59 µg/mL and is defined as ten times the standard deviation of the lowest vehicle/calibration standard because there was no blank response. This is equivalent to a formulation concentration of 59 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest vehicle/calibration standard with acceptable accuracy and precision, was 10.04 µg/mL.

4.3.6 Conclusions

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

5 FORMULATION STABILITY STUDIES

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

5.1 Study Design

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

5.2 Formulation Method

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

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

5.3 Analysis Method

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

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

5.4 Results

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

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

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

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

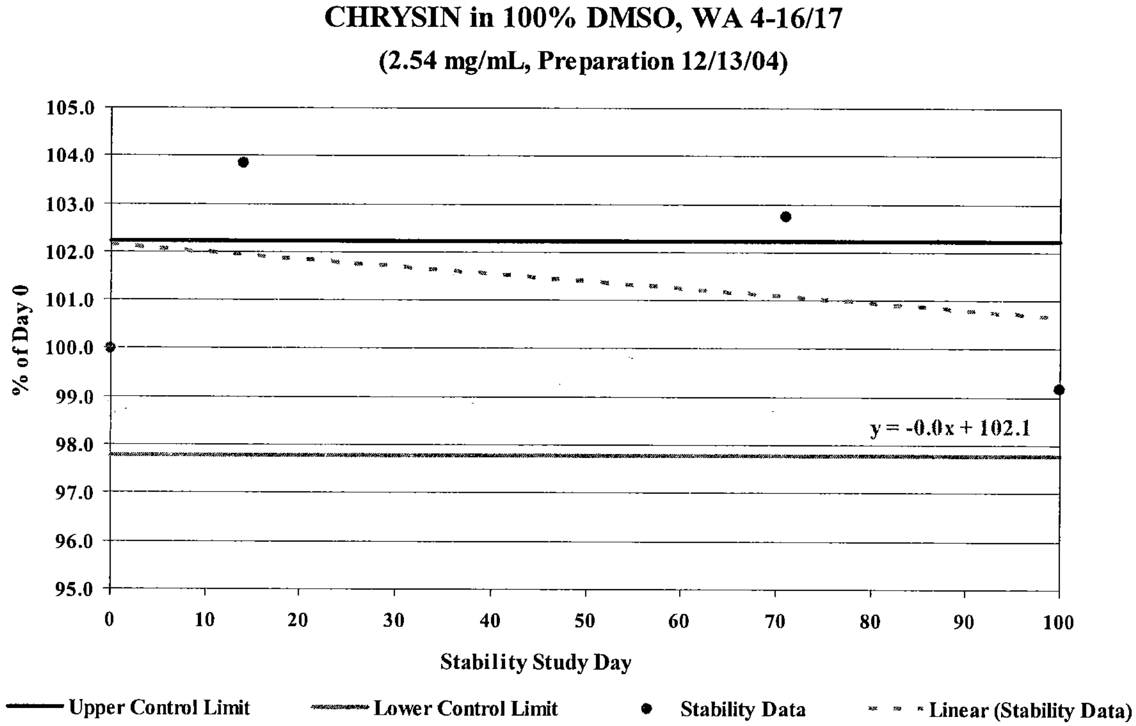

CHRYSIN in 100% DMSO, WA 4-16/17
(2.54 mg/mL, Preparation 12/13/04)

Figure 3 – Control Chart for Storage Stability Analysis

5.5 Discussion and Conclusions

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

6 FORMULATION PREPARATION AND ANALYSIS

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

6.1 Preparation of Formulation

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

6.2 Preparation of Standards and Blanks

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

6.3 Preparation of Formulation Samples

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

6.4 Analysis

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

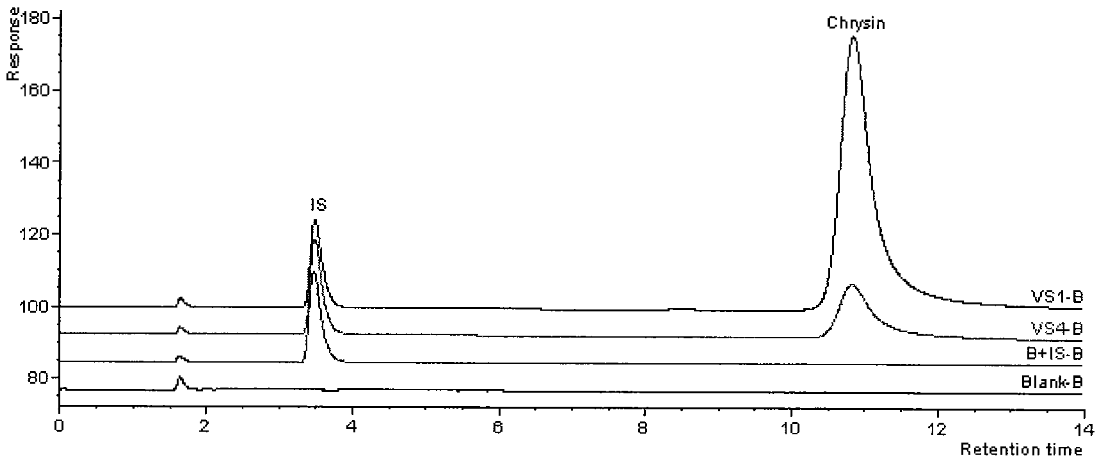


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

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

6.6 Results

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

Table 7 – Formulation Analysis Results

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

Table 8 – Regression Analysis Results

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

6.7 Conclusions

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

7 ACKNOWLEDGMENTS.

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

Battelle
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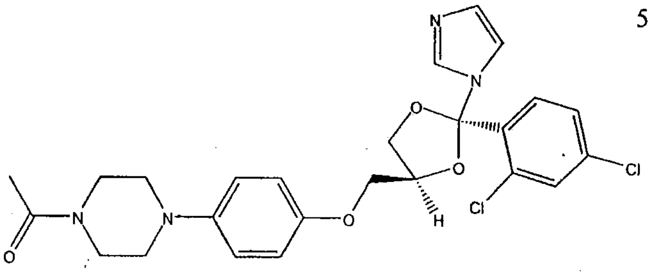
ANALYTICAL CHEMISTRY ACTIVITIES REPORT

KETOCONAZOLE

CAS No.: 65277-42-1
Receipt Date: 10/26/04
Appearance: Solid
Storage Conditions (@ Battelle): Refrigerated (~5°C)

Lot No.: 121H0524 (Sigma Aldrich)
Amount Received: 2.7 g
Vendor Purity: > 99% by TLC

STRUCTURE:



Mol. Wt.:
531.43 g/mol

Mol. Formula:
$C_{26}H_{28}Cl_2N_4O_4$

Prepared By:

Denise A. Contos 12/28/05

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

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

QUALITY ASSURANCE STATEMENT

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

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit study file	8/23/2005	8/23/2005
Audit analytical report	8/23/2005	8/23/2005
Audit study file	12/16/2005	12/16/2005
Audit analytical report	12/16/2005	12/16/2005

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

12-27-05

Quality Assurance Unit   Date

EXECUTIVE SUMMARY

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

Solubility of ketoconazole was determined to be acceptable in dimethylsulfoxide (DMSO) at a concentration of 5.31 mg/mL (0.01 M).

A ketoconazole formulation analysis method was validated on the previous EPA WA 3-10 study. The method was modified by including an additional 1:10 dilution of the formulation which resulted in a 1:200 final dilution of the 5.31 mg/mL (0.01 M) formulation prior to analysis. This modified method was used to analyze both stability and formulation analysis samples.

Storage stability was previously determined (EPA WA 3-10 study, Analytical Chemistry Activities Report, Ketoconazole, 2004) as 28 days when stored at approximately 5°C and protected from light at a target formulation concentration of 0.532 mg/mL in DMSO. In the current study, a formulation sample with a target concentration of 5.31 mg/mL in DMSO was stable when stored refrigerated and protected from light for 60 days.

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

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1 INTRODUCTION

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

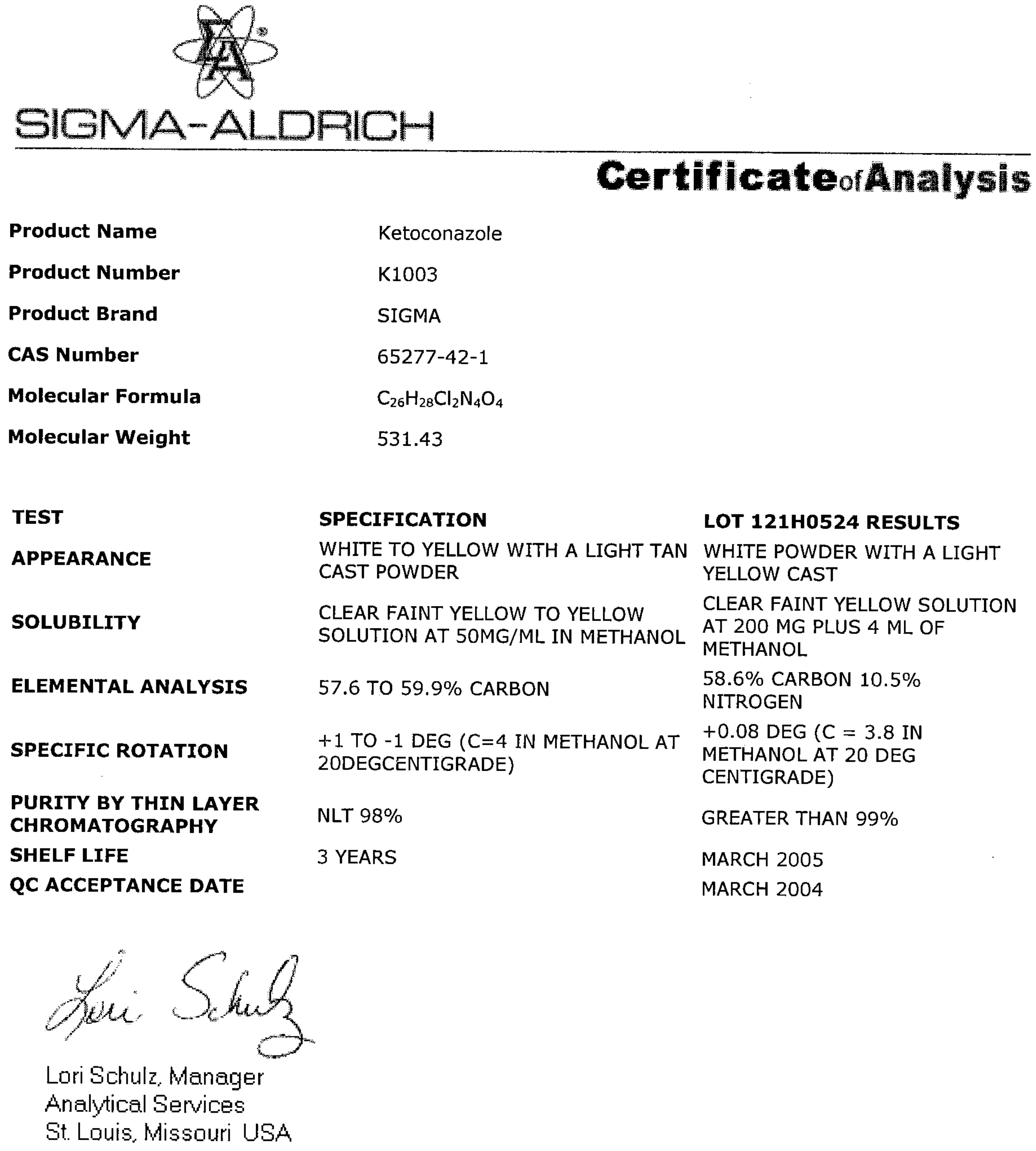
- Determining solubility in dimethylsulfoxide (DMSO).
- Preparing and analyzing a stock formulation and a formulation stability sample.

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

2 CHEMICAL RECEIPT AND STORAGE

One 15-mL amber glass bottle of ketoconazole, Lot No. 121H0524, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 26, 2004. The label amount indicated 2.7 grams was sent. The chemical was received and subsequently stored refrigerated.

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



SIGMA-ALDRICH

Certificate of Analysis

Product Name	Ketoconazole
Product Number	K1003
Product Brand	SIGMA
CAS Number	65277-42-1
Molecular Formula	$C_{26}H_{28}Cl_2N_4O_4$
Molecular Weight	531.43

TEST	**SPECIFICATION**	**LOT 121H0524 RESULTS**
APPEARANCE	WHITE TO YELLOW WITH A LIGHT TAN CAST POWDER	WHITE POWDER WITH A LIGHT YELLOW CAST
SOLUBILITY	CLEAR FAINT YELLOW TO YELLOW SOLUTION AT 50MG/ML IN METHANOL	CLEAR FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF METHANOL
ELEMENTAL ANALYSIS	57.6 TO 59.9% CARBON	58.6% CARBON 10.5% NITROGEN
SPECIFIC ROTATION	+1 TO -1 DEG (C=4 IN METHANOL AT 20DEGCENTIGRADE)	+0.08 DEG (C = 3.8 IN METHANOL AT 20 DEG CENTIGRADE)
PURITY BY THIN LAYER CHROMATOGRAPHY	NLT 98%	GREATER THAN 99%
SHELF LIFE	3 YEARS	MARCH 2005
QC ACCEPTANCE DATE		MARCH 2004

Lori Schulz

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

Figure 1 - Certificate of Analysis

3 SOLUBILITY STUDY

A solubility study was conducted to determine the solubility of ketoconazole in either 95% ethanol or 100% DMSO. Initially, both 95% ethanol and DMSO were used to prepare a 53.14 mg/mL (0.1 M) ketoconazole solution. Neither of the solvents dissolved the ketoconazole at the 0.1 M concentration. At the direction of the Task Leader, a 5.31 mg/mL ketoconazole solution (0.01 M) in 100% DMSO was prepared by weighing 0.05314 ± 0.00531 g into a

10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was capped and contents were mixed. The content of the flask was diluted to volume with DMSO, sealed, mixed and sonicated. The ketoconazole went into solution with minimal shaking and sonication. This experiment showed that DMSO was an acceptable solvent for a 5.31 mg/mL (0.01 M) formulation.

4 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on February 4, 2005 and June 29, 2005 according to SOP COMSPEC.II-018-02, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Ketoconazole in 100% DMSO." In addition, the February 4, 2005 formulation was re-analyzed to determine stability on April 5, 2005, 60 days after storage at approximately 5°C and protected from light. This section describes the method, results, and conclusions.

4.1 Preparation of Formulation

A ketoconazole formulation with a target concentration of 5.31 mg/mL (0.01 M) in DMSO was prepared on February 4, 2005 by accurately weighing 265.5 ± 5 mg of ketoconazole into a tarred 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The flask was sealed and sonicated for approximately 10 minutes then inverted ten times. The content of the flask was diluted to volume with DMSO, sealed, and mixed well by inverting at least ten times.

4.2 Preparation of High Performance Liquid Chromatography (HPLC) Mobile Phase

An accurate amount of ammonium acetate (3.0 g) was weighed into a 2000-mL HPLC mobile phase bottle. A 600 mL volume of Milli-Q water was added to the bottle and the contents were mixed well. A final volume of 1400 mL of methanol and 2.8 mL of diethanolamine were added to the bottle and the contents were mixed well. This produced a HPLC mobile phase containing 70:30:0.14 (v:v:v) of methanol:0.5% ammonium acetate: diethanolamine.

4.3 Preparation of Standards and Blanks

4.3.1 Internal Standard (IS)

Fifteen (15) ± 2 mg terconazole was added to a 50-mL volumetric flask. The content of the flask was diluted to volume with HPLC mobile phase, sealed, and mixed well. This produced a solution with a target concentration of 300 µg/mL.

4.3.2 Stock Standards

Two stock standards were prepared by accurately weighing 30 ± 3.0 mg of ketoconazole into two individual 200-mL volumetric flasks and dissolving in and diluting to volume with HPLC mobile phase. This produced stocks A and B with target concentrations of 150 µg/mL each.

4.3.3 Vehicle/Calibration Standards

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

Table 1 – Preparation of Vehicle/Calibration Standards

Vehicle Std	Target Final Conc (µg/mL)	Source	Source Volume (mL)	Internal Std (mL)	DMSO (mL)	Final Volume (mL)
VS1	60	A	4	1	0.05	10
VS2	45	B	3	1	0.05	10
VS3	30	A	2	1	0.05	10
VS4	15	B	1	1	0.05	10

4.3.4 Blanks

Triplicate blanks without IS were prepared by pipetting 0.05 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

Triplicate blanks with IS were prepared by pipetting 1 mL IS and 0.05 mL of DMSO into three individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.4 Preparation of Formulation and Formulation Stability Samples

Triplicate 1 mL aliquots of the formulation were pipetted into three individual 10-mL volumetric flasks, diluted to volume with HPLC mobile phase, sealed, and mixed well. A 0.5 mL aliquot of the diluted formulation and 1 mL of the IS were pipetted into individual 10-mL volumetric flasks. The contents of the flasks were diluted to volume with HPLC mobile phase, sealed, and mixed well.

4.5 Analysis

A portion of each vehicle/calibration standard, blank and sample were transferred to individual autoinjector vials and the vials were sealed. Single injections were made from each vial using the HPLC parameters for ketoconazole which are presented in Table 2.

Table 2 – HPLC System

Instrument System	Agilent (Palo Alto, CA); Waters (Milford, MA)
Column	Supelcosil LC-ABZ, 5 µm particle size, 250 mm × 4.6 mm (ID) (Supelco, St. Louis, MO)
Guard	C_{18} guard cartridge
Mobile Phase	70:30:0.14 (v/v/v) Methanol:0.5% Ammonium Acetate:Diethanolamine, Isocratic
Flow Rate	1.5 mL/minute
Injection Volume	20 µL
Detector Type	Ultraviolet (UV)
Detector Wavelength	245 nm

4.6 Calculations

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

4.7 Results

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

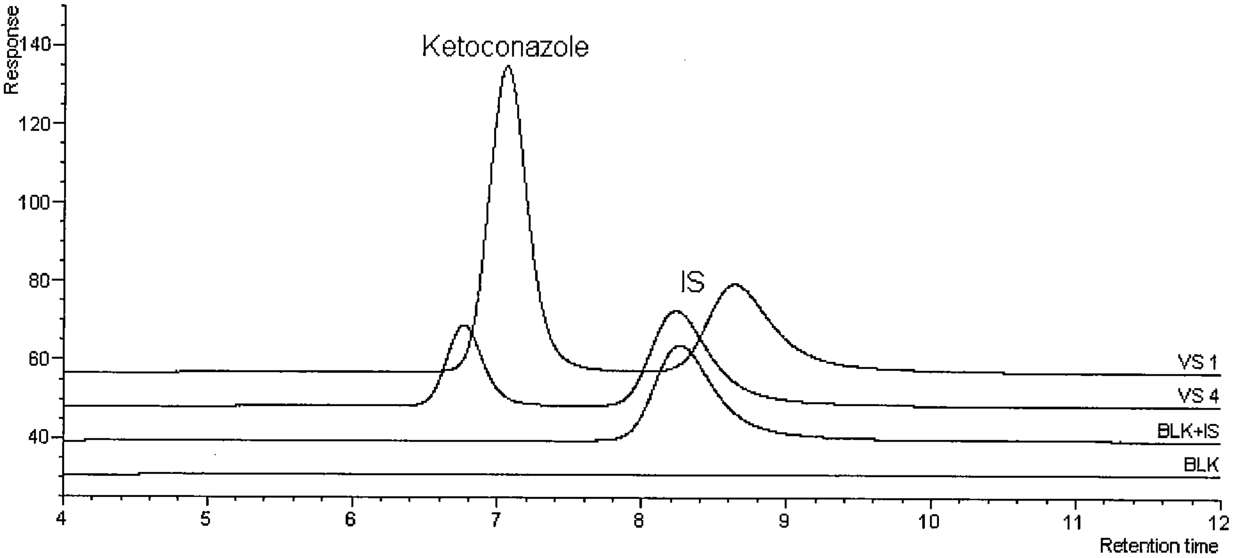


Figure 2 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from 2/4/2005 Analysis (Shown Top to Bottom)

The regression analysis results from the standard curve for February 4, 2005 analysis indicate linearity and are shown in Figure 3.

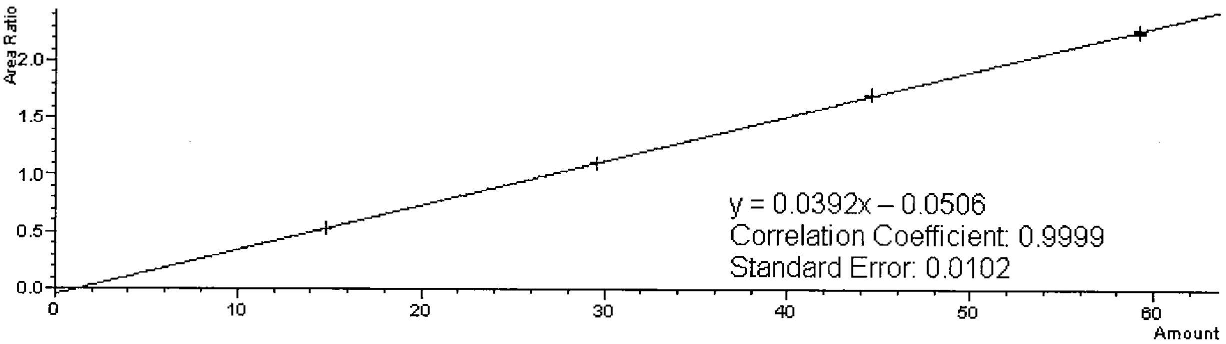


Figure 3 – Vehicle/Calibration Standard Curve for 2/4/2005 Analysis

The precision and accuracy of the vehicle/calibration standard results from February 4, 2005 analysis are shown in Table 3.

Table 3 –Vehicle/Calibration Standard Results for 2/4/2005 Analysis

Nominal Std Conc (µg/mL)	Det'd Conc (µg/mL)	Avg Det'd Conc (µg/mL)	s (µg/mL)	% RSD	% RE	Avg % RE
59.20	59.15	59.08	0.30	0.5	-0.1	-0.2
	59.33				0.2	
	58.75				-0.8	
44.60	44.98	NA	NA	NA	0.9	NA
29.60	29.64	NA	NA	NA	0.1	NA
14.87	14.69	14.85	0.14	0.9	-1.2	-0.1
	14.92				0.4	
	14.94				0.5	

The results of the formulation and formulation stability sample analysis are shown in Table 4 and 5. The formulation stability sample was the same formulation sample prepared and analyzed on February 4, 2005 that had been stored refrigerated for 60 days, protected from light in an amber glass bottle.

Table 4 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	RSD (%)
1-KET-1	2/4/2005	5.136	5.122	5.134	5.131	-3.4	0.1
2-KET-1	6/29/2005	5.458	5.487	5.464	5.470	3.0	0.3

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

Table 5 –Formulation Stability Analysis Results

Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % of Day 0 Conc ± s
4/5/2005	5.316	5.359	5.369	5.348	104.2 ± 0.5

The formulation stability sample analyzed on April 5, 2005 was within 4.2% of the Day 0 value (February 4, 2005 analysis value) and met acceptance criteria + 10 %.

4.8 Conclusions

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

The ketoconazole formulation at a target concentration of 5.31 mg/mL in DMSO was stable for 60 days when stored refrigerated and protected from light.

5 ACKNOWLEDGMENTS

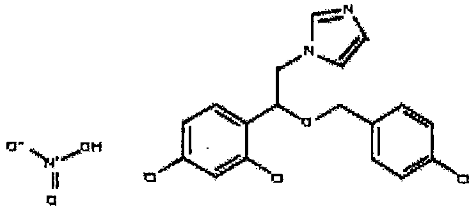
Analytical support for this work was provided by Jim Hoskinson and Tudor Fernando. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

ANALYTICAL CHEMISTRY ACTIVITIES REPORT

ECONAZOLE

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

STRUCTURE:



Mol. Wt.: 444.70 g/mol

Mol. Formula: $C_{18}H_{15}Cl_3N_2O{\cdot}HNO_3$

Prepared By:

Denise A. Contos 1/6/2006

Denise A. Contos, M.S.

Approved By:

Steven W Graves

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

QUALITY ASSURANCE STATEMENT

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

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

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

1-5-06

Quality Assurance Unit — Date

EXECUTIVE SUMMARY

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

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

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

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

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

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1 INTRODUCTION

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

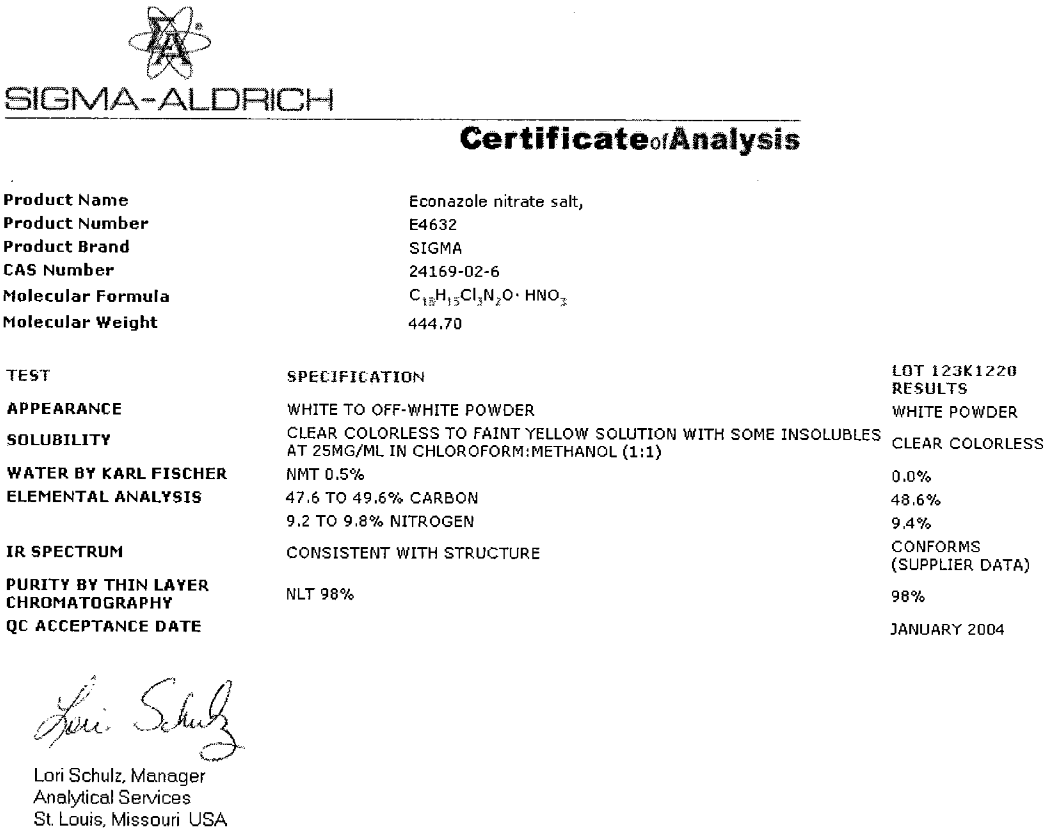
- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

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

2 CHEMICAL RECEIPT AND STORAGE

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

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



SIGMA-ALDRICH

Certificate of Analysis

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

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

Lori Schulz

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

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

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

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

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

4.1 Method Development

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

4.2 Method

The HPLC parameters for econazole are shown in Table 1.

Table 1 – HPLC System

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

4.3 Method Validation

Validation was accomplished using a single experiment.

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

4.3.1 Preparation of Mobile Phase

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

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

4.3.2 Preparation of Standards and Blanks

4.3.2.1 Internal Standard (IS)

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

4.3.2.2 Stock Standards

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

4.3.2.3 Vehicle/Calibration Standards

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

Table 2 – Preparation of Vehicle/Calibration Standards

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

4.3.2.4 Blanks

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

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

4.3.3 Analysis

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

4.3.4 Calculations

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

4.3.5 Results

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

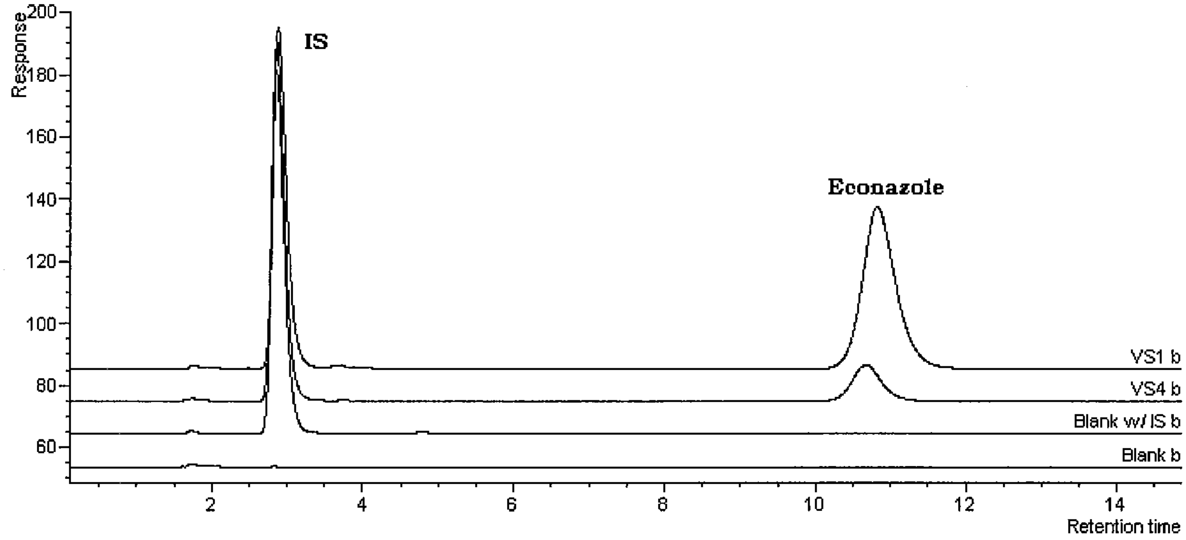


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Regression Analysis Results

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

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	% RSD	% RE	Avg % RE
512.6	513.2 512.7 511.7	512.5	0.8	0.1	0.1 0.0 -0.2	0.0
406.9	406.7	NA	NA	NA	0.0	NA
205.0	206.8	NA	NA	NA	0.9	NA
101.7	101.3 101.2 101.3	101.3	0.1	0.1	-0.4 -0.5 -0.4	-0.4

The method validation sensitivity was 0.1732 µg/mL, the LOD, which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 20 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 0.5774 µg/mL. This is equivalent to a formulation concentration of 60 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest vehicle/calibration standard with acceptable accuracy and precision, was 101.7 µg/mL.

4.3.6 Conclusions

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

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 44.47 mg/mL in DMSO for 56 days (8 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A formulation sample was analyzed on the day of preparation (Day 0) and Day 14. A second formulation sample was analyzed on the day of preparation (Day 0), Week 4 and Week 8. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on November 19, 2004, Day 0 of the storage stability study at a target concentration of 44.47 mg/mL in DMSO by accurately weighing 1110 ± 10 mg into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with DMSO. The flask was sealed and sonicated for approximately 5 minutes to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

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

A second formulation was prepared on February 7, 2005, Day 0, at a target concentration of 44.47 mg/mL in DMSO by accurately weighing 2.22350 ± 0.08894 g into a 50-mL volumetric flask. The flask was diluted to approximately 80% volume with DMSO, sealed and sonicated for approximately 5 minutes. The content of the flask was inverted 10 times, and diluted to volume with DMSO and shaken to mix well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Day 0, Weeks 4 and 8 for storage stability determination.

5.3 Analysis Method

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

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

5.4 Results

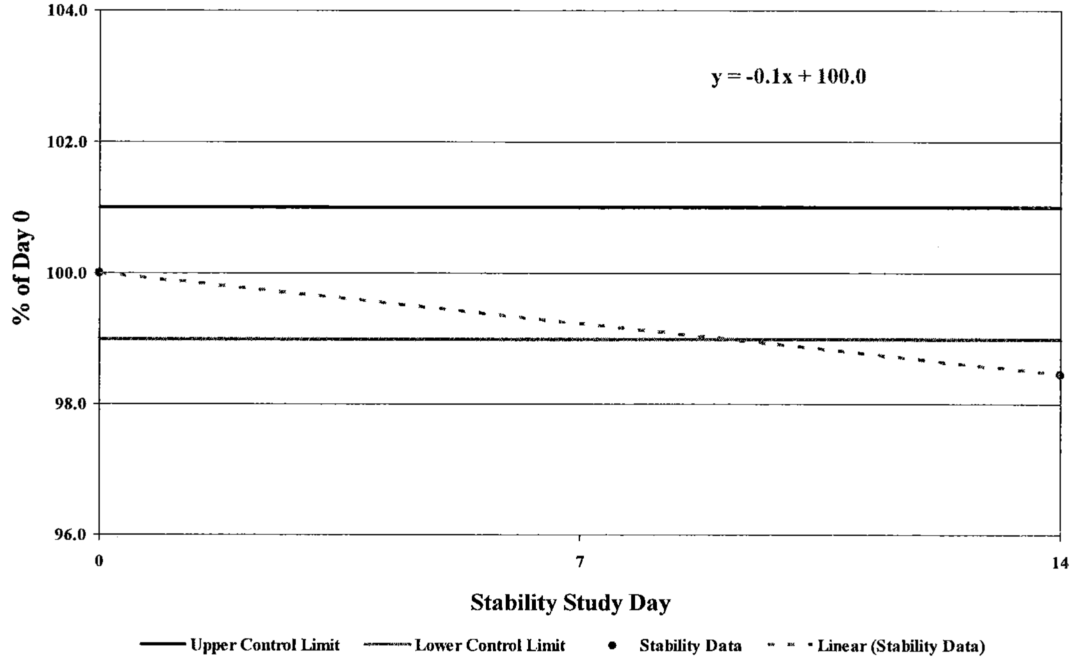
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (44.47 mg/mL)

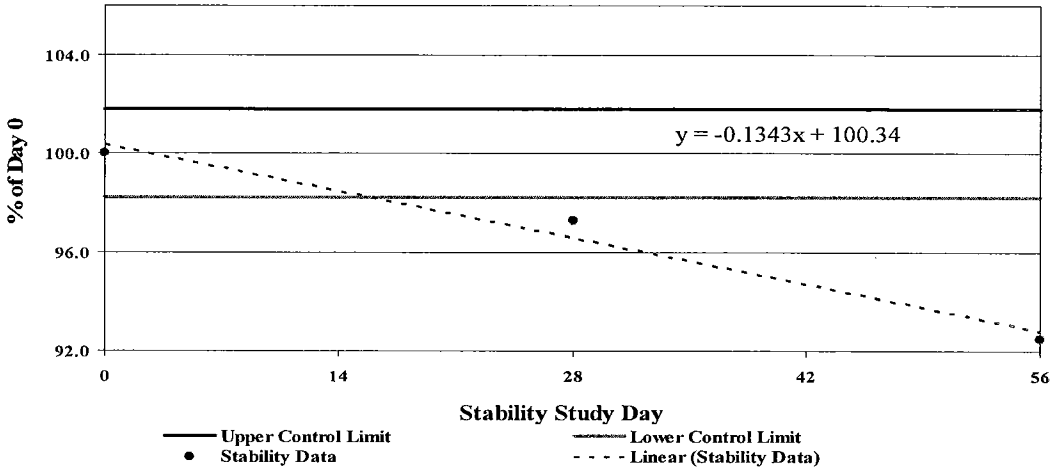
Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
11/19/04	11/19/04	0	46.91	46.37	46.47	46.58 ± 0.29	100.0 ± 0.6
11/19/04	12/3/04	14	45.91	45.88	45.80	45.86 ± 0.06	98.5 ± 0.1
2/7/05	2/7/05	0	46.24	46.08	45.89	46.07 ± 0.17	100.0 ± 0.4
2/7/05	3/7/05	28	45.03	44.74	44.66	44.81 ± 0.19	97.3 ± 0.4
2/7/05	4/4/05	56	42.95	42.92	41.94	42.60 ± 0.58	92.5 ± 1.3

For the formulation stability sample prepared on November 19, 2004, the pooled RSD of the analytical method was 0.44%. This means that there would have to be a difference of more than 1.01% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation stability sample prepared on February 7, 2005, the pooled RSD of the analytical method was 0.79%. This means that there would have to be a difference of more than 1.80% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.



ECONAZOLE in 100% DMSO
(46.58 mg/mL, Prepared 11/19/04)



ECONAZOLE in 100% DMSO
(46.07 mg/mL, Prepared 2/7/05)

Figure 3 – Control Charts for the Storage Stability Analysis

5.5 Discussion and Conclusions

The Day 0 determined value for the November 19, 2004 formulation was approximately 4.7% above the nominal value (the calculated concentration based on the weight of the chemical). The concentration of the sample stored at approximately 5°C protected from light in amber glass vial for Day 14 was below the lower significance level due to the tight precision of the assay but was within 1.5% of the Day 0 value (November 19, 2004) and met acceptance criteria ± 10%.

The Day 0 determined value for the February 7, 2005 formulation was approximately 3.5% above the nominal value (the calculated concentration based on the weight of the chemical). The concentrations of the sample stored at approximately 5°C protected from light in an amber glass vial for Days 28 and 56 was below the lower significance level due to the tight precision of the assay but was within 2.7 and 7.5%, respectively of the Day 0 value (February 7, 2005) and met acceptance criteria ± 10%.

These data indicate the formulation was stable when protected from light at approximately 5°C for 56 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on February 7, 2005 and August 8, 2005 according to SOP COMSPEC.II-031, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Econazole in 100% DMSO." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Econazole (2.22350 ± 0.08894 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were sonicated for approximately 5 minutes until the econazole dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

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

6.3 Preparation of Formulation Samples

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

6.4 Analysis

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

6.5 Calculations

The integration of the econazole and IS peaks by the chromatography data system was evaluated to assure it was correct in all chromatograms and manually reintegrated, if necessary. A linear regression equation was calculated relating the response ratio of econazole divided by the IS (y) to the concentration of the vehicle/calibration standards (x). The concentration of each vehicle/calibration standard was calculated using its individual response ratio and the regression equation. The percent RE for each vehicle/calibration standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation (s), and percent RSD were calculated for the vehicle/calibration standards and formulation samples when applicable.

6.6 Results

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

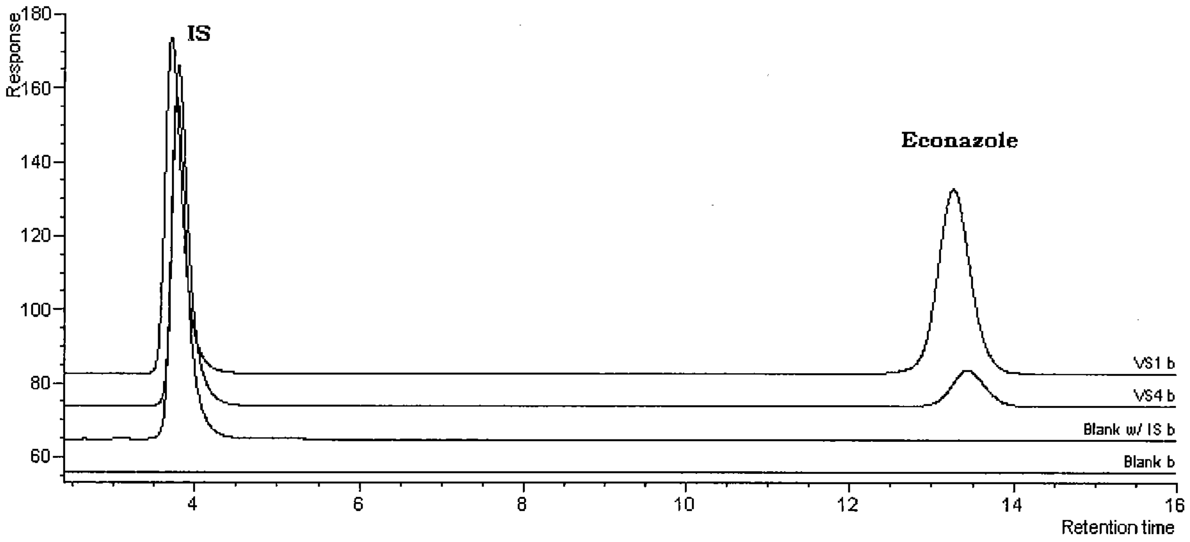


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

The vehicle/calibration standard curve and the results of the regression analysis indicated linearity and are shown in Figure 5.

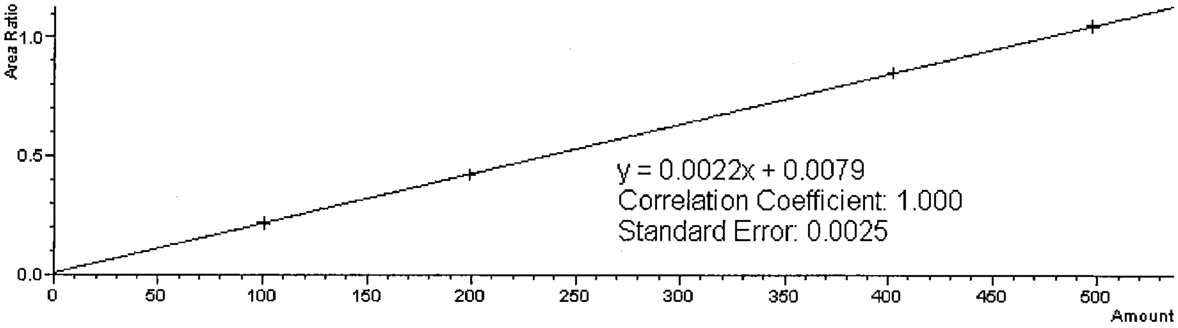


Figure 5 – Vehicle/Calibration Standard Curve and Regression Analysis Results

The results of the formulation analysis are shown in Table 6. The formulations met acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 6 – Formulation Analysis Results

Batch No.	Analysis Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg % RE	RSD (%)
1-ECON-1	2/7/05	46.24	46.08	45.89	46.07	3.5	0.4
2-ECON-1	8/8/05	44.58	44.49	44.14	44.40	-0.2	0.5

6.7 Conclusions

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

The econazole formulation at a target concentration of 44.47 mg/mL in DMSO was stable for 56 days when stored refrigerated and protected from light.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Christina Zielinski, Sandy Runyon, Tudor Fernando, Hans Whittenburg and Ron Haney. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

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ANALYTICAL CHEMISTRY ACTIVITIES REPORT

4-HYDROXYANDROSTENEDIONE (4-OH ASDN)

CAS No.: 566-48-3

Receipt Date: 10/22/04

Appearance: Solid

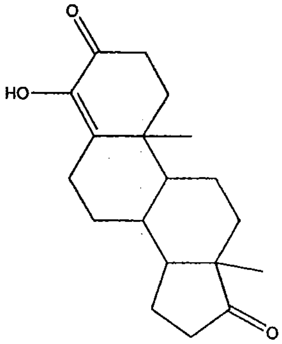
Storage Conditions (@ Battelle): Refrigerated (~5°C)

Lot No.: 063K4069 (Sigma Aldrich)

Amount Received: 3.1 g

Vendor Purity: 99% by TLC

STRUCTURE:



Mol. Wt.:

302.41 g/mol

Mol. Formula:

$C_{19}H_{26}O_3$

Prepared By:

Denise A. Contos 12/19/05

Denise A. Contos, M.S.

Approved By:

Steve W. Graves

Steven W. Graves, B.S.

Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

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

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

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

12-14-05

Quality Assurance Unit Date

EXECUTIVE SUMMARY

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

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

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

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

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

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1 INTRODUCTION

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

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

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

2 CHEMICAL RECEIPT AND STORAGE

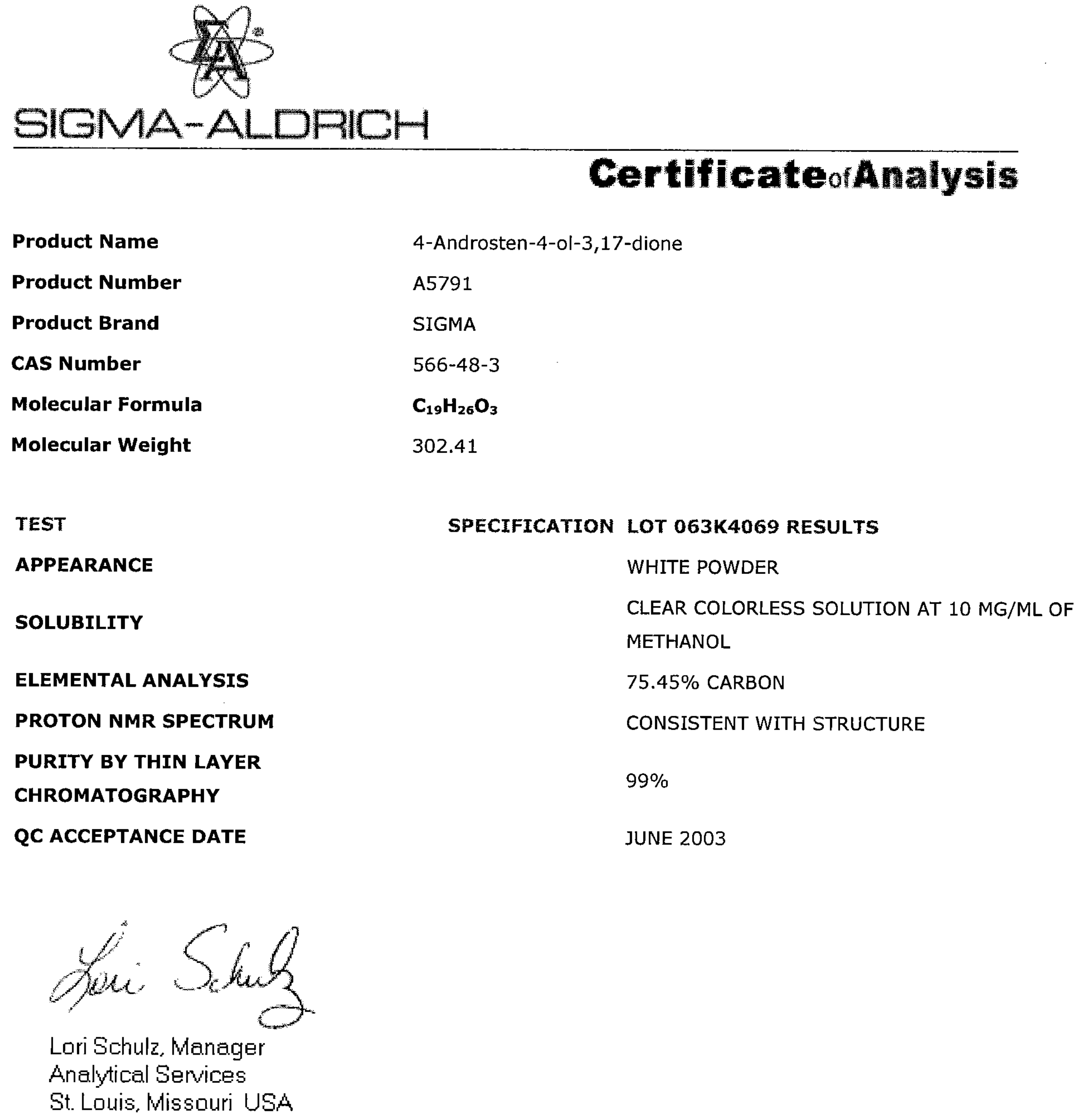
One 15-mL amber glass bottle of 4-hydroxyandrostenedione, 063K4069, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on October 22, 2004. The label amount indicated 3.1 grams was sent. The chemical was received and subsequently stored at approximately 5°C.

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

3 SOLUBILITY STUDIES

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

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



SIGMA-ALDRICH

Certificate of Analysis

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

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

Lori Schulz

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

Figure 1 – Certificate of Analysis

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

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

4.1 Method Development

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

4.2 Method

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

Table 1 – GC System

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

4.3 Method Validation

Validation was accomplished using a single experiment.

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

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

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

4.3.1.2 Stock Standards

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

4.3.1.3 Vehicle/Calibration Standards

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

Table 2 – Preparation of Vehicle/Calibration Standards

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

4.3.1.4 Blanks

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

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

4.3.2 Analysis

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

4.3.3 Calculations

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

4.3.4 Results

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

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

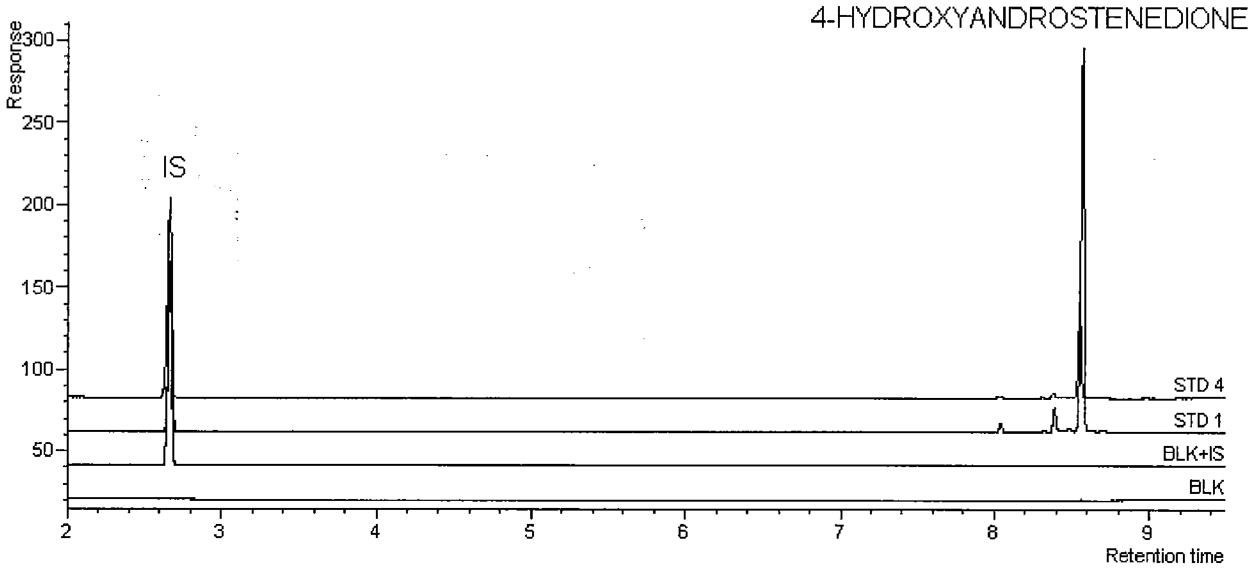


Figure 2 – Representative Overlaid Chromatograms from a Low and High Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

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

Table 3 – Regression Analysis Validation Results

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

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

Table 4 –Vehicle/Calibration Standard Validation Results

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

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

4.3.5 Conclusions

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

5 FORMULATION STABILITY STUDIES

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

5.1 Study Design

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

5.2 Formulation Method

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

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

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

5.3 Analysis Method

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

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

5.4 Results

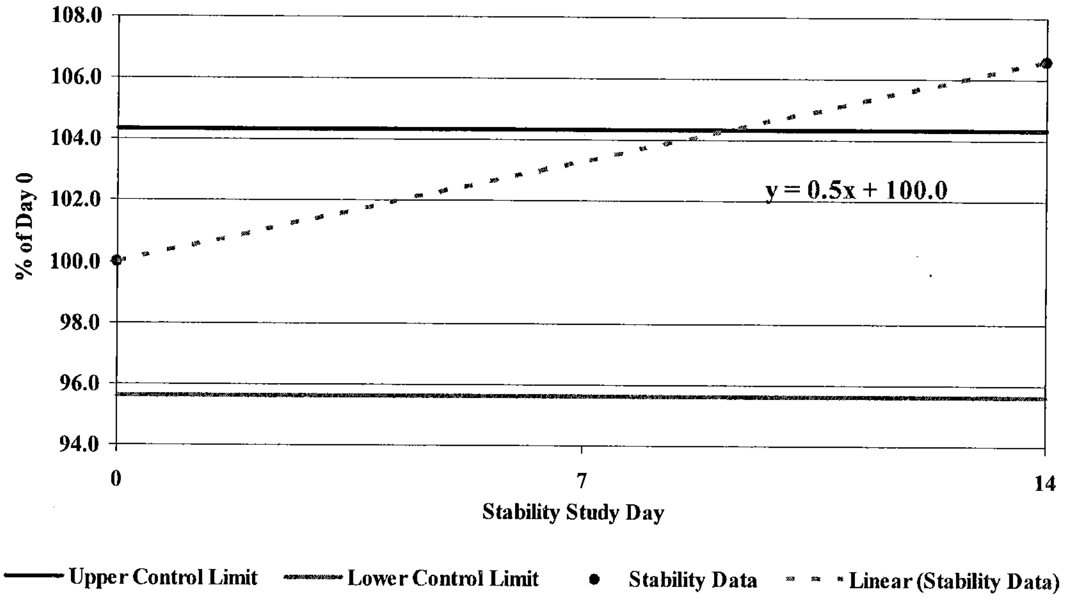
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

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

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

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

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


4-OH ASDN
(3.02 mg/mL Prepared 11-10-04)

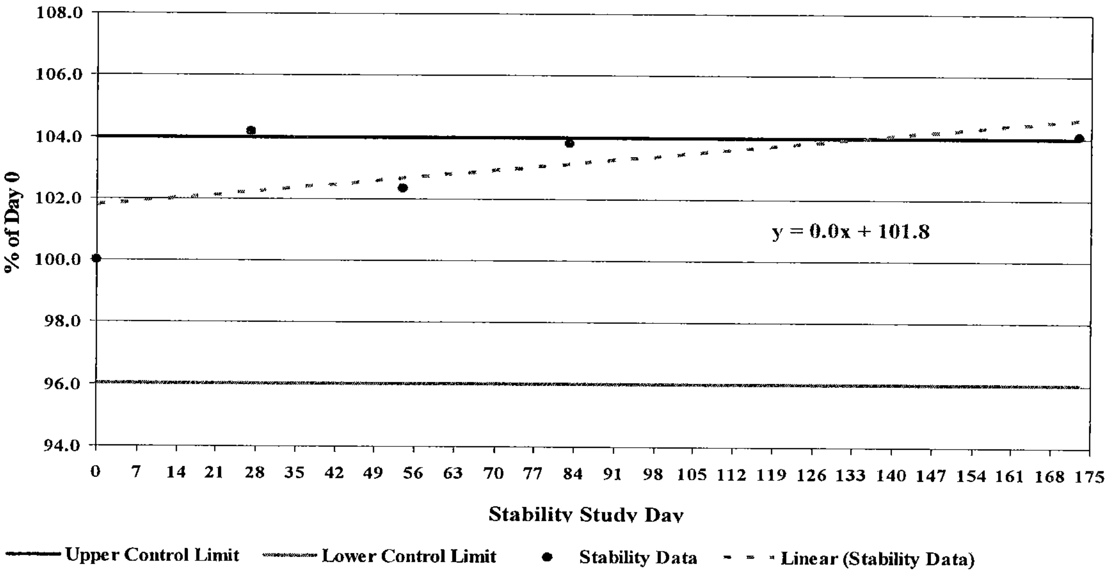

4-OH ASDN
(3.02 mg/mL Prepared 12-2-04)

Figure 3 – Control Charts for the Storage Stability Studies

5.5 Discussion and Conclusions

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

6 FORMULATION PREPARATIONS AND ANALYSES

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

6.1 Preparation of Formulations

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

6.2 Preparation of Standards and Blanks

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

6.3 Preparation of Formulation Samples

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

6.4 Analysis

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

6.5 Calculations

The peaks for 4-hydroxyandrostenedione and the IS were integrated for each injection by the chromatography data system. Any peak with improper integration was manually reintegrated. A linear regression equation weighted 1/x was calculated relating the response ratio (4-hydroxyandrostenedione/IS) (y) to the concentration of the vehicle/calibration standards (x). This regression equation and the response ratios were used to calculate the concentration in each standard and formulation sample. The percent RE for each standard was calculated by subtracting the nominal value from the determined value, dividing by the nominal value, and then multiplying by 100. The percent RE for each formulation sample was calculated by subtracting the target value from the determined value, dividing by the target value, and then multiplying by 100. The average determined concentration, standard deviation, and percent RSD were calculated for the vehicle/ calibration standards and formulation samples when applicable.

6.6 Results

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

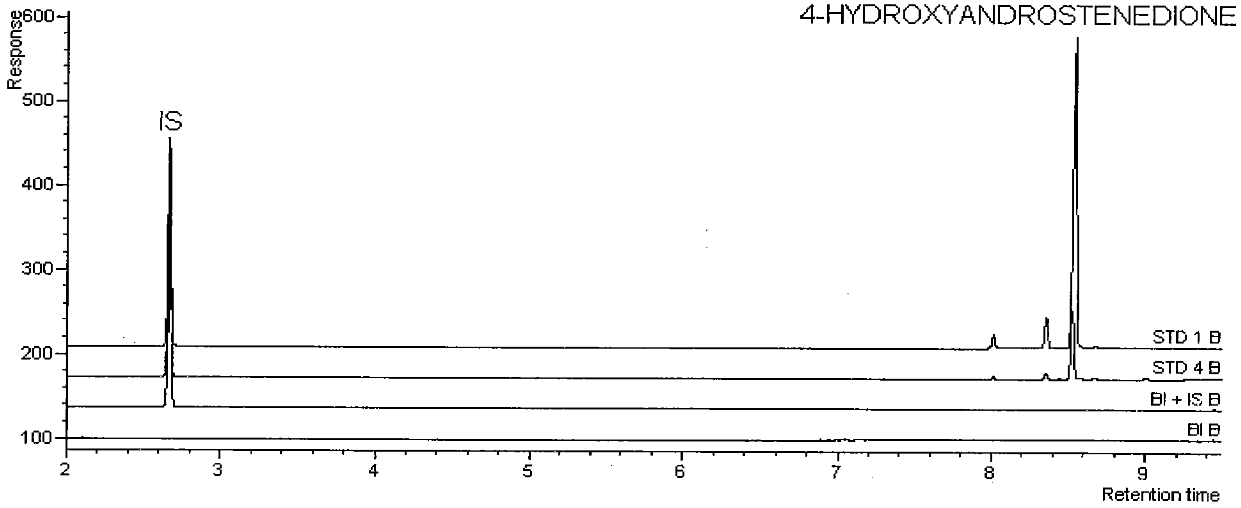


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from Formulation Analysis

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

Table 6 – Regression Analysis Results

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

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

Table 7 – Formulation Analysis Results

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

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

6.7 Conclusions

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

7 ACKNOWLEDGMENTS

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

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ANALYTICAL CHEMISTRY ACTIVITIES REPORT

LINDANE

CAS No.: 58-89-9

Lot No.: 14419EB (Sigma Aldrich)

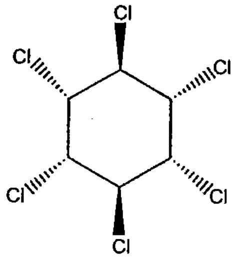
Receipt Date: 1/6/05

Amount Received: 10 g

Appearance: White Solid

Vendor Purity: 99.6% by GC

Storage Conditions (@ Battelle): Room temperature (~25°C)

STRUCTURE:	Mol. Wt.:	Mol. Formula:
	290.83 g/mol	$C_6H_6Cl_6$

Prepared By:

Denise A. Contos 1/6/2006

Denise A. Contos, M.S.

Approved By:

Steven W. Graves

Steven W. Graves, B.S.

Manager, Chemistry Technical Center

QUALITY ASSURANCE STATEMENT

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

Phase Inspected	Inspection Date	Date Reported to Study Director/Management
Test substance receipt*	10/26/2004	10/26/2004
Formulation preparation*	12/2/2004	12/2/2004
Dispensing*	12/2/2004	12/2/2004
Formulation analysis*	12/2/2004	12/2/2004
Audit analytical report	12/22/2005	12/22/2005
Audit study file	12/22/2005	12/22/2005

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

Kathleen E. Reed 1-6-06

Quality Assurance Unit Date

EXECUTIVE SUMMARY

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

Solubility of lindane was determined to be acceptable in dimethylsulfoxide (DMSO) for preparing formulations.

A formulation analysis method was developed and validated to analyze lindane in DMSO at a concentration of 29.08 mg/mL (0.1M). This method was used to analyze samples from both formulation and formulation storage stability studies at 29.08 mg/mL.

Storage stability study indicated that a 29.08 mg/mL formulation stored in sealed amber glass bottles and protected from light was stable for 168 days at approximately 5°C.

The formulations prepared for shipment to the testing laboratory were determined and met the established acceptance criteria.

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LIST OF FIGURES

1 INTRODUCTION

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

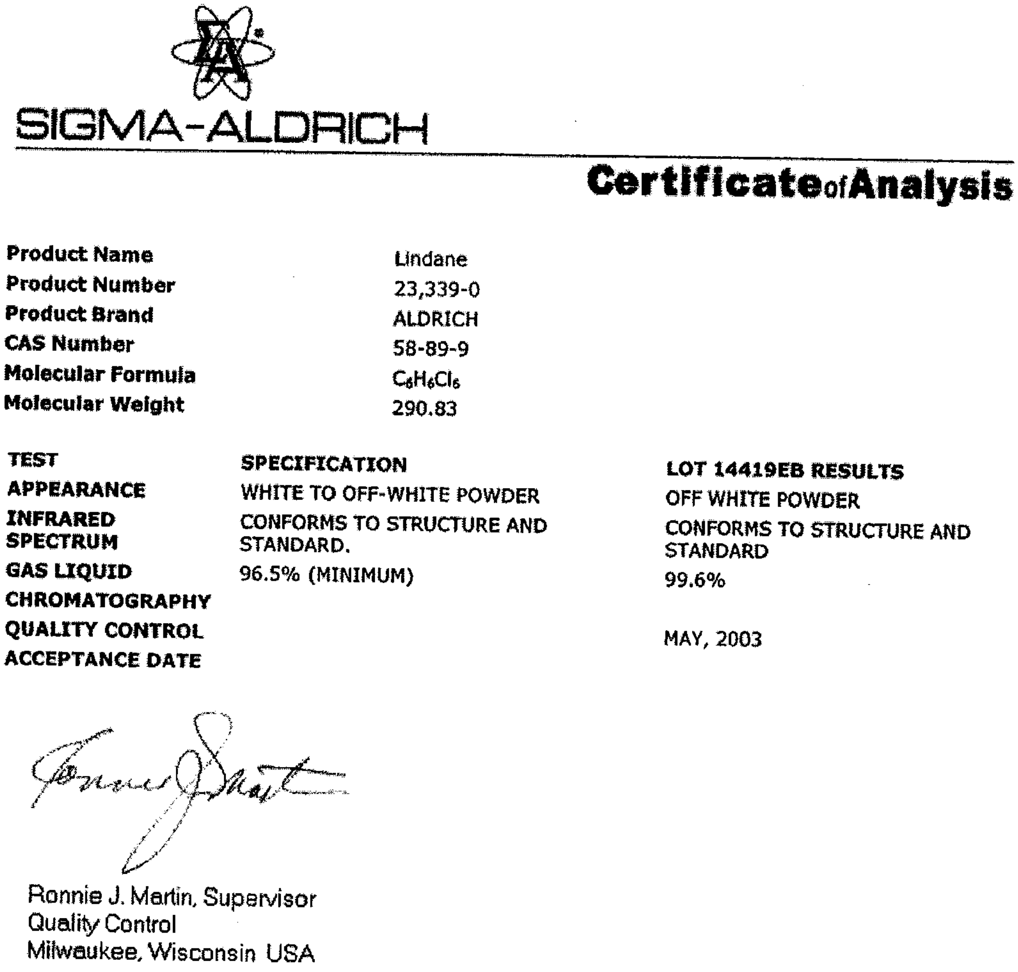
- Determining solubility in dimethylsulfoxide (DMSO).
- Developing and validating a formulation analysis method.
- Conducting a storage stability study.
- Preparing and analyzing a stock formulation.

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

2 CHEMICAL RECEIPT AND STORAGE

One 20-mL amber glass bottle of lindane, 14419EB, was received from the repository at Battelle's Marine Sciences Laboratory in Sequim, WA on January 6, 2005. The label amount indicated 10 grams was sent. The chemical was received and subsequently stored at room temperature.

A copy of the manufacturer's Certificate of Analysis for this lot is shown in Figure 1. This states that purity was 99.6% based on gas chromatography (GC).



SIGMA-ALDRICH

Certificate of Analysis

Product Name	Lindane
Product Number	23,339-0
Product Brand	ALDRICH
CAS Number	58-89-9
Molecular Formula	$C_6H_6Cl_6$
Molecular Weight	290.83

TEST	**SPECIFICATION**	**LOT 14419EB RESULTS**
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF WHITE POWDER
INFRARED SPECTRUM	CONFORMS TO STRUCTURE AND STANDARD.	CONFORMS TO STRUCTURE AND STANDARD
GAS LIQUID CHROMATOGRAPHY	96.5% (MINIMUM)	99.6%
QUALITY CONTROL ACCEPTANCE DATE		MAY, 2003

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

Figure 1 – Certificate of Analysis

3 SOLUBILITY STUDIES

A solubility study was conducted to determine the solubility of lindane in 100% DMSO, at a concentration of at least 29.08 mg/mL. Lindane (0.29080 ± 0.02908 g) was weighed into a 10-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well. The lindane went readily into solution. This experiment showed that DMSO was an acceptable solvent for the 29.08 mg/mL formulation.

4 FORMULATION ANALYSIS METHOD PERFORMANCE EVALUATION (MPE)

This section describes the evaluation of a method developed to analyze formulations of lindane in DMSO at a target concentration of 29.08 mg/mL for the stability study and the results and conclusions from this evaluation.

4.1 Method Development

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

4.2 Method

The GC parameters for lindane are presented in Table 1.

Table 1 – GC System

GC	Agilent 6890 (Palo Alto, CA)
Column	RTX-5, 30 m × 0.25 mm (ID), 0.25 µm film thickness (Restek, Bellefonte, PA)
Carrier Gas and Flow Rate	Helium at ~2 mL/minute
Oven Temperature	150°C, hold for ~2 minutes, increase at 20°C/minute to 300°C; hold for 2 minutes
Detector Type	Flame Ionization (FID)
Detector Flow Rates	Hydrogen at ~30 mL/minute; Air at ~380 mL/minute
Detector Temperature	320°C
Injector Temperature	285°C
Injection Volume	1 µL
Injection Mode	Split 5:1
Run Time	~12 minutes

4.3 Method Validation

Validation was accomplished using a single experiment.

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

4.3.1 Preparation of Standards and Blanks

4.3.1.1 Internal Standard (IS)

Approximately 25 ± 1 mg of phenanthrene was added to a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

The IS was prepared by pipetting 10 mL of stock IS into a 25-mL volumetric flask. The contents of the flask was diluted to volume with methanol, sealed, and mixed well.

4.3.1.2 Stock Standards

Two stock standards were prepared by accurately weighing 50 ± 2 mg of lindane each into two individual 25-mL volumetric flasks and dissolving in and diluting to volume with methanol. This produced stocks A and B with target concentrations of 2000 μg/mL each.

4.3.1.3 Vehicle/Calibration Standards

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

Table 2 – Preparation of Vehicle/Calibration Standards

Vehicle/Calibration Std	Target Final Conc (μg/mL)	Source	Source Volume (mL)	WIS (mL)	DMSO (mL)	Final Volume (mL)
VS1	800	A	4	1	0.1	10
VS2	600	B	3	1	0.1	10
VS3	400	A	2	1	0.1	10
VS4	200	B	1	1	0.1	10

4.3.1.4 Blanks

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

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

4.3.2 Analysis

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

4.3.3 Calculations

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

4.3.4 Results

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

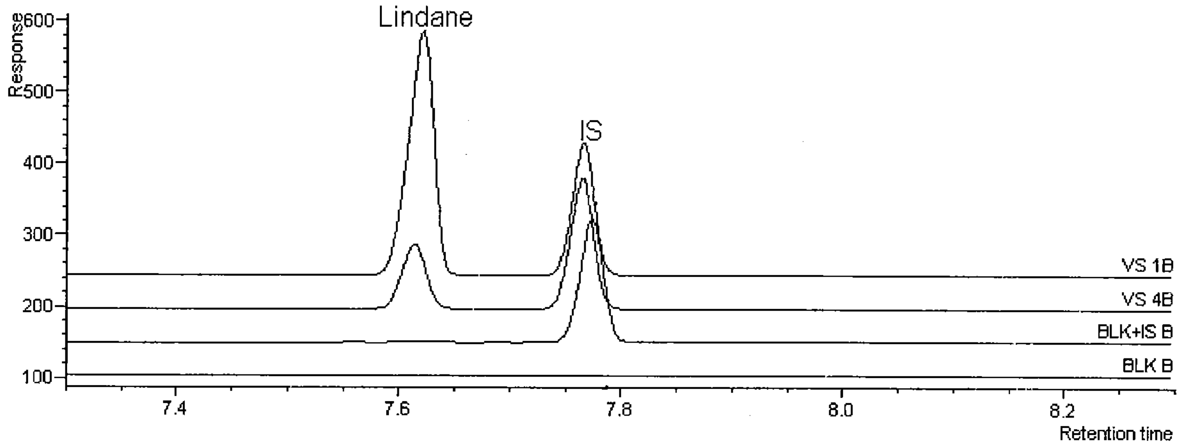


Figure 2 – Representative Overlaid Chromatograms from a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from the Validation (Shown Top to Bottom)

Table 3 – Method Validation Regression Analysis Results

Slope	y-Intercept	Correlation Coefficient	Standard Error
0.0027	-0.0351	1.000	0.0047

The precision and accuracy of the vehicle/calibration standard validation results are shown in Table 4.

Table 4 – Vehicle/Calibration Standard Validation Results

Nominal Std Conc (µg/mL)	Det'd Std Conc (µg/mL)	Avg Det'd Std Conc (µg/mL)	s (µg/mL)	%RSD	%RE	Avg %RE
776.3	777.3	776.8	1.1	0.1	0.1	0.1
	777.6				0.2	
	775.6				-0.1	
600.2	598.4	NA	NA	NA	-0.3	NA
388.2	387.0	NA	NA	NA	-0.3	NA
200.1	202.8	200.5	2.1	1.1	1.4	0.2
	200.1				0.0	
	198.6				-0.7	

The sensitivity of the method resulted in 6.4 µg/mL LOD which is defined as three times the standard deviation of the low vehicle/calibration standard. This is equivalent to a formulation concentration of 640 µg/mL when a formulation is diluted 1 to 100 for analysis. The LOQ, defined as ten times the standard deviation of the lowest standard because there was no blank response, was 21.3 µg/mL. This is equivalent to a formulation concentration of 2130 µg/mL when a formulation is diluted 1 to 100 for analysis. The estimated limit of quantitation (ELOQ), defined as the lowest standard with acceptable accuracy and precision, was 200.1 µg/mL.

4.3.5 Conclusions

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

5 FORMULATION STABILITY STUDIES

A formulation stability study was conducted at a target concentration of 29.08 mg/mL in DMSO for 168 days (24 weeks) in sealed, amber glass bottles stored at approximately 5°C.

5.1 Study Design

A single sample was analyzed on the day of preparation (Day 0), Day 14, Weeks 4, 8 and 12. A second formulation sample was prepared and analyzed on January 24, 2005 (Day 0) and on Week 24. Three aliquots were analyzed from each sample at each storage time.

5.2 Formulation Method

A formulation was prepared on January 13, 2005, Day 0 of the storage stability study at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 727 ± 7 mg of lindane into a 25-mL volumetric flask. The chemical was dissolved in and diluted to approximately three quarters of the total volume with

DMSO. The flask was sealed and manually shaken to mix the contents. The contents of the flask was diluted to volume with DMSO, sealed, and mixed well.

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

A second formulation (Batch 1-LIN-1) was prepared on January 24, 2005 (Day 0) at a target concentration of 29.08 mg/mL in DMSO by accurately weighing 1.45400 ± 0.058 g into a 50-mL volumetric flask. The content of the flask was diluted to approximately 80% volume with DMSO, sealed and mixed well. The contents of the flask was diluted to volume with DMSO and mixed well. Approximately 9 mL were dispensed into an amber glass bottle, sealed and stored refrigerated. A formulation sample aliquot was prepared for analysis on Days 0 and 168 for storage stability determination.

5.3 Analysis Method

Vehicle/calibration standards and blanks with and without IS were prepared as described in the validation experiment (Section 4.3.1) of this report.

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

5.4 Results

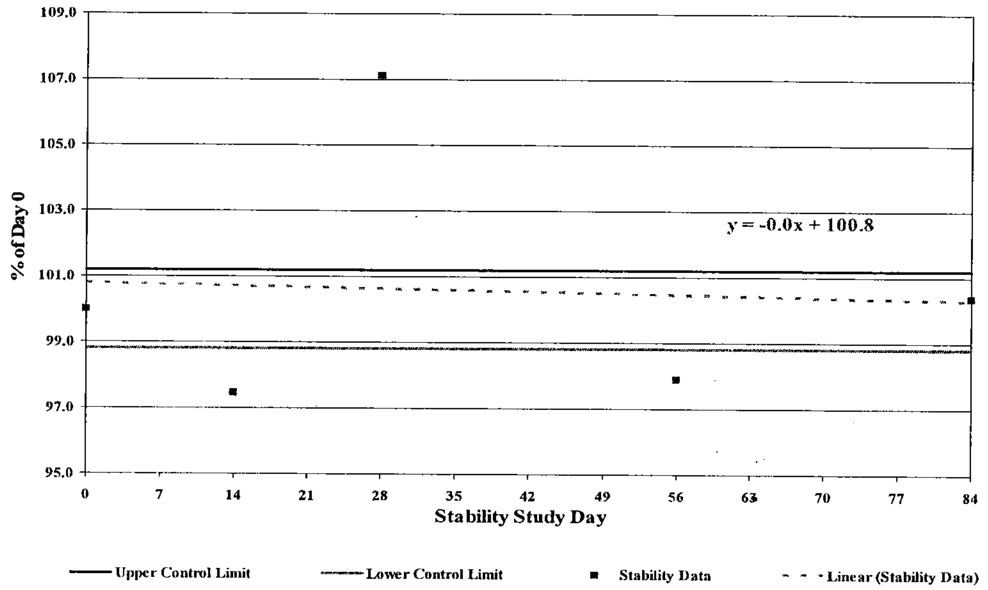
The results from the storage stability study are shown in Table 5 and presented in control chart format in Figure 3.

Table 5 – Formulation Storage Stability Results (29.08 mg/mL)

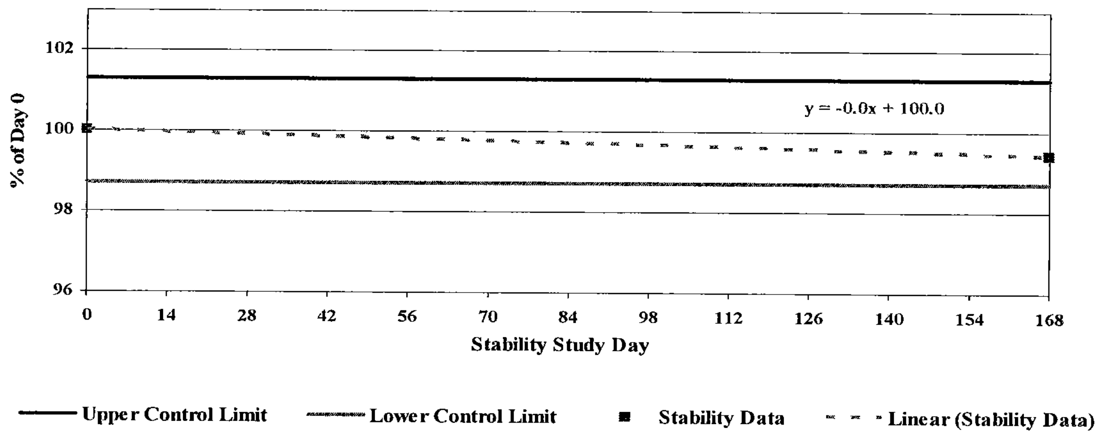
Preparation Date	Analysis Date	Day	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL) ± s	% of Day 0 Conc ± s
1/13/05	1/13/05	0	29.38	29.48	29.18	29.35 ± 0.15	100 ± 0.5
1/13/05	1/27/05	14	28.56	28.56	28.67	28.60 ± 0.06	97.4 ± 0.2
1/13/05	2/10/05	28	31.36	31.30	31.64	31.43 ± 0.18	107 ± 0.6
1/13/05	3/10/05	56	28.77	28.76	28.65	28.73 ± 0.07	97.9 ± 0.2
1/13/05	4/7/05	84	29.22	29.67	29.47	29.45 ± 0.23	100 ± 0.8
1/24/05	1/24/05	0	30.02	29.88	29.93	29.95 ± 0.07	100 ± 0.2
1/24/05	7/11/05	168	29.64	29.72	29.95	29.77 ± 0.16	99.4 ± 0.5

For the formulation sample prepared on January 13, 2005, the pooled relative standard deviation of the analytical method was 0.5%. This means that there would have to be a difference of more than 1.2% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.

For the formulation sample prepared on January 24, 2005, the pooled RSD of the analytical method was 0.6%. This means that there would have to be a difference of more than 1.3% from the Day 0 value for the difference to be statistically significant at a 95% confidence level.



LINDANE in 100% DMSO
(29.08 mg/mL, Prepared on 1/13/05)



LINDANE in 100% DMSO
(29.08 mg/mL, Prepared on 1/24/05)

Figure 3 – Control Chart for the Storage Stability Study

5.5 Discussion and Conclusions

The Day 0 determined value for the formulation prepared on January 13, 2005 was approximately 1.0% above nominal (the calculated concentration based on the weight of the chemical). The concentrations of the samples stored at approximately 5°C protected from light in amber glass vials for Days 14 and 56 were below the lower significance level and for Day 28 it was above the upper significance level due to the tight precision of the assay. The average concentrations of the samples were within 2.6% (Day 14), 7.1% (Day 28), 2.1% (Day 56), and 0.4% (Day 84) of the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C for 84 days.

The formulation stability sample prepared on January 24, 2005 (Day 0) and analyzed on Day 0 and Day 168 (July 11, 2005) was approximately 3.0% above nominal for Day 0 (the calculated concentration based on the weight of the chemical) and for Day 168, 0.6% below the Day 0 value and met acceptance criteria of ± 10%. These data indicate the formulation was stable at approximately 5°C protected from light for 168 days.

6 FORMULATION PREPARATION AND ANALYSIS

Formulations were prepared and analyzed on January 24, 2005, March 21, 2005 and July 1, 2005, according to SOP COMSPEC.II-029, "Standard Operating Procedure (SOP) for the Formulation and Analysis of Lindane in 100% Dimethylsulfoxide (DMSO)." This section describes the method, results, and conclusions.

6.1 Preparation of Formulation

Lindane (1.45400 ± 0.058 g) was weighed into a 50-mL volumetric flask. DMSO was added until the flask was approximately 80% full. The contents were mixed until the lindane dissolved. The contents of the flask were diluted to volume with DMSO, sealed, and mixed well.

6.2 Preparation of Standards and Blanks

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

6.3 Preparation of Formulation Samples

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

6.4 Analysis

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

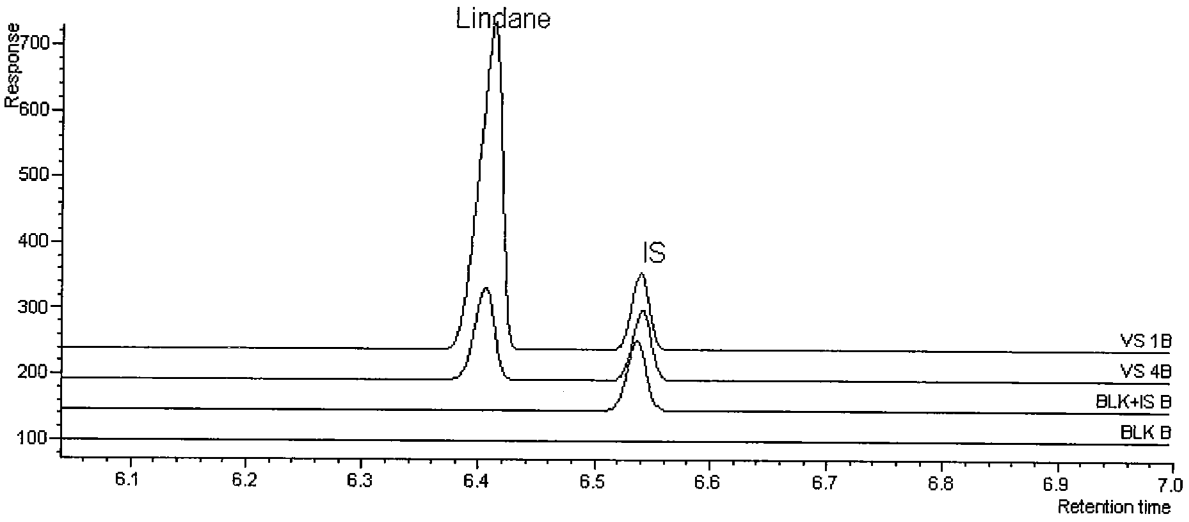


Figure 4 – Representative Overlaid Chromatograms of a High and Low Vehicle/Calibration Standard, Blank with IS, and Blank from a Formulation Analysis (Shown Top to Bottom)

6.5 Calculations

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

6.6 Results

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

Table 6 – Formulation Regression Analysis Results

Formulation Date	Slope	y-Intercept	Correlation Coefficient
1/24/05	6.8029	-0.0081	1.000
3/21/05	7.2898	-0.0197	1.000
7/1/05	6.8477	-0.1022	1.000

The results of the formulation analysis are shown in Table 7. Formulations met all acceptance criteria (RE within 10% of target and RSD of ≤ 10%).

Table 7 – Formulation Analysis Results

Formulation Date	Det'd Conc (mg/mL)			Avg Det'd Conc (mg/mL)	Avg %RE	%RSD
1/24/05	30.02	29.88	29.93	29.95	3.0	0.2
3/21/05	29.23	29.67	29.20	29.37	1.0	0.9
7/1/05	29.32	29.26	29.63	29.40	1.1	0.7

6.7 Conclusions

The average concentration of the formulations and its percent RSD were within acceptance criteria. Therefore the formulation was suitable for use.

7 ACKNOWLEDGMENTS

Analytical support for this work was provided by Barb Harritos, Darren Brown, John Kelly, Christina Zielinski, Jim Hoskinson, Melinda Pauff, Tudor Fernando, and Sandy Runyon. The report was written by Denise Contos. Review of the data and report for completeness and accuracy was performed by Maria Evascu.

Appendix F

Radiochemical Analysis Report

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
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Columbus, OH 43201-2693

Performing Laboratory:
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FINAL REPORT

Title: PLACENTAL AROMATASE VALIDATION STUDY
[^{3}H]ASDN Radiochemical Purity Determination

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Battelle

Analysis Date: January 5, 2005

Final Report Date September 28, 2005

Author:
Sherry J 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
K. Collier
Quality Assurance Specialist

9/28/2005
Date

Approval:

Carrie Ingalls
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).

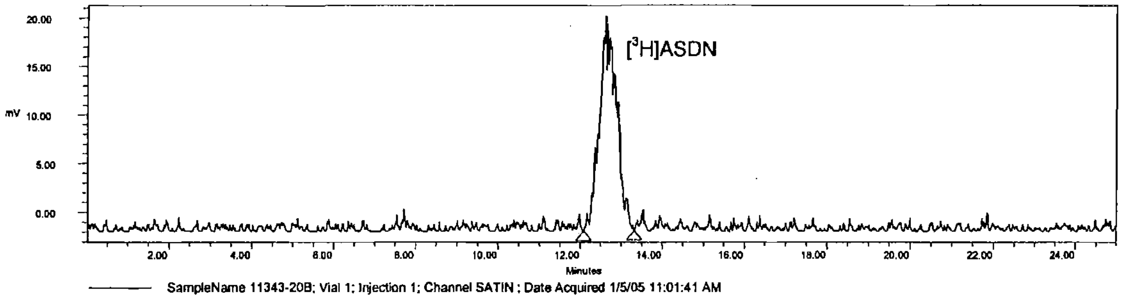
The radiochemical purity of the [^{3}H]ASDN (1:100 dilution in ethanol) was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consists of a Waters 2690 Separations Module, a Waters 2487 Dual λ Absorbance Detector and a β-RAM Model 3 flow-through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

Analysis Report

Report on the Statistical Analysis for WA 4-16, Task 5

1.0 Concentration Response Fits for the Reference Chemicals (as described in Section 6.2.1 of the protocol)

From the model fits to the response curves for each replicate within each chemical, a dataset was prepared containing the $\log_{10}(IC50)$, slope and their corresponding standard errors for each of the 3 replicates within each of the 4 reference chemicals. For each chemical, the geometric mean of IC50 was calculated as:

$$geo\_mean\_IC50 = 10^{\overline{X}_{\log 10(IC50)}}$$

where $\overline{X}_{\log 10(IC50)}$ is the mean of the estimated $\log_{10}(IC50)$ values across the 3 replicates within the chemical. The values for means of the estimated $\log_{10}(IC50)$ values and the estimated geometric means of IC50 for each chemical are presented in Table 1. The geometric means for IC50 are very close for aminoglutethimide and chrysin, while ketoconazole is a bit higher but of the same magnitude. Econazole has a geometric mean much smaller in magnitude than the other 3 reference chemicals.

Table 1. Means of $\text{Log}_{10}(IC50)$ and Geometric Means of IC50 by Chemical.

Chemical	Mean $\text{Log}_{10}(IC50)$	Geometric Mean IC50
Aminoglutethimide	-5.36904	4.27519E-06
Chrysin	-5.35445	4.42135E-06
Ketoconazole	-5.14342	7.18761E-06
Econazole	-8.67850	2.09650E-09

A mixed effects model was fit to the $\log_{10}(IC50)$ and slope estimates from the response curve model fits. The chemical type was assigned as the fixed effect and replicate was assigned as the random effect, with homogeneous covariance parameters within the chemicals. SAS PROC MIXED was used to obtain estimates of the replicate-to-replicate variation within each chemical by entering the square of the standard errors from the response curve model parameter estimates as the variation estimates for the within replicate (within chemical) variance components. The estimated variance components from the mixed effects models are provided in Table 2. The replicate-to-replicate variation estimates for slope were constrained to be no lower than 1E-30 in order to allow the mixed effects model to converge and provide estimates of the variance components. The estimates of replicate-to-replicate variation of slope for chrysin and econazole show a value of 1E-30, indicating that those variance components are estimated to be zero, but for modeling purposes were not allowed to go below 1E-30.

Table 2. Estimated Replicate-to-Replicate Variation by Chemical

Response Curve Parameter	Chemical	Rep-to-Rep Variation
Log_{10}(IC50)	Aminoglutethimide	0.000227
	Chrysin	0.002023
	Ketoconazole	0.000170
	Econazole	0.003294
Slope	Aminoglutethimide	8.05E-21
	Chrysin	1.00E-30
	Ketoconazole	0.000836
	Econazole	1.00E-30

2.0 Graphical and Analysis of Variance Comparisons Among Concentration Response Curve Fits (as described in Section 6.2.2 of the protocol)

Graphs were prepared for the parameter estimates from the response curve model fits to visually assess the estimates and their variations. Exhibits 1 through 4 display the means of $\log_{10}$(IC50) by replicate within each chemical and their associated 95% confidence intervals, as indicated by the vertical lines in the graphs. The dotted horizontal reference lines indicate the overall mean of $\log_{10}$(IC50) for the chemical and the 95% confidence intervals for the chemical mean, incorporating the within-replicate and replicate-to-replicate variance components from the mixed effects model ANOVA results in section 6.2.1. Exhibits 5 through 8 display the corresponding means and 95% confidence intervals for slope by replicate and chemical. Table 3 displays the response curve model fit estimates, standard errors and associated 95% confidence interval bounds. A t-statistic with 5 degrees of freedom and a 2-sided alpha value of 0.05 was used to calculate the confidence interval bounds for each replicate within each chemical. The standard errors of the parameter estimates of $\log_{10}$(IC50) for aminoglutethimide and ketoconazole are fairly consistent across the replicates. The standard errors of estimates of $\log_{10}$(IC50) for chrysin show a decreasing value across the replicates.

Table 4 shows the overall means by chemical for $\log_{10}$(IC50) and slope, along with their corresponding standard errors and 95% confidence interval bounds. Aminoglutethimide, chrysin and ketoconazole have similar values for the overall mean of $\log_{10}$(IC50) but the standard error for chrysin is about 4 times larger than the standard errors for aminoglutethimide and ketoconazole. The slope estimates for aminoglutethimide, ketoconazole and econazole are similar in value but the standard errors of aminoglutethimide and econazole are about half the size of the standard error for ketoconazole. The slope estimate for chrysin is about half the value of the other slope estimates and has a standard error more than 3 times the size of the standard errors for the slope estimates of the other chemicals.

Table 3. Summary Statistics for Log_{10}(IC50) and Slope by Replicate and Chemical

Response Curve Parameter	Chemical	Replicate	Mean	Standard Error	95% CI Lower Bound	95% CI Upper Bound
Log_{10}(IC50)	Aminoglutethimide	1	-5.389	0.016	-5.429	-5.349
		2	-5.374	0.016	-5.415	-5.333
		3	-5.351	0.011	-5.379	-5.323
	Chrysin	1	-5.427	0.085	-5.646	-5.208
		2	-5.260	0.071	-5.441	-5.079
		3	-5.388	0.062	-5.548	-5.228
	Ketoconazole	1	-5.128	0.010	-5.154	-5.102
		2	-5.164	0.014	-5.199	-5.129
		3	-5.143	0.011	-5.171	-5.115
	Econazole	1	-8.584	0.054	-8.723	-8.445
		2	-8.676	0.008	-8.697	-8.655
		3	-8.732	0.007	-8.750	-8.714
Slope	Aminoglutethimide	1	-0.970	0.019	-1.018	-0.922
		2	-0.958	0.025	-1.021	-0.895
		3	-0.990	0.019	-1.038	-0.943
	Chrysin	1	-0.589	0.064	-0.753	-0.426
		2	-0.577	0.066	-0.747	-0.407
		3	-0.598	0.061	-0.755	-0.440
	Ketoconazole	1	-1.047	0.017	-1.090	-1.004
		2	-0.993	0.022	-1.048	-0.937
		3	-0.987	0.018	-1.032	-0.941
	Econazole	1	-1.081	0.122	-1.396	-0.766
		2	-1.037	0.016	-1.077	-0.997
		3	-1.023	0.013	-1.057	-0.989

Table 4. Summary Statistics for Log_{10}(IC50) and Slope by Chemical

Response Curve Parameter	Chemical	Mean	Standard Error	95% CI Lower Bound	95% CI Upper Bound
Log_{10}(IC50)	Aminoglutethimide	-5.36904	0.01184	-5.39634	-5.34175
	Chrysin	-5.35445	0.04879	-5.46696	-5.24193
	Ketoconazole	-5.14342	0.01004	-5.16656	-5.12027
	Econazole	-8.67850	0.03632	-8.76227	-8.59474
Slope	Aminoglutethimide	-0.97533	0.01159	-1.00207	-0.94860
	Chrysin	-0.58859	0.03669	-0.67321	-0.50398
	Ketoconazole	-1.00976	0.01987	-1.05557	-0.96394
	Econazole	-1.02916	0.01007	-1.05239	-1.00593

3.0 Graphical and Analysis of Variance Comparisons of FEAC, BAC, and Positive and Negative Control Percent of Control across Reference Chemicals and Replicates (as described in Section 6.2.3 of the protocol)

The means of the FEAC control values within each replicate and chemical were calculated as the reference value for 100% of control for the replicate and chemical. The percent of control for each repetition within each replicate and chemical was calculated as the ratio of the repetition value divided by the corresponding 100% of control reference value. Graphs of the percent of control values indicating the repetition portion by replicate and chemical for BAC, FEAC, negative and positive controls are presented in Exhibits 9 through 12. In Exhibit 9, the percent of control values for BAC are fairly consistent and close to 0% of control across the replicates and chemicals except for ketoconazole replicate 2 and econazole replicates 1 and 2. In Exhibit 10, the percent of control values for FEAC are fairly consistent and close to 100% of control across the replicates and chemicals except for econazole replicate 1. Exhibit 11 shows that the percent of control values of the negative control are fairly consistent and close to 100% of control except for econazole replicate 1. Exhibit 12 shows that the percent of control values of the positive control are fairly consistent and close to 45% of control except for ketoconazole replicate 2 and econazole replicate 1. Exhibits 13 through 16 display the differences of the means of the beginning repetitions and the means of the end repetitions within each replicate and chemical. The differences appearing above the zero reference line in Exhibit 14 indicate that the beginning percent of control values were consistently larger than the end percent of control values for FEAC. The same pattern of consistently larger beginning values compared to the end values of percent of control also appears in Exhibits 15 and 16 showing the differences of portion means for negative and positive controls.

A mixed effects model was fit to the percent of control values for each control. The fixed effects were assigned as the chemical type, portion, and portion by chemical interaction. The random effects were assigned as replicates within reference chemical and portion by replicate interaction within chemical. Tables 5 and 6 present the results of fitting the mixed effects model on percent of control using SAS PROC MIXED. Table 5 shows the results of significance tests for the fixed effects using a F-test statistic. A p-value less than 0.05 corresponds to a significant test result with at least 95% confidence. For BAC, none of the fixed effects had a significant effect on the variation in the percent of control values. For the FEAC, negative and positive controls, portion had a significant effect on the variation in the percent of control values. Table 6 shows the results of significance tests for the random effects using a Wald test statistic. For BAC and FEAC, both the replicate within chemical and portion by replicate within chemical covariance parameters were estimated to be zero, leaving the remainder of the variation in the residual term. There was a significant amount of variation in percent of control attributed to variation between the replicates within the chemicals for negative control and an indication of some variation in percent of control, but not quite statistically significant, attributed to variation between the replicates within chemicals for positive control.

Table 5. Fixed Effects Results of Mixed Effects Model on Percent of Control

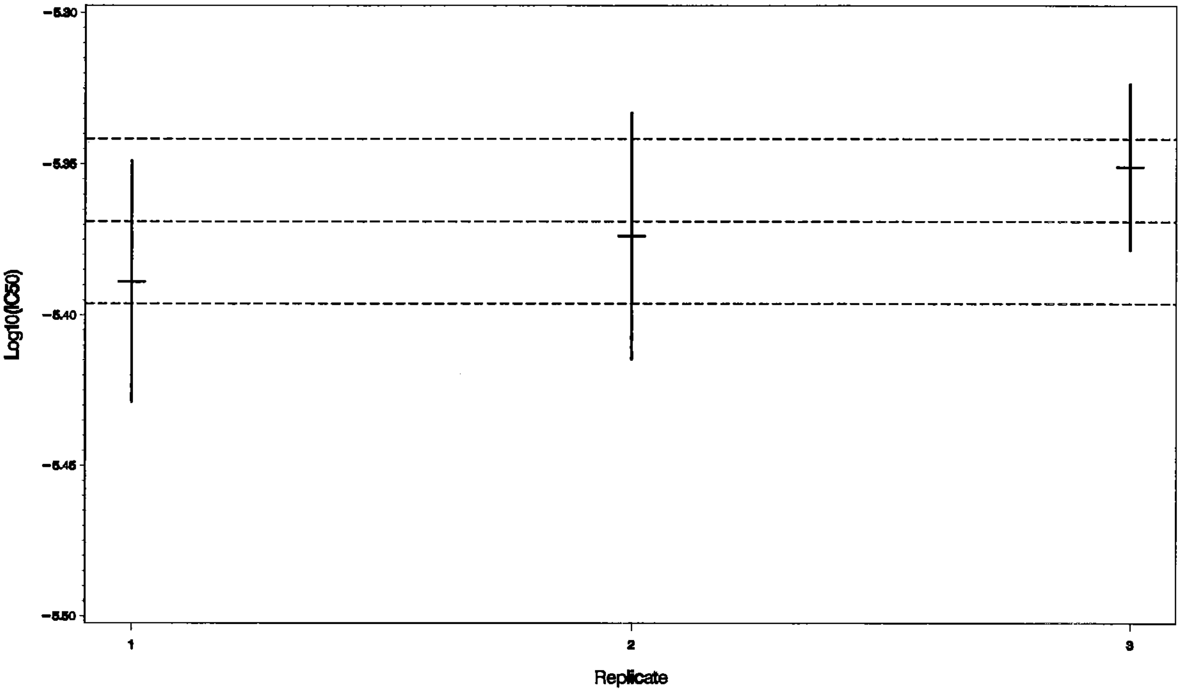
Control	Fixed Effect	F Test	P-value
Background control	Chem_code	0.00	1.0000
	Portion	0.09	0.7665
	Chem_code*Portion	1.77	0.2301
Full activity control	Chem_code	0.00	1.0000
	Portion	6.24	0.0370
	Chem_code*Portion	1.03	0.4300
Negative Control	Chem_code	0.83	0.5121
	Portion	79.06	<.0001
	Chem_code*Portion	1.66	0.2507
Positive control	Chem_code	2.16	0.1706
	Portion	8.63	0.0188
	Chem_code*Portion	1.35	0.3264

Table 6. Random Effects Results of Mixed Effects Model on Percent of Control

Control	Covariance Parameter	Estimate	Standard Error	WaldZ Test	P-value
Background control	Rep(Chem_code)	0.0000	.	.	.
	Portion*Rep(Chem_code)	0.0000	.	.	.
	Residual	0.0699	0.0156	4.4721	0.0000
Full activity control	Rep(Chem_code)	0.0000	.	.	.
	Portion*Rep(Chem_code)	0.0000	.	.	.
	Residual	164.6110	36.8081	4.4721	0.0000
Negative Control	Rep(Chem_code)	211.1848	106.2736	1.9872	0.0235
	Portion*Rep(Chem_code)	0.2268	1.5363	0.1476	0.4413
	Residual	4.9782	1.4371	3.4641	0.0003
Positive control	Rep(Chem_code)	21.2021	13.8596	1.5298	0.0630
	Portion*Rep(Chem_code)	1.2824	6.6182	0.1938	0.4232
	Residual	20.9757	6.0552	3.4641	0.0003

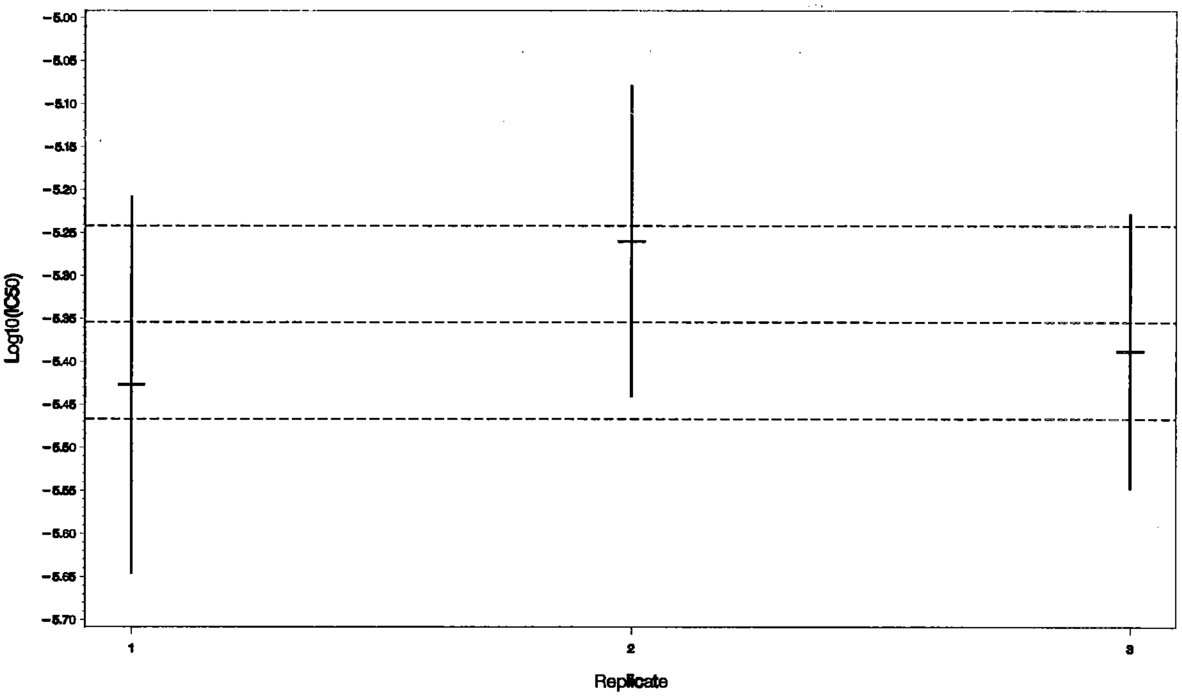
Exhibit 1

Means and 95% Confidence Intervals of Aminoglutethimide by Replicate and Overall



RTI Project 08055.003.032: WA 4–16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Means and 95% Confidence Intervals of Chrysin by Replicate and Overall

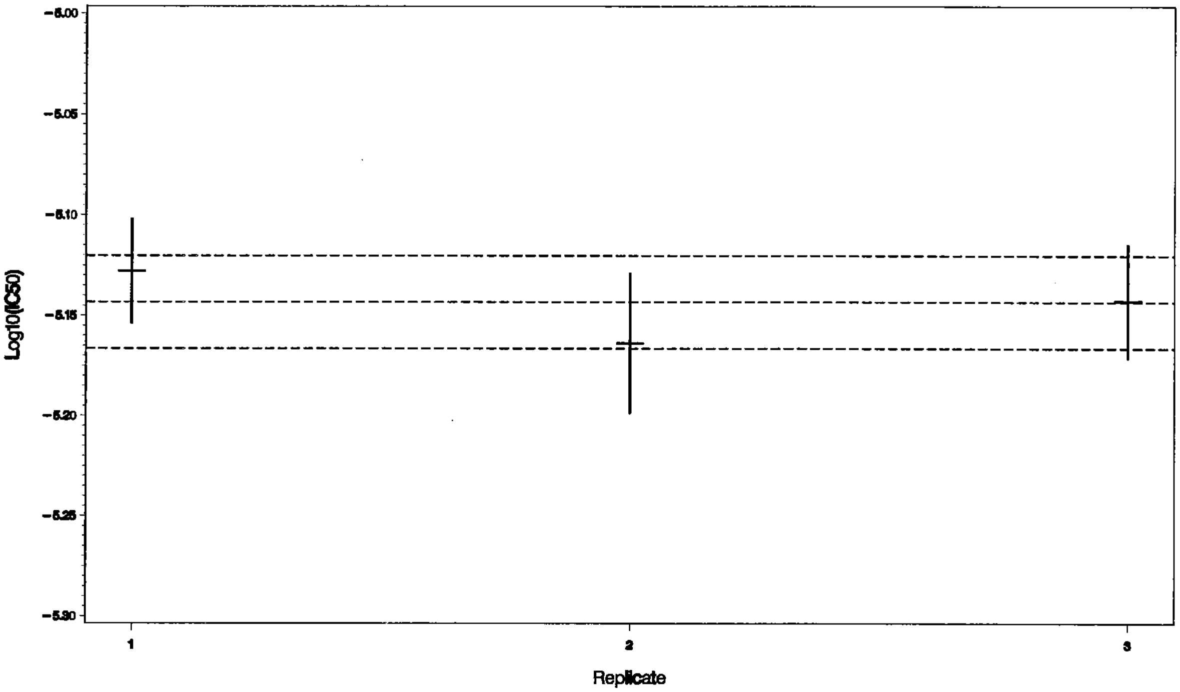


RTI Project 08055.003.032: WA 4–16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Exhibit 2

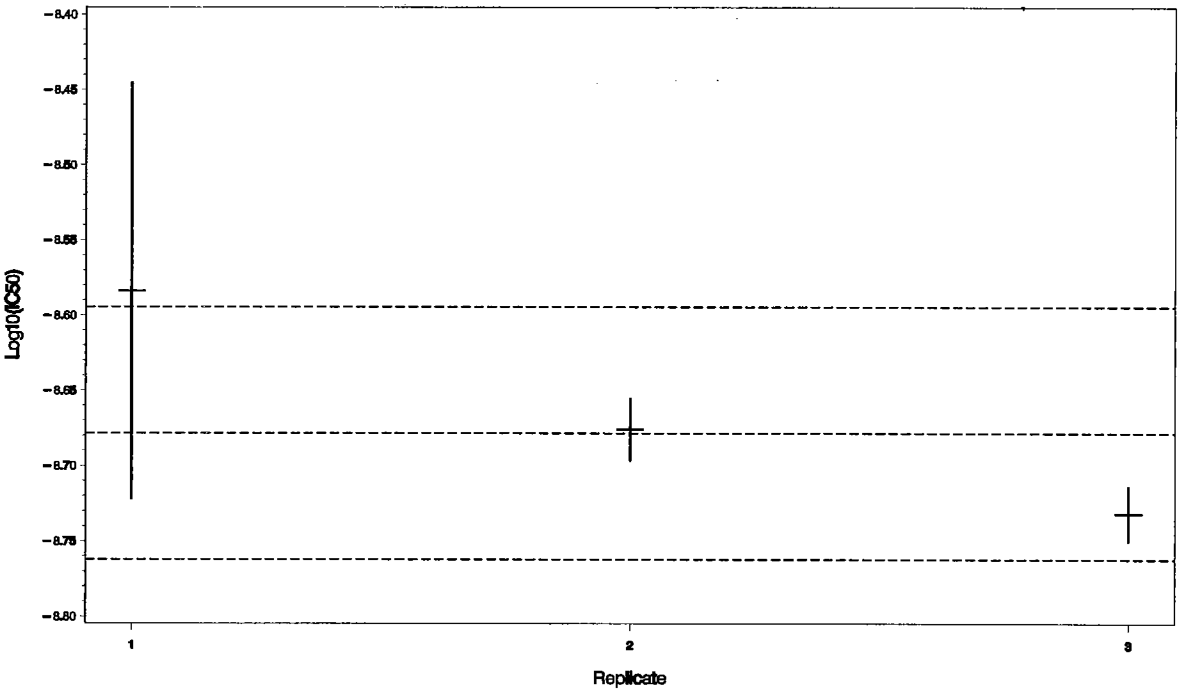
Exhibit 3

Means and 95% Confidence Intervals of Ketoconazole by Replicate and Overall



RTI Project 08(65.003.032: WA 4−16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Means and 95% Confidence Intervals of Econazole by Replicate and Overall



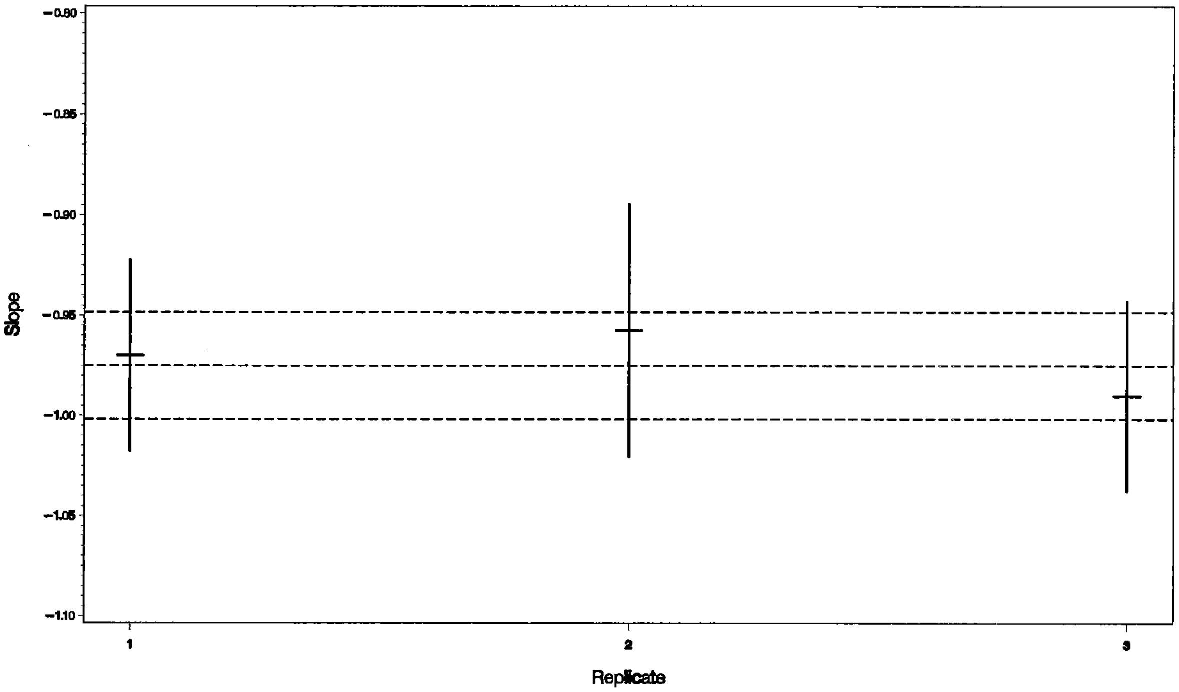
RTI Project 08065.003.032: WA 4−16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Exhibit 4

Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole

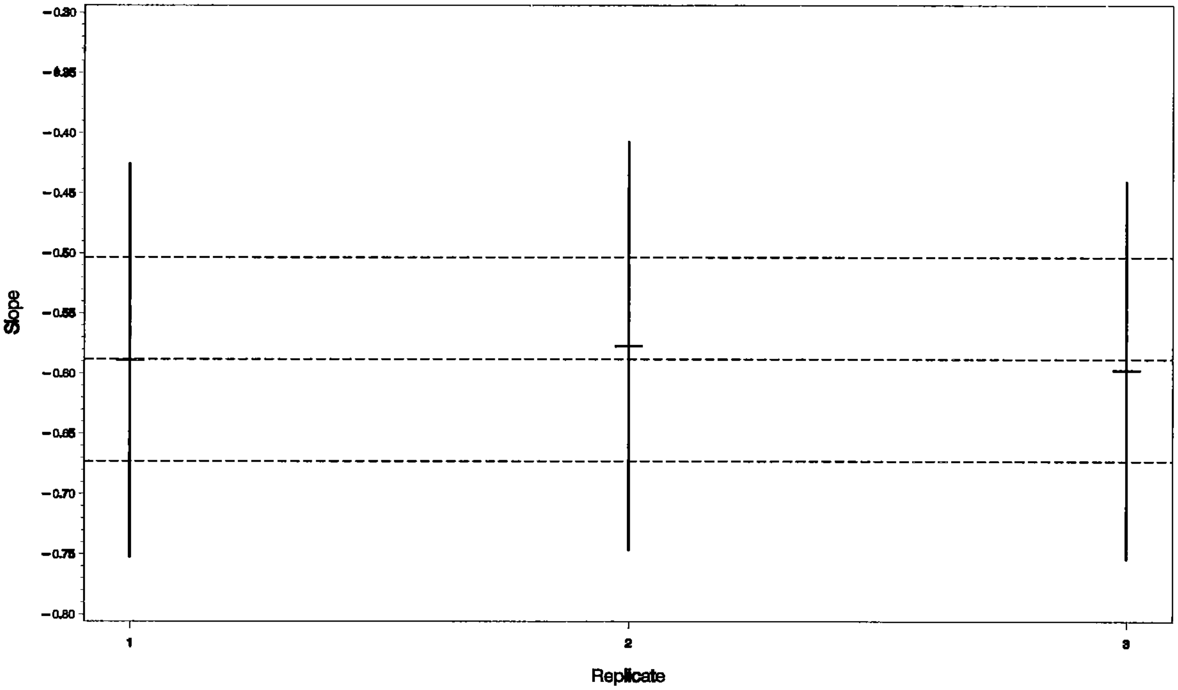
Exhibit 5

Means and 95% Confidence Intervals of Aminoglutethimide by Replicate and Overall



RTI Project 08055.003.032: WA 4-16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Means and 95% Confidence Intervals of Chrysin by Replicate and Overall

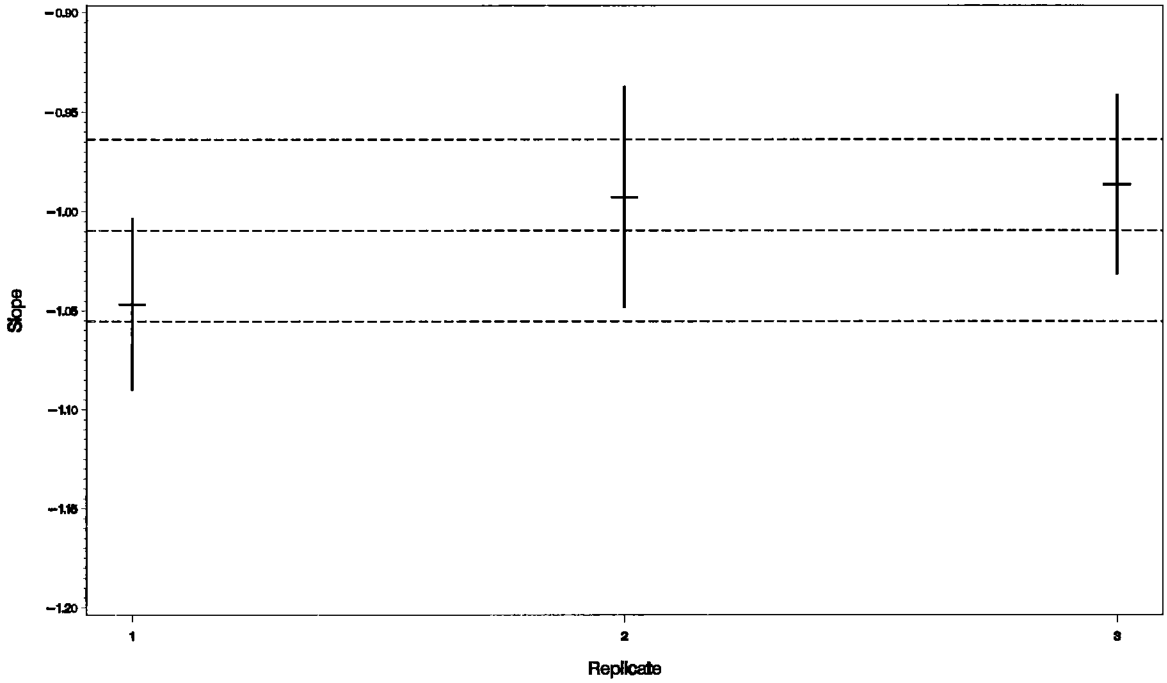


RTI Project 08055.003.032: WA 4-16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Exhibit 6

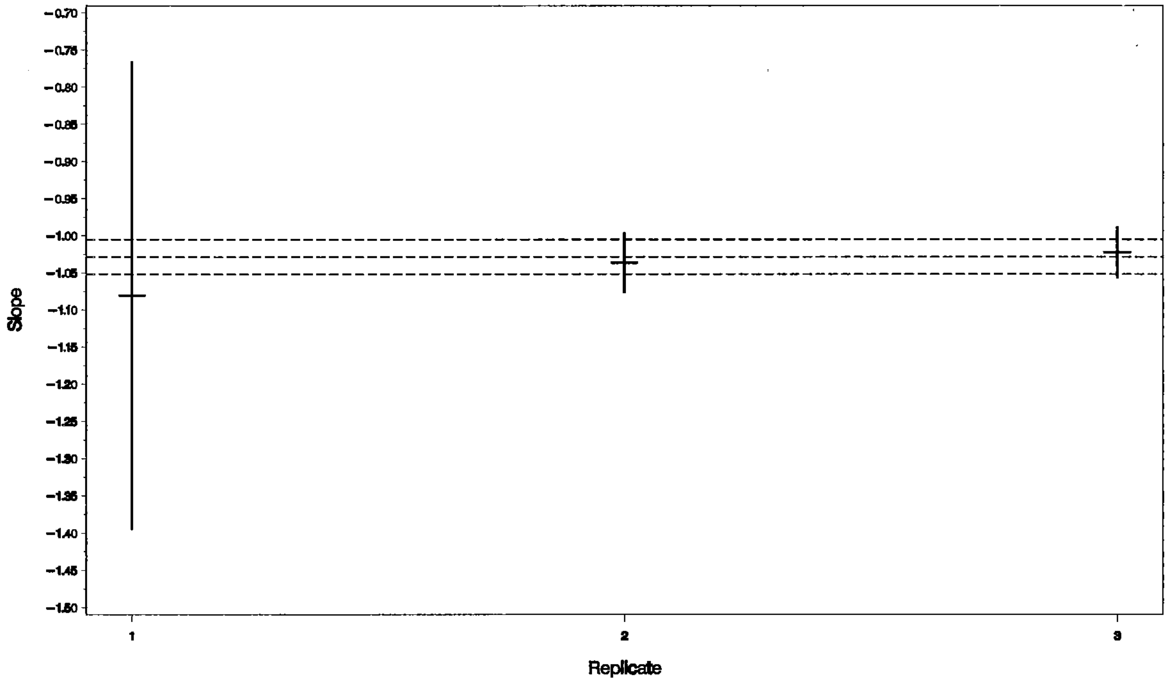
Exhibit 7

Means and 95% Confidence Intervals of Ketoconazole by Replicate and Overall



RTI Project 08055.003.032: WA 4–16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

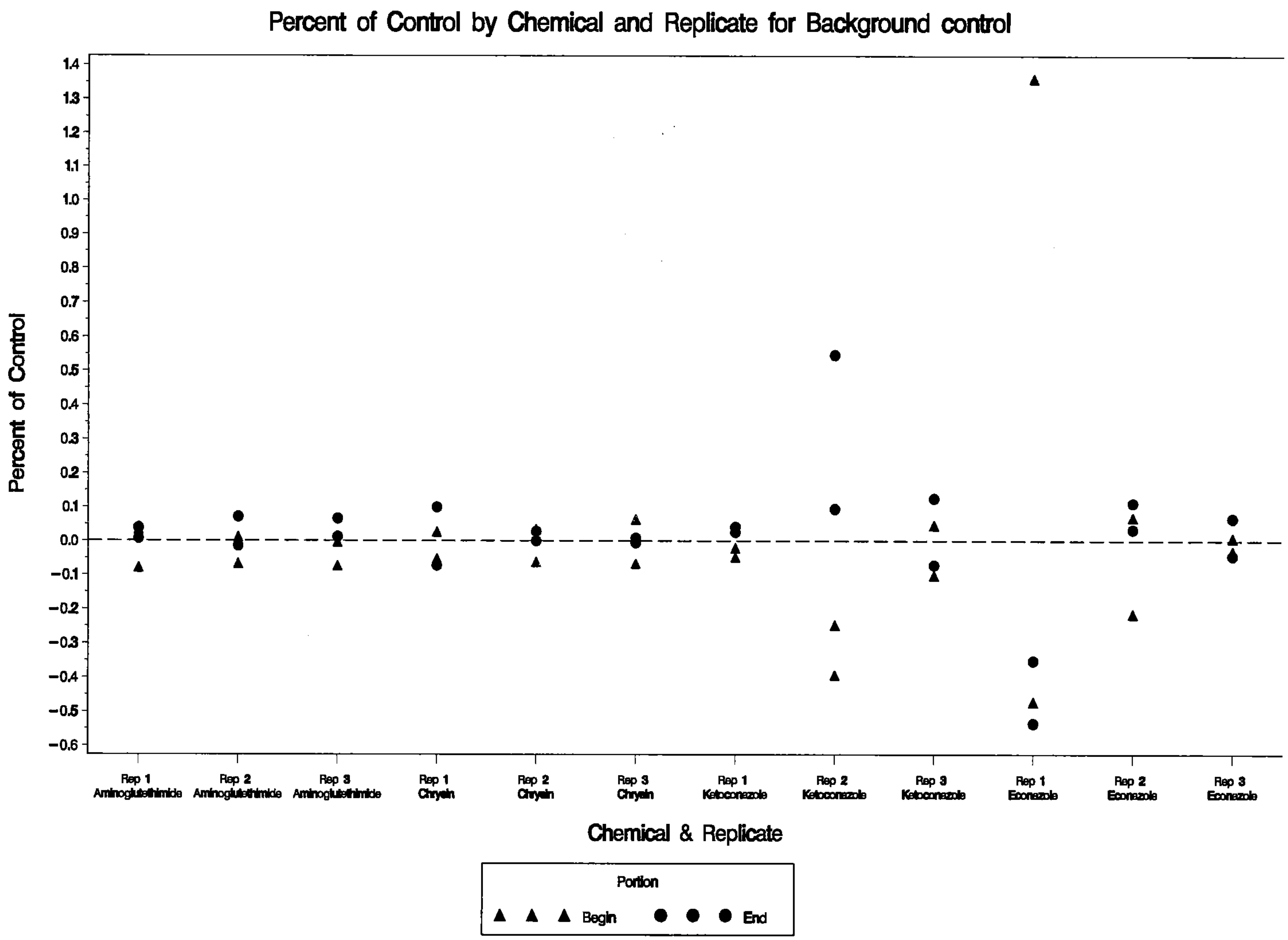
Means and 95% Confidence Intervals of Econazole by Replicate and Overall



RTI Project 08055.003.032: WA 4–16 Task 5, response_curves_analysis_revised_newdata.sas (23JAN06)

Exhibit 8

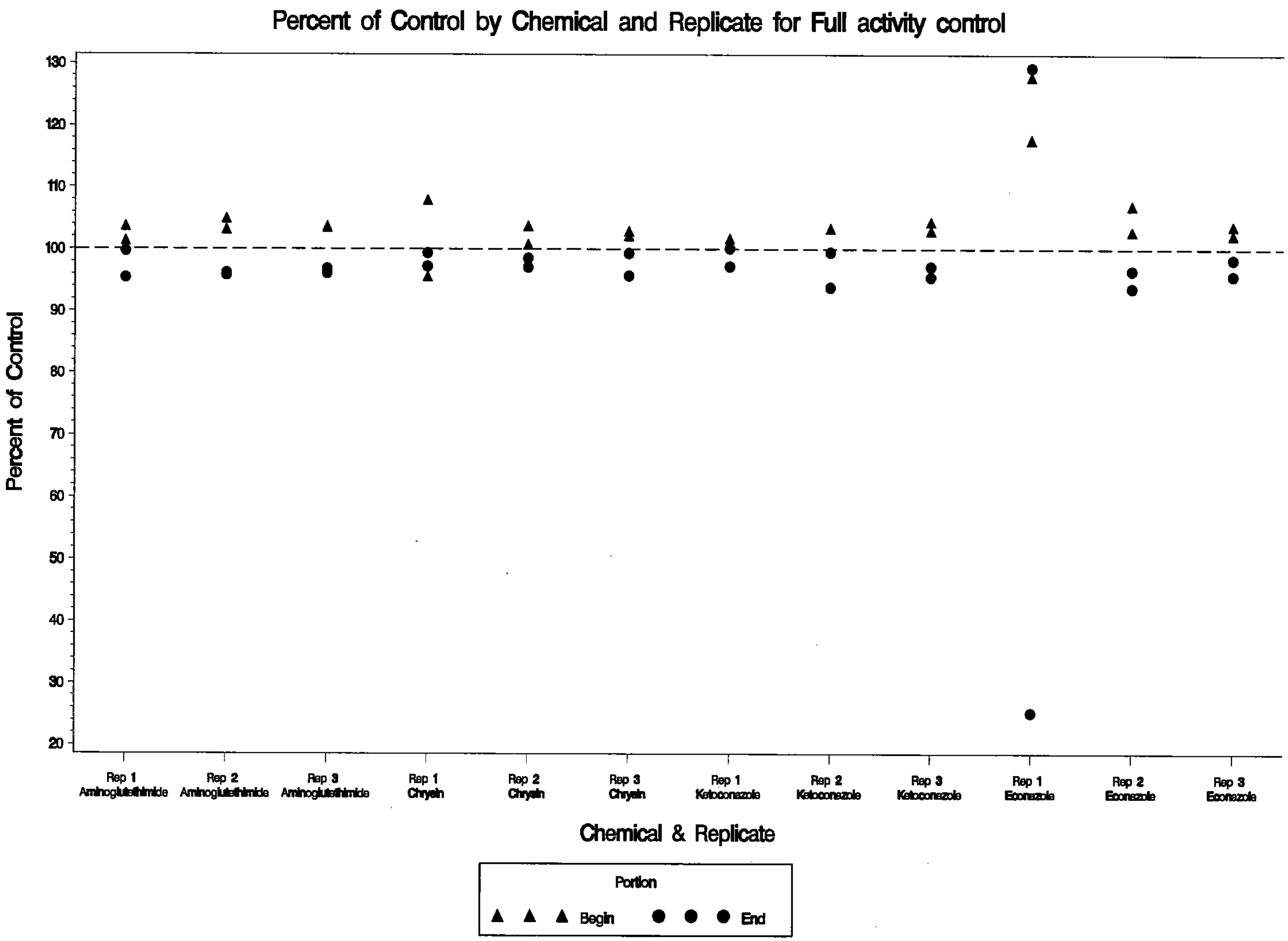
Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Exhibit 9

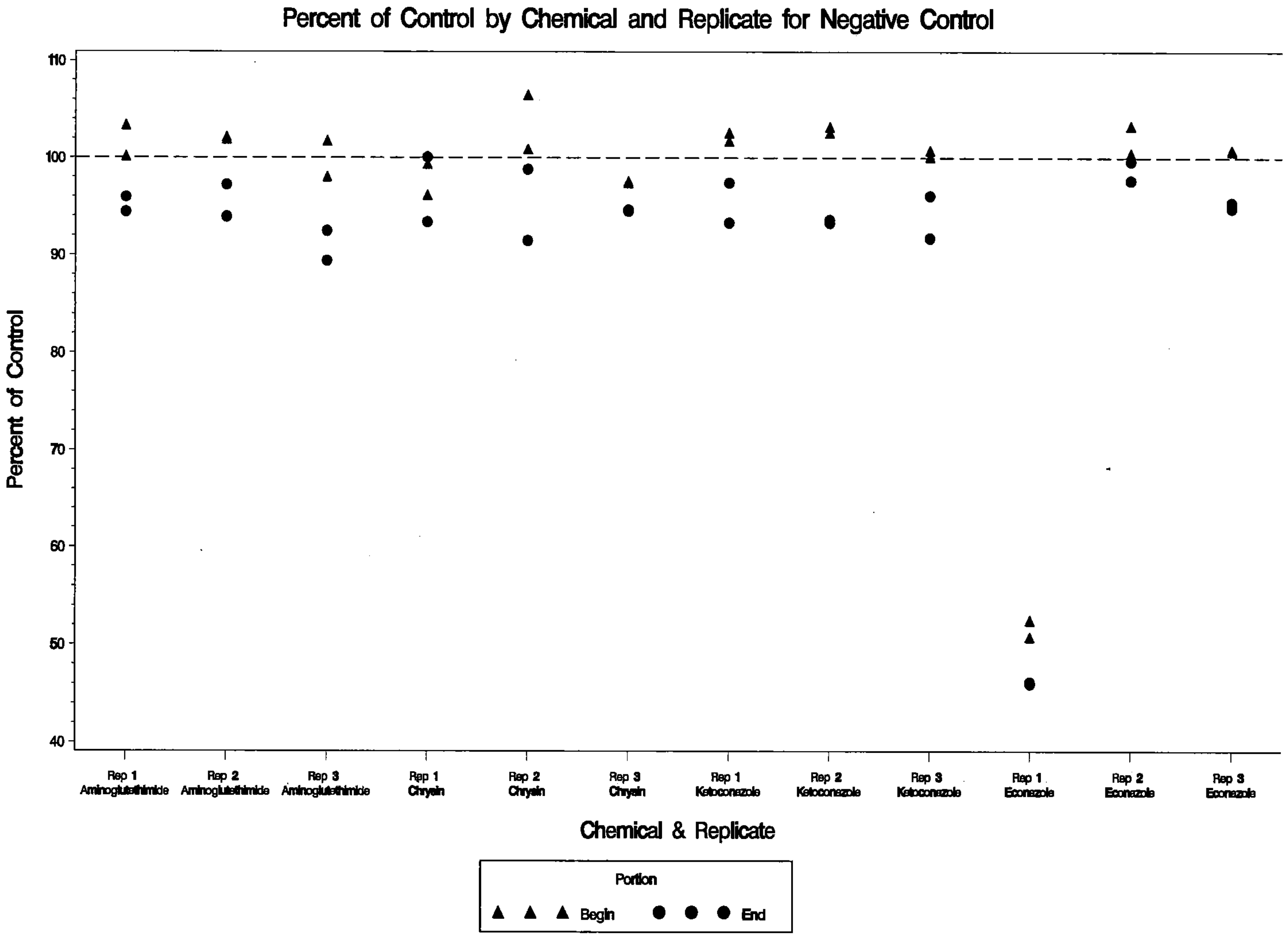
Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Exhibit 10

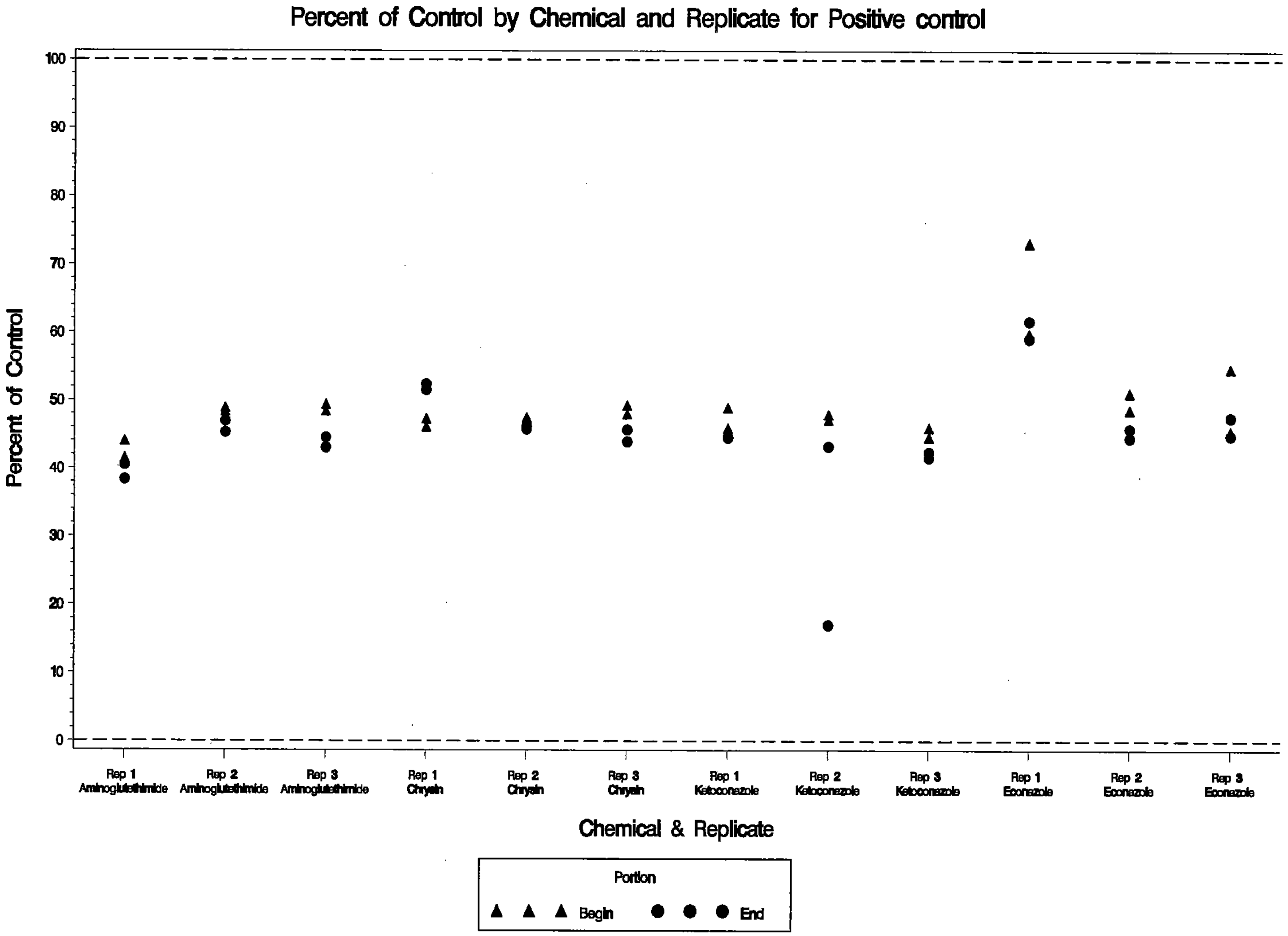
Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Exhibit 11

Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole



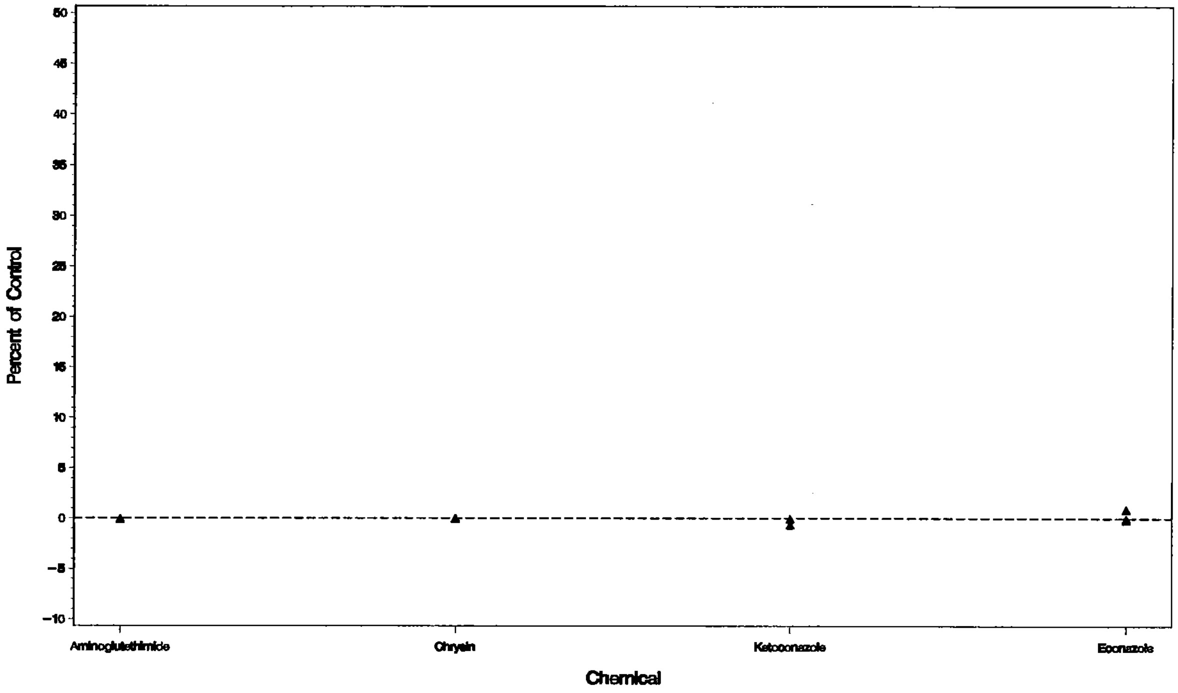
RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Exhibit 12

Note: Data presented as Rep 3, Ketoconazole is actually Rep 4, Ketoconazole and that presented as Rep 1, Econazole is actually Rep 4, Econazole

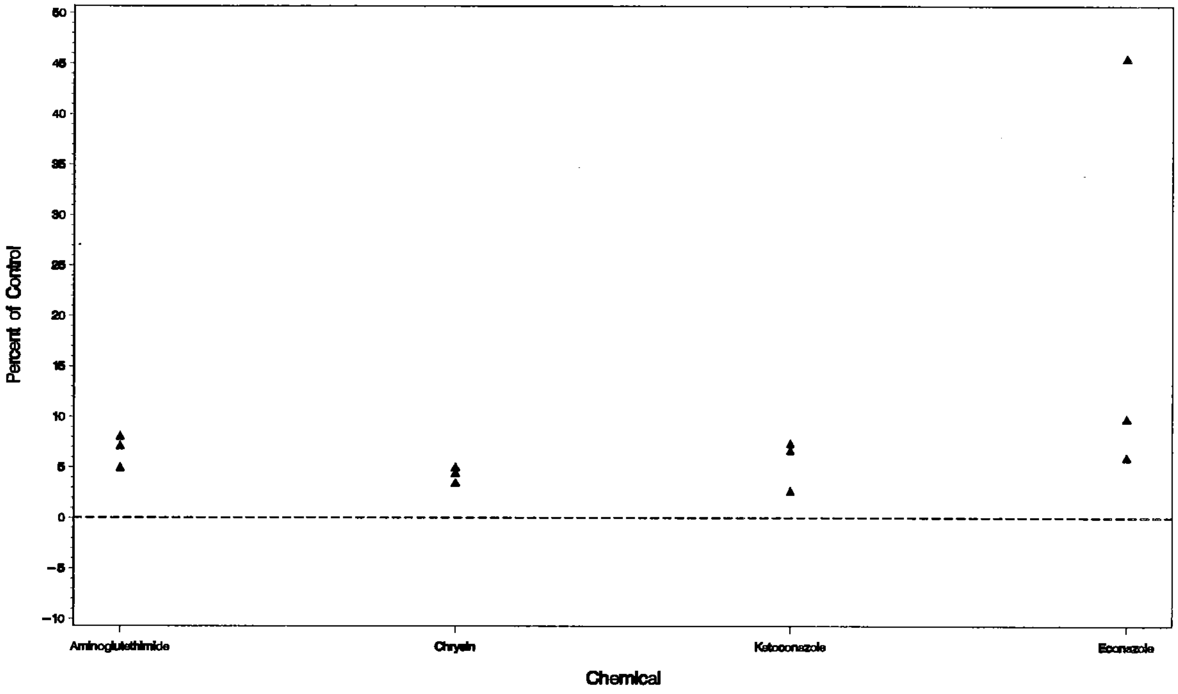
Exhibit 13

Difference of Portion Means by Chemical for Background control



RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Difference of Portion Means by Chemical for Full activity control

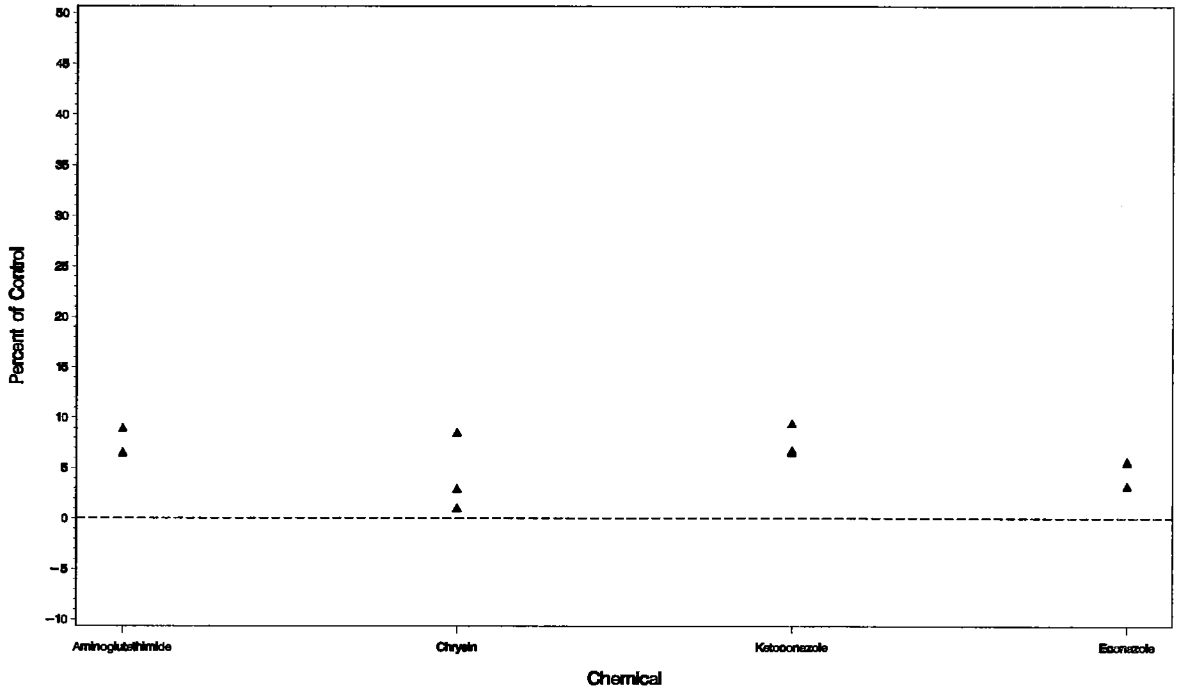


RTI Project 08055.003.032: WA 4-16 Task 5, control_analysis.sas (11MAY05)

Exhibit 14

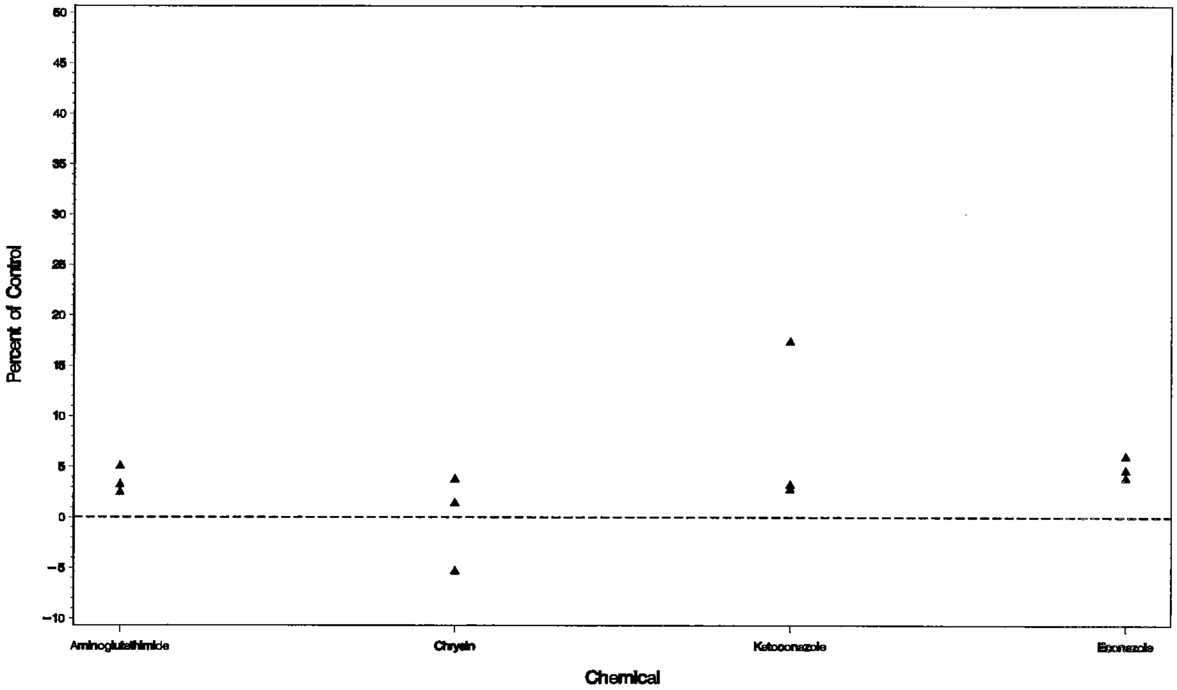
Exhibit 15

Difference of Portion Means by Chemical for Negative Control



RTI Project 08055.003.032: WA 4−16 Task 5, control_analysis.sas (11MAY05)

Difference of Portion Means by Chemical for Positive control



RTI Project 08055.003.032: WA 4−16 Task 5, control_analysis.sas (11MAY05)

Exhibit 16