FINAL REPORT

on

MICROSOMAL AROMATASE PREVALIDATION SUPPLEMENTARY STUDY: TASK 6- SUMMARIZATION OF FINDINGS AND REVISED AROMATASE PROTOCOL

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Prepared for:

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Final Report

Microsomal Aromatase Prevalidation Supplementary Study

WA 4-10, Task 6
Summarization of Findings and Revised Aromatase Protocol

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FINAL REPORT

Title: **Microsomal Aromatase Prevalidation Supplementary Study: WA 4-10, Task 6: Summarization of Findings and Revised Aromatase Protocol**

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1.0 INTRODUCTION

The experiments conducted in WA 4-10 investigated the technician-to-technician and day-to-day variability of the aromatase tritiated water assay using both recombinant and human placental microsomes with 4-hydroxyandrostenedione as the test inhibitor (Task 3). In addition, IC_{50}s were established for ketoconazole and econazole using both microsome types (Task 4). Finally, assay mixtures were assayed for estrone content by RIA and the results were compared with those obtained using the tritiated water assay (Task 5).

Two reports detailing the experiments and their results, a Task 3 and a combined Task 4 and 5 report, have been prepared and submitted separately. The results of these Tasks are summarized below. A protocol describing all aspects of the aromatase assay which incorporates the findings of both WA 2-24 and WA 4-10 has been prepared and is included in this report. The summarization of Tasks 3, 4, and 5 as well as preparation of a finalized protocol meets the deliverable requirements for Task 6 of WA 4-10.

2.0 DETERMINATION OF DAY-TO-DAY AND TECHNICIAN VARIABILITY (TASK 3)

Preliminary aromatase activity values determined for previously thawed and refrozen stocks of human placental and human recombinant microsomes showed that aromatase activity was well preserved (113% and 98% of previously determined average activities for placental and recombinant preparations, respectively) through at least one freeze/thaw cycle.

The aromatase assay was conducted using both microsome types in order to examine technician-to-technician and day-to-day variation in enzyme activity. Aromatase activity was also measured in the presence of eight different levels of the aromatase inhibitor, 4-OH ASDN, and variance was assessed. For the variability assessment, independent sets of assays were conducted by three technicians on three days each. Each set of assays included positive control samples that represented 100% aromatase activity. IC_{50}s were calculated for 4-OH ASDN in each microsome type and these were examined for technician-to-technician variation.

Statistically significant variances in positive control activities were seen for technician-to-technician, day-to-day, and portion (beginning vs. end) interactions. The source of this variation is not clear, but it may be related to small discrepancies in the preparation of the final microsomal protein dilutions. The protein concentrations used in the assay were very low, and small variances in actual protein concentration could have a large effect on apparent aromatase activity. Generally the activities of the two portion replicates of a given Study were very similar, and therefore, it does not appear that the variances are due to an experimental error. Also, the positive control activities for a given day were generally comparable to those activities measured in the presence of the lowest concentration of 4-OH ASDN for a given day.

Technician-to-technician variation in the aromatase activity of either microsome type in the presence of varying concentrations of 4-OH ASDN was not statistically significant. Day-to-day variance within a technician was statistically significant ($p<0.05$) for both microsome types.

IC_{50} values were calculated for each Study by nonlinear regression of the percent of control activity remaining at each of the 4-OH ASDN concentrations. An analysis of variance was carried out on the log(IC_{50}), slope and their associated standard errors for technician-to-technician variation. No significant technician-to-technician variation was found in any of those parameters. The mean IC_{50} for the human placental microsome assay was 56.0 ± 10.3 nM (range 44.5-68.9 nM) and that for the human recombinant microsome assay was 65.2 ± 10.5 nM (range 54.7-83.5 nM). These IC_{50}s are comparable to the literature values of 30-50 nM cited in the WA 2-24 protocol and those previously determined in WA 2-24, which were 41 and 53 nM for placental and recombinant preparations, respectively.

3.0 INHIBITION CURVES AND IC_{50}s FOR TWO REFERENCE CHEMICALS (TASK 4) AND A COMPARISON OF ESTRONE AND TRITIATED WATER MEASUREMENT METHODS (TASK 5)

Complete inhibition curves for the interaction of the inhibitors ketoconazole and econazole with human placental and recombinant aromatase (CYP19) activity was established. Ketoconazole was tested over the range 8E-4 to 1E-8 M; econazole was assayed over the range 1E-7 to 1E-12 M. The measured IC_{50} for ketoconazole in the recombinant assay was 10.08 ± 1.85 μM, while that for the placental assay was 15.00 ± 1.67 μM. These values are about one-sixth to one-fourth the literature value of > 65 μM reported in the protocol to WA 2-24. However, White et al (1999) reported an IC_{50} for ketoconazole of 6 μM and noted that literature values vary widely (7-60 μM). Rowlands et al (1995) reported an IC_{50} for ketoconazole in human placental microsomes of 15 μM while Ayub and Levell (1988) reported an IC_{50} of 7.3 μM for this inhibitor. The measured IC_{50} for econazole in the recombinant assay was 1.93 ± 0.06 nM, while that for the placental assay was 2.79 ± 0.60 nM. These values are an order of magnitude less than the literature values of 30-50 nM reported in the protocol for WA 2-24. There was little variance in the IC_{50} measured for a particular inhibitor using either microsome type.

Estrone content of samples from the recombinant aromatase assay was measured by RIA and compared with the nmol ^3H_2O formed in the tritiated water assay. One mole of ^3H_2O is formed for each mole of estrone formed in the aromatization of androstenedione. Generally, the amount of tritiated water measured in the assays was about 3-fold higher than the amount of estrone measured. At some high concentrations of inhibitor, the ratio was reduced, perhaps due to inhibition of another enzyme that may be involved in the further metabolism of estrone. It is possible that estrone may be further metabolized to another component that may not be

detectable using RIA. Further investigations would be necessary to determine the source of the apparent discrepancy between the amount of estrone and ^3H_2O formed in microsomes.

Aromatase activity in the recombinant microsomes in the presence of ketoconazole or econazole was calculated based on estrone content measured by RIA. IC_{50}s calculated based on these activities were similar to those obtained based on the ^3H_2O release assay. Therefore, it appears that application of either method (RIA or ^3H_2O) gives similar results.

4.0 REFERENCES

Ayub, M., and Levell, M. J., (1988) Structure Activity Relationships of the Inhibition of Human Placental Aromatase by Imadazole Drugs Including Ketoconazole. *J Steroid Biochem*, **31**, 65-72.

Rowlands, M. G., Barrie, S. E., Chan, F., Houghton, J., Jarman, M., McCague, R., and Potter, G. A., (1995) Esters of 3-Pyridylacetic Acid that Combine Potent Inhibition of 17 alpha-Hydroxylase/C17,20-lyase (Cytochrome P45017 alpha) with Resistance to Esterase Hydrolysis. *J Med Chem*, **38**, 4191-4197.

White, E. L., Ross, L. J., Steele, V. E., Kelloff, G. J., and Hill, D. L. (1999) Screening of potential cancer preventing chemicals as aromatase inhibitors in an in vitro assay. *Anticancer Res*, **19**, 1017-1020.

5.0 FINALIZED AROMATASE ASSAY PROTOCOL (TASK 6)

The finalized assay protocol is attached on the following pages.

EPA Contract No.:
EPA Work Assignment No.:

TITLE: Microsomal Aromatase Assay

SPONSOR:

TESTING FACILITY:

PROPOSED EXPERIMENTAL START DATE:

PROPOSED EXPERIMENTAL END DATE:

AMENDMENTS:

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

Approved By:

Principal Investigator	Date	WAL/Study Director	Date
Work Assignment Manager	Date	Program Manager	Date
Project Officer	Date		

Reviewed By:

Quality Assurance Specialist	Date	Quality Assurance Manager	Date
Work Assignment Manager	Date	Program Manager	Date

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1.0 OBJECTIVES

The objective of this protocol is to describe procedures for conduct of the aromatase assay using either human placental or human recombinant microsomes.

This protocol describes:

- Substrate Characterization
- Preparation of microsomes from human placenta
- Sources of human recombinant microsomes
- Preliminary experiments to confirm P450 content and aromatase activity in both microsome preparations.
- Experimental details for the aromatase assay
- Use of the aromatase assay to determine the inhibitory potential of test substances, including methods for calculation of IC_{50}s.

2.0 MATERIALS RECEIPT AND/OR PREPARATION

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 (Lot # 072K1134) was obtained from Sigma, St. Louis, MO and has a reported purity of 99 percent. The radiolabeled androstenedione ([1β-^{3}H]-androstenedione, [^{3}H]ASDN, Lot # 3474115), was obtained from Perkin Elmer Life Science, Boston and has a reported specific activity of 25.3 Ci/mmol. Radiochemical purity was determined to be 98%. The radiochemical purity of the [^{3}H]ASDN (and of any additional lots that are purchased) will be assessed as described in Section 2.1.2 prior to use of the material in the aromatase assay.

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 β-RAM Model 3 flow-through radioactivity detector (IN/US, Inc., Tampa, FL) with a 250 µL glass scintillant cell. Data will be collected using Waters Millennium^{32} Client/Server Chromatography Data System Software, Version 4.0.

The HPLC method uses a Zorbax SB-C_{18} column (4.6 x 250 mm) with a mobile phase of 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol and a flow rate of 1 mL/min. The eluant will be monitored by UV absorbance at 240 nm and by a flow-through radiochemical detector. Eluant fractions will be collected manually into vials containing ca.

10 mL Ultima Gold and assayed for radiochemical content by liquid scintillation spectrometry (LSS). A reference standard of nonradiolabeled ASDN will be analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN will be confirmed.

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

2.2 Test Substances

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

2.2.1 4-Hydroxyandrostenedione (4-OH ASDN)

CAS No.: 566-48-3
Molecular Formula/Weight: $C_{19}H_{26}O_3$; 302.4 g/mol
Supplier: Sigma
Lot No: 063K4069
Purity: 99%
Storage Conditions: 2-8°C

2.2.2 Test Substance Formulation and Analysis

Test substance solutions will be prepared fresh on each day of use. The formulations for each test substance will not be analyzed. 4-OH ASDN will be formulated in absolute ethanol. Test substances may be formulated in water, DMSO or ethanol. The total volume of test substance formulation used in each assay should be no more than 1% of the total assay volume (i.e., 20 µL in a 2 mL assay) in order to minimize the potential of the solvent to inhibit the enzyme.

2.3 Microsome Preparation

2.3.1 Human Placentas

2.3.1.1 Source of the Placentas. Human placenta will be obtained from a local hospital. The exact source of placentas will be documented in the study records. Human placentas are to be from non-smoking, 21-40 year old mothers with full term deliveries. Within 30 minutes of the delivery of the placenta by the mother, it will be placed in a tissue bag, sealed, and packed in wet ice in an insulated shipping container. Placenta tissue bags will be labeled with date and time of delivery. Laboratory personnel will be on-call and will be responsible for transporting placentas to their laboratory for processing into microsomes, as described below. Efforts will be made to minimize the time from delivery to the initiation of microsome preparation.

2.3.1.2 Placental Microsome Preparation. While keeping the placenta chilled on ice, the membrane and fibrous material are dissected, removed and discarded, and the tissue is then homogenized in portions using a Polytron and/or Potter Elvejhem homogenizer in a buffer (2:1, w:v) containing 0.25 M sucrose, 0.05 M sodium phosphate (pH 7.0), and 0.04 M nicotinamide. Next, the microsomal preparation is isolated by differential centrifugation procedures. The tissue homogenate is centrifuged at a setting of 10,000 g for 30 minutes in an IEC B-22M centrifuge; the supernatant is then centrifuged at a setting of 35,000 rpm (which is equivalent to approximately 100,000 g) in a Beckman L5-50B Ultracentrifuge for one hour to obtain the crude microsomal pellet. The supernatant is removed, the microsomal pellet is resuspended in 0.1 M sodium phosphate buffer (pH 7.4), and recentrifuged at a setting of 35,000 rpm (ca. 100,000g, Beckman L5-50B) for one hour to wash the microsomes. This washing procedure is repeated one additional time. The twice-washed microsomal pellet is resuspended in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol, and 0.05 mM dithiothreitol. The microsomes will be aliquoted into labeled vials that are convenient for conducting the aromatase assay. The vials will be flash frozen in liquid nitrogen and then stored at approximately -70°C until removed for use.

2.3.2 Human Recombinant Microsomes

2.3.2.1 Source of the Human Recombinant Microsomes. Human recombinant microsomes will be obtained from Gentest™ (Woburn, MA; www.gentest.com). The product name is Human CYP19 (Aromatase) Supersomes™ and the catalog number is 456260. The Supersomes™ package size is 0.5 nmoles cytochrome P450 in 0.5 mL. The cytochrome P450 content is 1000 pmol/mL. The representative total protein concentration is 4.0 mg/mL in 100 mM potassium phosphate (pH 7.4). The representative aromatase activity is 1200 pmol product/(min x mg protein); 5 pmol product/pmol P450/ min. [Supplier-provided values for protein concentration, cytochrome c reductase activity, and aromatase activity will be found on the data sheet accompanying each shipment and will be included in the report.] The Supersomes™ will be stored at approximately -70°C.

2.3.2.2 Human Recombinant Microsome Preparation. Preparation of the human recombinant microsomal preparation will involve thawing the microsomes rapidly in a 37 ± 1°C water bath and then keeping them on ice until used. The first time that the microsomes are thawed, they will be aliquoted into individual vials to minimize the freeze-thawing cycles. The assay uses approximately 0.004 mg/mL (final concentration) of microsomal protein. After aliquoting the microsomes into individual vials, the vials not planned for immediate use will be flash frozen in liquid nitrogen and then returned to the freezer for storage (approximately -70°C) until removed for use in the future.

3.0 PRELIMINARY EXPERIMENTS TO DETERMINE SUITABILITY OF MICROSOME PREPARATIONS

3.1 Protein Determination

The protein concentration of both microsome preparations will be determined for each batch of microsomes prepared or received, and 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.

3.2 Cytochrome P450 Content

Cytochrome P450 content will be determined in order to demonstrate that the microsome preparations have cytochrome P450 present prior to initiating enzyme activity assays. Using the Carbon Monoxide (CO) spectrum assay of Omura and Sato (1964), a single experiment using each of the preparations will be conducted as described below.

A sample of each microsomal preparation will be diluted in 0.1 M phosphate buffer (pH 7.4) to a protein concentration of approximately 1 mg/mL. The diluted sample, 4 mL, will be gently bubbled with carbon monoxide for approximately 10 sec. and then divided between a pair of matched cuvettes (1 mL/cuvette). Next, a few grains of solid sodium dithionite will be added to the sample cuvette with gentle mixing. The spectrum will then be recorded from 400 to 500 nm using a split-beam spectrophotometer.

Results will be presented as cytochrome P450 concentration and specific content. The concentration (nmoles/mL) will be calculated according to Beer's Law using an extinction coefficient value for cytochrome P450 of 100 mM^{-1} cm^{-1}. The specific content (nmol/mg protein) will be calculated by multiplying the cytochrome P450 concentration (nmol/mL) times the dilution factor and dividing this product by the protein content (mg/mL) of the original sample.

3.3 Cytochrome P450 (CYP19) Aromatase Activity

Aromatase activity of the microsome preparations will be determined prior to their use in IC_{50} determination studies to demonstrate that they have sufficient activity to conduct the current experiments. Each preparation will be run in triplicate tubes in the aromatase assay using the

optimized conditions as presented below in Table 1 and using the method described in Section 4.0.

3.4 Acceptance Criteria for Total Protein Yield and Aromatase Activity

It is essential that, for each microsomal preparation, enough protein be on hand for all of the planned studies. The microsomal preparations must also demonstrate acceptable aromatase activity.

The minimum acceptable aromatase activity in human placenta microsomes is set at 0.03 nmol product/mg protein/min. If the aromatase activity for any human placenta microsomal preparation is below the minimum acceptable level, then this preparation will not be used in further studies. In this case, new microsomal preparations would be made from additional placenta(s).

The minimum acceptable aromatase activity in human recombinant microsomes is set at 0.1 nmol product/mg protein/min. If the aromatase activity for the recombinant microsomal preparation is below the minimum acceptable level, then this preparation will not be used in further studies. In this case, a new batch of recombinant microsomes would be ordered and assayed for aromatase activity.

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and microsomal preparations from the human placentas, as well as human recombinant microsomes, will be obtained prior to initiation of the first set of experiments to ensure that sufficient quantities are available to conduct the studies. All reagent solutions will be prepared fresh daily, with the exception of assay buffer, which may be stored for up to one month in the refrigerator.

4.0 AROMATASE ASSAY METHOD

The assays will be performed in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. Propylene glycol (100 µL), [^{3}H]ASDN, NADPH, and buffer (0.1 M sodium phosphate buffer, pH 7.4) will be combined in the test tubes (total volume 1 mL). The final concentrations for the assay components are presented in Table 1. The tubes will be placed at 37 ± 1°C in the water bath for five minutes prior to initiation of the assay by the addition of 1 mL of the diluted microsomal suspension. The total assay volume will be 2.0 mL, and the tubes will be incubated for 15 min. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca. 5 s and placed on ice. The tubes are then vortex-mixed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6R centrifuge with GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layer will be removed and discarded; the aqueous layers are extracted again with methylene chloride (2 mL). This extraction procedure will be performed one additional time, each time discarding the methylene chloride layer. The aqueous layers will be transferred to vials and duplicate aliquots (0.5 mL) will be transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial

and shaken to mix the solution. The radiochemical content of each aliquot will be determined as described below.

Table 1. Optimized Aromatase Assay Conditions

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

[a] Final concentrations

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

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

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

5.1 Assay Conduct

Each study will test the response of aromatase activity to the presence of eight concentrations of potential inhibitor. Each concentration of inhibitor will be run in triplicate tubes in each Study. The concentrations of inhibitor to be used will be determined in consultation with the Sponsor and in consideration of literature data. If no information regarding the inhibition of aromatase by the test inhibitor is available, initial experiments should use inhibitor concentrations (final) ranging from 10^{-3} to 10^{-9} M. Positive and negative control samples will be included for each study. Positive controls will contain substrate, NADPH, propylene glycol, buffer, vehicle (used for preparation of inhibitor solutions) and microsomes. Negative controls contain all positive control assay components except NADPH and serve as assay blanks. Four positive and four negative controls are included with each Study and are treated the same as the other samples. The controls sets will be split so that two tubes (of each positive and negative controls) are run at the beginning and two at the end of each study set.

The assay will be conducted as described in Section 4.0 with the following modification. Inhibitor 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.

5.2 Data Analysis and Presentation

The data to be reported will include the following information: assay date and run number, recombinant or placental assay, technician, chemical and log chemical concentration, total DPM-background DPM, and % activity.

The spreadsheet calculates DPM/mL for each aliquot of extracted aqueous incubation mixture and average DPM/mL and total DPM for each aqueous portion (after extraction). Multiplication of the volume (mL) of substrate solution added to the incubation by the substrate solution specific activity (DPM/mL) yields the total DPM present in the assay tube at initiation. The total DPM remaining in the aqueous portion after extraction divided by the total DPM present in the assay tube at initiation times 100 yields the percent of the substrate that was converted to product. The total DPM remaining in the aqueous portion after extraction is corrected for background by subtracting the average DPM present in the aqueous portion of the negative control tubes (for that day/assay). This corrected DPM is then converted to nmol product formed by dividing by the substrate specific activity (DPM/nmol). The activity of the enzyme reaction is expressed in nmol (mg protein)$^{-1}$min^{-1} and is calculated by dividing the amount of estrogen formed (nmol) by the product of mg microsomal protein used times the incubation time, e.g. 10 minutes. Average activity in the positive control samples for a given Study is calculated. Percent of control activity remaining in the presence of various inhibitor concentrations is calculated by dividing the aromatase activity at a given concentration by the average positive control activity and multiplying by 100.

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

$$Y=\text{Bottom} + (\text{Top}-\text{Bottom})/(1+10^{((\text{Log}IC_{50}-X)*\text{HillSlope})})$$

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

The data will be formatted as follows:

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

- Another table will present the results of the analysis of variability of the assay and will include :
  (1) the variation between replicates within a single assay,
  (2) the day to day (study-to-study) variation, and
  (3) technician variation

- Graphs of activity versus log chemical concentration.

- Table of IC_{50}s by date, run, technician, assay method.

6.0 STATISTICAL ANALYSES

6.1 Quality Control Comparisons

For purposes of comparison, the control responses will be combined across batches and an analysis of variance will be carried out on the data. The factors in the analysis will be technician, batch, portion of batch (beginning or end) and the interaction between batch and portion of batch. The response will be enzymatic activity rate. If a daily batch is in control, the average of the first two (beginning) control runs should be statistically equivalent to the average of the second two (end) control runs. The estimated variance between the two replicate runs/batch will be based on the pooled value across the quadruplicate replicate runs on a given day for a given technician. In addition, the data will be analyzed by multiple comparisons and graphical analyses.

6.2 Variability Assessment

Differences among technicians and day-to-day within technicians for enzymatic activity at various concentration levels of an inhibitor will be estimated. The variance components will be compared across the assays to determine their comparability. Analyses will be based on mixed effects analysis of variance models using PROC MIXED or PROC GLM in SAS Version 8.2. Technician and inhibitor concentrations will be treated as fixed effects; day-to-day variation will be treated as a random effect. Variation between replicates within a single assay will be examined using the Levene's test for homogeneity of variance. Graphical analyses will be used to examine the changes in the variances across technician, day and inhibitor concentration.

6.3 Concentration Response Curve Fits

Concentration response curves will be fitted to the percent control activity values at each inhibitor concentration level. Plots will be prepared displaying the individual percent control activity determinations and the concentration response curve fits. A model will be developed for the relationship between percent of control aromatase activity and inhibitor concentration. A nonlinear model will be fitted by least squares regression for each technician, technician and day, and for the pooled values at each inhibitor concentration. If the results of the variability

assessment indicate significant changes in variation by the levels of the inhibitor concentration, weighted least squares regression will be used.

Analysis of variance tests will be carried out on log10(IC_{50}), slope, and associated within day standard errors. Technician-to-technician variation will be determined for each of the parameters and will be tested for significance.

7.0 RETENTION OF RECORDS

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

8.0 QUALITY CONTROL/QUALITY ASSURANCE PROCEDURES

Quality control (QC) and quality assurance (QA) procedures will follow those outlined in the Quality Assurance Project Plan (QAPP) that will be prepared for this study.

9.0 STUDY RECORDS TO BE MAINTAINED

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