PRE-VALIDATION STUDY PLAN AND STUDY PROTOCOL FOR THE AROMATASE ASSAY USING HUMAN, BOVINE, AND PORCINE PLACENTA, AND HUMAN RECOMBINANT MICROSOMES

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Aromatase Pre-validation Study Plan

INTRODUCTION

The objective of the aromatase assay is to detect the ability of environmental chemicals to inhibit the enzyme aromatase. Estradiol is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase and the levels of estrogens are controlled by the extent of formation of estrogens from androgens. The levels of estradiol, the most potent endogenous estrogen in humans, are critical for female reproduction and other hormonal effects in females. Alterations in the amount of aromatase present or in the catalytic activity of the enzyme will alter the levels of estrogens in tissues and dramatically disrupt estrogen hormone action. Inhibition of aromatase alters the catalytic activity of the enzyme and results in a rapid decrease in the levels of estrogens.

An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. If the 20-day pubertal female assay is not selected as a primary Tier 1 Screening Method, the 20-day male pubertal assay will be used as an alternative. The aromatase assay would be used to complement the male pubertal assay. An *in vitro* aromatase assay could easily be utilized to assess the effects of various environmental toxicants on aromatase activity. Environmental chemicals and various natural products can inhibit aromatase activity through a direct alteration of the catalytic activity of the enzyme, resulting in a rapid decrease in the levels of estrogens.

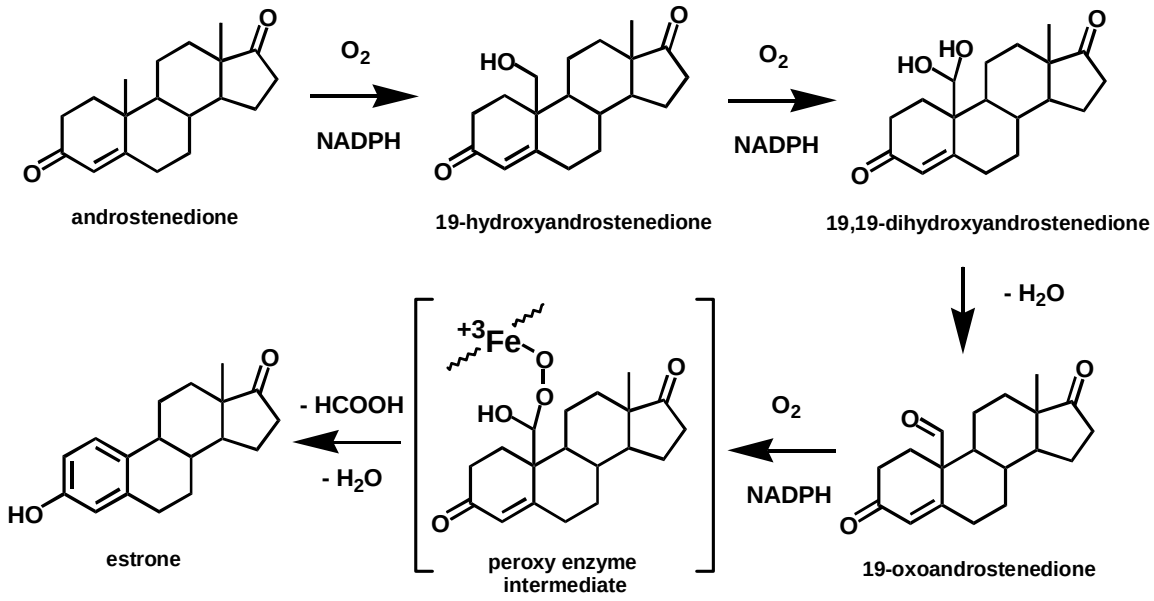
The pre-validation study plan addresses the optimization of the aromatase assay using human placental microsomal preparations, bovine placental microsomal preparations, porcine placental microsomal preparations, and recombinant human aromatase microsomal preparations. This pre-validation study plan has been developed to address the following issues:

- the endpoints to be measured
- the protocol issues needing resolution
- the study design to address the protocol issues
- recommended test substances and concentrations to be used and the justification for each recommendation
- the detailed study protocols
- statistical methods for comparing the performance of the assays.

1.0 ENDPOINT MEASUREMENTS

1.1 AROMATASE REACTION

Aromatase is the enzyme complex responsible for the conversion of androgens to estrogens during steroidogenesis (Simpson et al., 1994). The enzyme complex is bound in the endoplasmic reticulum of the cell and is comprised of two major proteins (Simpson et al., Kellis and Vickery, 1987). One protein is cytochrome $P450_{arom}$, a hemoprotein that converts C_{19} steroids (androgens) into C_{18} steroids (estrogens) containing a phenolic A ring. The second protein is NADPH-cytochrome P450 reductase, which transfers reducing equivalents to cytochrome $P450_{arom}$. Three moles of NADPH and three moles of oxygen are utilized in the conversion of one mole of substrate into one mole of estrogen product (reaction is illustrated below). Aromatization of androstenedione, the preferred substrate, proceeds via three successive oxidation steps, with the first two being hydroxylations of the angular C-19 methyl group. The final oxidation step, whose mechanism remains for complete elucidation, proceeds with the aromatization of the A ring and loss of the C-19 carbon atom as formic acid.



Reaction Mechanism for Estrogen Biosynthesis by Aromatase

1.2 HUMAN PLACENTAL MICROSOMAL AROMATASE ASSAY

The human placental microsomal assay is commonly used for measuring the inhibition of aromatase activity by various compounds, including potential therapeutic agents and various endocrine disruptors (Lephart and Simpson, 1991). The source of the aromatase is a microsomal preparation isolated from human term placenta. Normal placenta is obtained after delivery at a local hospital, is delivered on ice to the laboratory, and then processed at 4°C following a standard procedure. The microsomal preparation is isolated by differential centrifugation procedures. This microsomal preparation consists of the endoplasmic reticulum membrane of the cell and contains the membrane-bound cytochrome $P450_{arom}$ and the NADPH-cytochrome P450 reductase. Complete enzyme activity requires the addition of either NADPH or an NADPH-generating system, and the activity is measured using either the product isolation method or the radiometric method. The highest tissue levels of human aromatase are present in term placenta and the placenta is discarded after birth, thus providing a rich and inexpensive source of the enzyme. Consequently, the human placental microsomal aromatase assay is used extensively in academic labs and pharmaceutical firms as the initial biological evaluation for potential steroidal and nonsteroidal aromatase inhibitors.

1.3 OTHER MICROSOMAL AROMATASE ASSAYS

Aromatase has also been well studied in other mammalian species (rodents, cows, pigs, horses) and in non-mammalian vertebrates. In many species, aromatase expression and/or activity is restricted to the gonads and the brain. Placental aromatase is also found in other primates, cattle, horses, sheep, and pigs. Microsomal preparations from placental tissues from these animals are used in investigations of comparative biochemical endocrinology, gene expression studies, and structure-function studies of cytochrome $P450_{arom}$. Similar to humans and other primates, cattle and sheep have a single functional CYP19 gene and the gene is regulated by tissue-specific promoter regions (Vanselow, et al., 2001). Pigs, on the other hand, have several aromatase proteins that are produced by three distinct genes (Conley et al., 1996; Corbin et al., 1995; Corbin et al., 1999).

Biochemical studies have shown species differences in regards to substrate specificity and/or inhibition by various steroidal and nonsteroidal agents, suggesting catalytic differences at the enzyme active site as a result of amino acid sequence differences. In pigs, testosterone is aromatized more efficiently (apparent K_m for testosterone is 33 nM, apparent K_m for androstenedione is 77 nM) and differences in sensitivity to imidazole inhibitors have been reported (Corbin et al., 1995; Conley et al., 1997). Differences in inhibition by aniline inhibitor analogs have been observed in bovine vs. human placental enzymes (Kellis and Vickery, 1985). Similarly, imidazole and indolizinone derivatives have different aromatase inhibition kinetics in equine vs. human placental preparations (Auvray et al., 1999; Moslemi and Seralini, 1997; Moslemi et al., 1998).

Recombinant systems for expression of aromatase have also been developed using a cDNA clone of human placental aromatase containing a baculovirus expression system in insect

cell suspension cultures (Lahde et al., 1993; Sigle et al., 1994; Amarneh and Simpson, 1995). In general, these systems produce catalytically active aromatase enzyme, and the levels of enzyme activity in insect microsomal preparations are similar to the levels in human placental microsomal preparations. Such systems are primarily used to generate sufficient aromatase protein for enzyme purification. A commercial microsomal preparation of recombinant human aromatase from stably-transfected insect cells is available and has been used in a potential high-throughput assay (McNamara et al., 1999).

1.4 METHODS FOR MEASURING AROMATASE ACTIVITY

The method employed for measuring aromatase activity *in vitro* and *in vivo* is critical. This assay endpoint, i.e., measurement of aromatase activity, must be accurate and reproducible. The endpoint is accurate when the assay measurement is in agreement with the accepted reference value. The endpoint is reproducible when the same findings occur under the same conditions within a single laboratory (intra-laboratory) and among other laboratories (inter-laboratory). The two methods that are utilized extensively for the determination of aromatase activity in both *in vitro* and *in vivo* assays are the product isolation method and the radiometric method (Lephart and Simpson, 1991).

The most rigorous method is the product isolation technique. The method involves administration of a substrate such as androstenedione or testosterone, incubation or treatment for a designated time, isolation of the estrogen products formed, and measurement of the amount of estrogens. One common method uses radiolabeled substrate (either ^{3}H or ^{14}C) in the aqueous incubation medium. At the end of the incubation or treatment period, isolation of the radiolabeled steroids is accomplished by organic solvent extraction techniques. The radiolabeled substrate and products are separated using either thin layer chromatography or high-pressure liquid chromatography (HPLC) and analysis of the quantity of estrogen products formed using liquid scintillation counting. This method is best suited for *in vitro* assays using subcellular enzyme preparations, tissue incubations, or cell culture systems. Variations of the product isolation assay method include unlabeled substrate (or endogenous substrate in an *in vivo* study) and other methods for measurement and quantification. These other measurement methods include mass spectrometry, radioimmunoassays (RIA), or enzyme-linked immunoassays (EIA).

The radiometric method is also utilized for *in vitro* measurements of aromatase activity. This method is also referred to as the $^{3}H_2O$ assay. The level of aromatase activity is determined by measuring the amount of $^{3}H_2O$ released from [1b-^{3}H]-androstenedione substrate. The basis of this assay is that the aromatization of the A-ring catalyzed by aromatase involves the stereospecific cleavage of the covalent bond between the carbon atom at position 1 and the hydrogen on the b face of the steroid ring system. The procedures for this method are similar to the product isolation method but do not involve any chromatography to separate steroid substrate and products. Rather, the amount of $^{3}H_2O$ released is measured in the aqueous phase following rigorous extractions with organic solvents and/or dextran-coated charcoal. This method is very straightforward and more rapid. However, the results of the radiometric method must be confirmed for the specific aromatase assay conditions using the product isolation assay before

extensive use. This radiometric method has been confirmed for *in vitro* assays using human placental microsomes, ovarian microsomes and tissues, cell culture systems (including human breast cancer cells and human choriocarcinoma cells), and isolated human breast or ovarian cells. This comparison of the results from the radiometric method with the product isolation assay is critical because other biochemical pathways that involve extensive androgen metabolism without aromatization of the androgen can produce false positive measurements. Examples are determinations of ^3H_2O released by liver or prostate cells, tissues, or tissue homogenates, which results in much higher activity measurements than is observed with a product isolation method as a result of androgen metabolism, and presence of a 19-hydroxylase activity in adrenal tissues which does not result in A ring aromatization and formation of estrogens.

2.0 PROTOCOL RESOLUTION ISSUES/STUDY DESIGN

2.1 PRE-OPTIMIZATION EXPERIMENTS

The pre-optimization experiments are designed to assess the chemical and biological properties of the critical components that are used in the aromatase assay. These experiments include characterizing the radiolabeled substrate and preparation of the placental microsomes. In addition, the microsomal preparations, including the human recombinant microsomes, will be analyzed for protein concentration, cytochrome P450 content, and aromatase activity. The P450 content measurement will provide assurance that the enzyme is present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. A single aromatase activity assay using each type of microsomal preparation was included as a pre-optimization experiment in order to determine whether the preparations are of sufficient activity to conduct the optimization experiments. The outcome of the pre-optimization experiments will be to know that the placental and recombinant microsomes and substrate are sufficient in quantity and activity to conduct the optimization experiments.

2.2 AROMATASE ASSAY OPTIMIZATION EXPERIMENTS

The assay optimization experiments are designed to optimize the chemical and biological components of the microsomal aromatase assay. These experiments involve a factorial design approach to include evaluation of co-factor concentration, enzyme concentration, protein concentration, substrate concentration, and incubation time using human placental microsomal preparations, bovine placental microsomal preparations, porcine placental microsomal preparations, and recombinant human aromatase microsomal preparations. This experiment will include four parts.

- Part 1, 2^{6-1} factional runs, will permit the estimation of all linear main effects and linear by linear interactions to be determined.

- Part 2, center point run, will provide an estimate of the response in the center of the design space and an overall indication of the goodness-of-fit to the linear trend assumptions.

- Part 3, axial point runs, will provide an estimate of the quadratic main effects for each of the factors.

- Part 4, replicate runs, will provide an estimate of the reproducibility of the response at the center of the design space and at various extremes of the design space.

In addition, this stage of the study plan will also provide an estimate of the variability of the optimized assay in each of the different microsomal preparations. Thus, the outcome of the optimization experiments will be to know the optimal conditions and variability of the aromatase assay in each of the microsomal preparations in order to test the assay using selected test substances.

3.0 OPTIMIZED ASSAY EXPERIMENTS USING SELECTED TEST SUBSTANCES

Various substances will be tested in the optimized aromatase assays using human placental microsomal preparations, bovine placental microsomal preparations, porcine placental microsomal preparations, and recombinant human aromatase microsomal preparations. The results of these evaluations will be compared to determine whether the assays were similar or not in their response to detect an effect of the test substance.

For each of the four optimized assays, seven test substances that exhibit aromatase inhibition to varying degrees will be evaluated:

- Aminoglutethimide: This compound is a non-steroidal aromatase inhibitor exhibiting moderate inhibitory activity. It is referred to as a first-generation aromatase inhibitor and was the first agent used for inhibition of aromatase in clinical trials. Aminoglutethimide is commonly used in various *in vitro* assays as a standard inhibitor to compare results with other non-steroidal agents. IC_{50} values for aromatase inhibition by aminoglutethimide in *in vitro* assays typically range from 1.0 to 6.0 µM. This agent is commercially available.

- Letrozole or Anastrozole: These compounds are non-steroidal aromatase inhibitors exhibiting potent inhibitory activity. Letrozole is marketed by Novartis under the trade name Femara, and anastrozole is marketed by Astra Zeneca under the trade name Arimidex. These agents are referred to as third-generation aromatase inhibitors. Both drugs are approved as first-line therapy in women with advanced hormone-dependent breast cancer and as second-line therapy in women with hormone-dependent breast cancer who have failed tamoxifen treatment. IC_{50} values for aromatase inhibition by these two agents in *in vitro* assays have been reported in the low nanomolar range (1 –

15 nM), and both agents suppress estrogen biosynthesis *in vivo* by greater than 96%. The only sources for these agents are the pharmaceutical manufacturers, and it may be difficult to obtain these agents for that reason.

- 4-Hydroxyandrostenedione: This compound is a steroidal aromatase inhibitor exhibiting potent inhibitory activity. It is referred to as a second-generation aromatase inhibitor and was the second agent used for inhibition of aromatase in clinical trials. 4-Hydroxyandro-stenedione (4-OH-A, formestane) is commonly used in various *in vitro* assays as a standard inhibitor to compare results with other steroidal agents. IC_{50} values for aromatase inhibition by 4-OH-A in *in vitro* assays typically range from 30.0 to 50.0 nM. This agent is commercially available.

- Chrysin: This compound is a flavonoid natural product isolated from various plant sources. It exhibits moderate aromatase inhibitory activity in *in vitro* assays, with IC_{50} values for aromatase inhibition typically ranging from 0.5 to 10.0 µM. This agent is commercially available.

- Genistein: This compound is an isoflavonoid natural product isolated from various plant sources. It exhibits weak aromatase inhibitory activity in *in vitro* assays, with IC_{50} values for aromatase inhibition typically ranging from 30.0 to 100.0 µM. Genistein at micromolar concentrations exhibits many other biological activities as well, including weak estrogenic activity, binds to estrogen receptor a and estrogen receptor b, and inhibits protein tyrosine kinase activity. This agent is commercially available.

- Econazole: This compound is an imidazole anti-fungal agent and is marketed by numerous pharmaceutical or agricultural firms. It exhibits potent aromatase inhibitory activity in *in vitro* assays, with IC_{50} values for aromatase inhibition typically ranging from 30.0 to 50.0 nM. This agent is commercially available.

- Ketoconazole: This compound is also an imidazole anti-fungal agent and is marketed by numerous pharmaceutical or agricultural firms. It exhibits weak aromatase inhibitory activity in *in vitro* assays, with IC_{50} values for aromatase inhibition greater than 65.0 µM. This agent is commercially available.

For each of the four optimized assays, five test substances that do not exhibit aromatase inhibition will be evaluated:

- Atrazine: This compound is a 2-chloro-s-triazine herbicide. Atrazine does not inhibit aromatase enzymatic activity. Atrazine and related 2-chloro-s-triazine herbicides have been reported to affect aromatase gene expression and result in induction of the enzyme in cell cultures administered the agent. This agent is commercially available.

- Mono-(2-ethylhexyl)phthalate: This agent (MEHP) is the biologically active metabolite of di-(2-ethylhexyl) phthalate (DEHP). Phthalates are plasticizers and are added to polyvinyl chloride-based products to increase flexibility of the materials. MEHP does not inhibit aromatase enzymatic activity. However, MEHP has been reported to affect aromatase gene expression and results in suppression of the enzyme in cultured granulose cells. This agent is commercially available.

- Nonylphenol: Nonylphenol is a degradation product of alkylphenol polyethoxylate detergents, and nonylphenol exhibits weak estrogenic activity through interactions with the estrogen receptor. Nonylphenol does not inhibit aromatase enzymatic activity. This agent is commercially available.

- Lindane: This agent is an organochlorine insecticide, and lindane and its isomers affect the functions of steroid acute regulatory protein (StAR) and alter cholesterol metabolism. Lindane does not inhibit aromatase enzymatic activity. This agent is commercially available.

- TCDD: TCDD, a dioxin, is a highly toxic contaminant produced as a by-product during the manufacture of chlorinated phenols and phenoxyherbicides. One of TCDD's effects are to alter the expression of other cytochrome P450 genes involved in drug metabolism (such as CYP1A1). TCDD does not inhibit aromatase enzymatic activity. This agent is commercially available, but its use is restricted due to its highly toxic effects.

4.0 STUDY PROTOCOL

The study protocol is included in this study plan as an attachment (See Appendix).

5.0 STATISTICAL METHODS

5.1 OPTIMIZATION EXPERIMENTS

5.1.1 Experimental Factors and Response

The object of the experiment is to identify the combination(s) of experimental conditions that will maximize the rate of the aromatase reaction. The combination of experimental factors that maximizes this response will be experimentally determined. The experimental factors to be studied and their ranges of variation are as follows:

Factor Identification[a]	Units	Factor Designation	Factor Levels (Conc.) 1	2	3	4	5
NADP	mM	X_1	0.1	0.5	1	2	4
G-6-P	mM	X_2	0.1	1	2	3	4
G-6-P dehydrogenase	units	X_3	0.1	0.5	1	2	4
Androstenedione (substrate)	nM	X_4	10	25	50	100	500
Protein	mg/mL	X_5	0.01	0.05	0.1	0.5	1
Incubation time	min	X_6	10	20	30	60	120

[a] Glucose-6-phosphate (G-6-P)

It is assumed that all combinations of these factors are *a priori* possible. Since each of the factors range across one or more orders of magnitude, consideration will be given to expressing response relations in terms of transformations of the factors, such as logarithmic transformations. All factors are assumed to be *a priori* equally important so the suggested design is symmetric in each of the factors. This assumption may be modified after analyzing the results of the initial experiments.

5.1.2 Design Considerations for the Microsomal Preparations

The microsomal preparations that will be used in the pre-validation plan are:

- Human placenta
- Bovine placenta
- Porcine placenta
- Human recombinant microsomes.

Separate experiments will be conducted to optimize the aromatase assay for each of the microsomal preparations. Initially a 51 run experiment will be conducted to optimize the human placenta Aromatase assay. Based on the results of the experiments using the human placental microsomes, the optimization experiments using the bovine and porcine placental microsomes, as well as the human recombinant microsomes, may require further experiments to estimate more complex structure (e.g. higher order trends of interactions) than initially assumed. Alternatively, the response structure of the assay using the human placental microsomes may be simpler than initially assumed. In this instance, the designs for the other microsomal preparations could then be reduced, thereby decreasing the level of effort and time required to complete the optimization experiments for all four microsomal preparations.

5.1.3 Experimental Design Matrix

The suggested experimental design matrix is based on a factorial design that simultaneously varies each of the six experimental factors. The design permits the estimation of

linear and quadratic main effects for each of the factors (averaged across all the other factors), as well as linear by linear two factor interactions among all pairs of factors. It is initially assumed that these trends and interactions describe the principal structure in the response trends. This assumption will be re-evaluated after the initial test results are determined. The initial design will be supplemented if appropriate.

Factorial designs are preferable to one-factor-at a time experiments because they utilize all the data to estimate each of the effects, rather than just the portion of the data pertaining to a single factor. They also permit estimation of interaction effects, which indicate if the response to varying one factor depends on the levels of one or more other factors.

It is assumed that each of the factors can be varied with equal ease. Thus the experimental runs will be completely randomized across the 51 runs. The experimental matrix is divided into four parts but the runs in these parts will be intermixed when conducted in the laboratory.

- Part 1 - 2^{6-1} factional factorial design (32 runs). These runs will estimate the linear main effects and linear by linear interactions.
- Part 2. Center Point design (1 run). This run will estimate the response in the center of the design space and will provide an overall indication of goodness-of-fit to the linear trend assumptions.
- Part 3. Axial Point design (12 runs). These runs will estimate the quadratic main effects for each of the factors.
- Part 4. Replicate design (6 runs). These runs will estimate the reproducibility of the response at the center of the design space and at various extremes of the design space.

5.1.4 Statistical Analysis

Statistical analysis will be based on multiple regression analysis. Preliminary graphical displays will be used to identify the nature of the trends, the nature of the response variability, and the need for transformations of the response or of the primary experimental variables X_1, ..., X_6 full quadratic response surface model will be fitted to the data. Residuals from the model will be examined graphically and numerically to identify outlying observations, heterogeneity of variability, and departures from model assumptions.

If several individual factors or combinations of factors exhibit particularly strong influence on the response outcome, then consideration will be given to augmenting the design with supplemental runs to study the trends or interactions in these directions in greater detail. If the trends or interactions associated with several of the experimental factors are small or not significant, then consideration will be given to omitting them from subsequent experiments with the other microsomal preparations.

The final response surface model will be optimized to determine the experimental conditions associated with the optimum response. A confidence region will be constructed around the optimum by considering the set of experimental factors whose associated responses do

not differ significantly from that at the maximum. The optimum condition may occur at the interior of the design space or at a boundary. In the former case, the optimum and an associated confidence region will be reported. In the latter case, it may be possible to further improve the efficiency of the reaction by extrapolating outside the design space. Consideration will be given to extending the experimental region in the direction of the (increasing) response gradient to determine the extent of possible improvement in efficiency.

Sensitivity analysis will be conducted in the region of the optimum to assess the effects of perturbations in experimental factors from their optimum values on the reaction efficiency. The individual factors X_1, ...,X_6 will be varied by differing amounts, e.g. ± 5%, ± 10%, ± 20% to determine the influence on reaction rate. The linear combination of experimental factors corresponding to the eigenvector associated with the minimum (i.e. the most negative) eigenvalue of the matrix of normalized second partial derivatives at the maximum will also be studied to assess the effect on reaction sensitivity of perturbations in experimental conditions in the direction of greater rate of change of the response surface.

5.1.5 Determine Variability of the Optimized Assays

After optimum conditions have been determined for the assay using the four different microsomal preparations, each assay will be rerun at its optimum conditions to assess variability of results. Each assay will be conducted independently by three technicians and at three separated times per technician. This permits the estimation of the technician-to-technician and the within technician components of variation. These components of variation can be compared with that determined from the regression fit to assess their statistical and biological significance.

The variance components will be compared across the assays to determine their comparability.

5.2 OPTIMIZED ASSAY USING SELECTED TEST SUBSTANCES

5.2.1 Test Run Composition

This testing stage of the study will be carried out in a single laboratory, under homogeneous conditions. The term “run” will be used to describe when the assay is used to measure aromatase activity of a single sample. Multiple runs will be conducted each day and the multiple runs will be referred to as a “batch.” The size of a batch is up to 48 runs and the order of the runs will be randomized within a batch.

On a given day, the batch of runs will include:

- Four replicates (4 runs) of the substrate standard (androstenedione) at the highest tested concentration. This sample will provide the lowest measure of aromatization due to greater substrate-enzyme catalysis of the non-radiolabeled substrate relative to the radiolabeled substrate. It is designated at the NSB (non-specific binding) sample.

- One replicate of the substrate standard at each of the seven other concentrations (7 runs)

- Four replicates (4 runs) of the vehicle. The test substances that are run within the same batch will be grouped so that they use the same vehicle.

  The vehicle replicates will provide the highest measure of aromatization (assuming the vehicle is inert) since it will not reduce radiolabeled substrate-enzyme catalysis.

- One replicate of each test substance (up to four a day) at each of the eight concentrations tested (up to 32 runs).

5.2.2 Quality Control Comparisons

The quadruplicate replicate runs on a given day for the vehicle control(s) and androstenedione (highest concentration only) will be used for quality control comparisons. On a given day, the batch of up to 48 runs will include half of the quadruplicate replicate runs at the beginning and half at the end for the vehicle control and androstenedione. A two-way analysis of variance will be carried out on the data. This same comparison will be made for the androstenedione. The variation between the two replicate runs at the beginning of the batch and the two replicate runs at the end will be based on the pooled variation across batches.

By definition the four NSB values must average 0 percent aromatization and the four vehicle values must average 100 percent aromatization. It follows that for these endpoints, a comparison can be made for the average of the first two values to 0 percent or 100 percent, respectively. If the test conditions remain constant across the duration of the runs within a batch, then the NSB average within each batch should be statistically equivalent to 0, and the vehicle average within each batch should be statistically equivalent to 100. This structure will be examined by analysis of variance, multiple comparisons, and graphical analysis.

For each of the responses, the standard deviation within batches will be compared between the first and last tests and across batches by analysis of variance and graphical techniques in a manner similar to the average values.

5.2.3 Concentration Response Curve Fits to the Standard Tests

Competitive concentration response curves will be fitted to the standard substrate test results within each batch. The NSB runs will be included in the fits but the vehicle runs will not be included. Responses will be normalized to “percent aromatization”, based on the NSB values and the ”corresponding vehicle” values. Percent aromatization is defined as:

[Observed DPM – NSB DPM] / [Vehicle DPM – NSB DPM] x 100

If $Y \equiv$ percent aromatization and $x \equiv \log_{10}$ (concentration), then the competitive

concentration response relation between Y and x can be described by the model

$$Y = b + \frac{t - b}{1 + 10^{\beta(x-\mu)}}$$

where t and b are the "top" and the "bottom" of the curve (approximately 100% and 0% respectively), β is the slope, and μ is the $\log_{10}$ (IC_{50}). The model will be fitted by weighted least squares. The weights are inversely proportional to the predicted amount of ^3H_2O as measured by the DPM count. Namely,

$$DPM\ pred = NSB\ DPM + (\tfrac{\hat{Y}}{100})[SPC\ DPM]$$

where NSB DPM is the "nonspecific DPMs" associated with the NSB concentration of standard substrate and SPC DPM is the "specific DPMs", i.e. vehicle DPM – NSB DPM:

$$Weight = \frac{K}{DPM\ \ pred}$$

The concentration response relation will be fitted within each test batch. Since the replicate determinations within a single test run involve only replication of the scintillation counter readings, their differences do not reflect all of the variation. Thus, the model will be fitted to the average of the replicate determinations at each concentration. This process will be repeated in each test batch.

Univariate one way random effects analyses of variance will be carried out on the results of the fits. The responses will be t, b, μ, β, std err μ, std err β. The standard errors are the within batch standard errors. Test batch will be treated as a random effect. Batch-to-batch variation will be determined for each of the parameters and will be tested for significance. It is anticipated that the batch-to-batch variation will be specific to the assay. If the batch-to-batch variation is significant it will be incorporated into the standard error of the corresponding parameter and associated confidence intervals.

5.2.4 Concentration Response Curve Fits to the Test Substances

Concentration response curves will be fitted to the results from each test substance within each batch. Plots will be prepared displaying the individual percent aromatization determinations and the concentration response curve fits. The form of the model is the same as for the standard tests, namely

$$Y = b + \frac{t - b}{1 + 10^{\beta(x-\mu)}}$$

where t and b are "top" and the "bottom" of the curve (approximately 100% and 0% respectively), β is the slope, and μ is the $\log_{10}$ (IC_{50}). The "top" and "bottom" parameters t and b

will be constrained to correspond to those of the standard curve within the same run, resulting in a two parameter model fit.

The model will be fitted by weighted least squares. The weights are inversely proportional to the predicted DPM count. Namely,

$$DPM\ pred = NSB\ DPM + (\frac{\hat{Y}}{100})[SPC\ DPM]$$

where NSB DPM is the "nonspecific DPM" associated with the NSB concentration of standard substance and SPC DPM is the "specific DPM", i.e. vehicle DPM – NSB DPM.

$$Weight = \frac{K}{DPM\ pred}$$

Based on the results of the fit within each run the aromatase inhibition activity will be expressed as the IC_{50} (concentration corresponding to 50 percent inhibition, μ).

The concentration response relation for each test substance will be fitted within four independent test batch. Since the replicate determinations within a test run involve only replication of scintillation counter readings their differences do not reflect all of the within run variation. Thus, the model will be fitted to the average of the replicate determinations at each concentration.

Univariate one way random effects analyses of variance will be carried out on $\log_{10}(IC_{50})$ and their associated within batch standard errors. Test batches will be treated as a random effect. Batch-to-batch variation will be determined for each of the parameters and will be tested for significance. It is anticipated that the batch-to-batch variation will be specific to the test substance and the assay. If the batch-to-batch variation is significant it will be incorporated into the standard error of the corresponding parameter and associated confidence intervals.

5.2.5 Comparison of Optimized Assays

For each test compound $\log_{10}(IC_{50})$ and its associated standard error, degrees of freedom, and confidence interval will be determined with each assay. The estimates and their standard errors will be compared among assays by two-sample heterogeneous variances t-tests, adjusting for simultaneous inferences. Consistent patterns across test substances of inequalities among assays will be examined.

5.2.6 Comparisons Based on Reduced Numbers of Test Concentrations

The comparisons among assays discussed above were based on IC_{50} determinations obtained with a range of eight concentrations from 10^{-9} M to 10^{-3} M. These comparisons will be

repeated with a subset of the data, with a range of three concentrations at 0.1, 1, and 10 μM (10^{-7}M to 10^{-5}M). Estimates of $\log_{10}(IC_{50})$ within each test run will be carried out as if the available data were limited to:

- test concentrations from 10^{-7}M to 10^{-5} M for each test substance
- test concentrations from 10^{-7}M to 10^{-5} M for the standard substrate
- NSB concentration of 10^{-3} M for the standard substrate
- the vehicles.

The determinations of the IC_{50}s and their standard errors and confidence intervals for each test substance will be repeated in the manner discussed above, but based only on the available data. Comparisons among assays will be carried out in the same manner as discussed above.

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