FINAL REPORT

Pre-Validation of the Aromatase Assay Using Human, Bovine, and Porcine Placental Microsomes and Human Recombinant Microsomes

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Study Initiation Date:

November 14, 2002

Experimental Dates:

December 30, 2002 - December 19, 2003

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FINAL REPORT

Pre-validation of the Aromatase Assay Using Human, Bovine, and Porcine Placental Microsomes and Human Recombinant Microsomes

1.0 ABSTRACT

Human, porcine and bovine placentas were obtained and microsomes were prepared. Human recombinant microsomes with aromatase activity were purchased from a commercial supplier. Each microsomal preparation was assayed for cytochrome P450 (P450) and protein content and aromatase (CYP19) activity. Concerns regarding the difficulty of ensuring a suitable supply of porcine and bovine tissue within a close proximity of testing laboratories coupled with the difficulty experienced in processing these tissues into microsomes with sufficient aromatase activity led to the decision by the Endocrine Disruptor Method Validation Subcommittee (EDMVS) to abandon further development of the aromatase assay using those tissues.

The aromatase assay was optimized for both the human placental and human recombinant microsomes. The determined optimal conditions were: 100 nM androstenedione, 0.3 mM NADPH, and a 15 min incubation time with 0.004 and 0.0125 mg/mL of recombinant and placental microsomal protein, respectively.

The optimized assay was tested for its ability to identify aromatase inhibitors and non-inhibitors. The assay performed as expected and correctly identified known aromatase inhibitors and non-inhibitors. Experimentally determined IC_{50} values were in the range of the literature values in most cases. The placental and recombinant assays yielded similar, but in some cases statistically significantly different, IC_{50} values for each test substance. The two microsomal preparations showed similar sensitivity to the inhibitory effects of the test substances.

2.0 OBJECTIVE

The objective of the pre-validation protocol for the aromatase assay was to design experiments that would identify the optimal factors and conditions for the assay. Preoptimization experiments were conducted with human, bovine and porcine placental microsomes and human recombinant microsomes. The assay was optimized and the response to inhibition of this activity by various test substances was assessed in two preparations, i.e., human placental microsomes and human recombinant microsomes.

Three sets of experiments were conducted to achieve the objectives:

- Pre-Optimization Experiments
- Assay Optimization Experiments using a Factorial Design
- Optimized Assay Experiments using Selected Test Substances.

The basis for each of these experiments is explained and described in the following paragraphs.

2.1 Pre-Optimization Experiments

The pre-optimization experiments were designed to assess the chemical and biological properties of the critical components that are used in the aromatase assay. These experiments included characterizing the radiolabeled substrate and preparation of the placental microsomes. Placental tissue was obtained from three species (human, porcine and bovine) and microsomes were prepared. Human recombinant CYP19 (expressed in baculovirus infected insect cells) microsomes were purchased from a commercial source. In addition, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and the human recombinant microsomes) were analyzed for protein concentration, cytochrome P450 content, and aromatase activity. The P450 content measurement was used to assure that the enzyme was present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. Finally, a single aromatase activity assay run using each type of microsomal preparation was included as a pre-optimization experiment in order to determine whether the preparations were of sufficient activity to conduct the optimization experiments. These experiments are described in Sections 3.1-3.5.

2.2 Assay Optimization Experiments Using a Factorial Design

The assay optimization experiments were designed to (1) identify the combination(s) of experimental factors and conditions that would maximize the rate of the aromatase reaction in each of the microsomal preparations, and (2) after the optimal factor levels and conditions were determined, the optimized assays were used to assess variability of the results. These experiments are described in Section 3.6.

The experimental factors and conditions to be optimized in the assay included the incubation time and concentrations of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, androstenedione (substrate) and protein (microsomal preparation). Each of these factors was tested at five different levels using a factorial experimental design. The factorial experimental design consists of four parts. Part 1, 26-1 factorial runs, permitted an estimation of all linear main effects and linear by linear interactions to be determined. Part 2, center point run, provided 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, provided an estimate of the quadratic main effects for each of the factors. Part 4, replicate runs, provided an estimate of the reproducibility of the response at the center of the design space and at various extremes of the design space.

Finally, after optimal factors and conditions had been determined, the optimized assay using each type of microsomal preparation was used to assess variability. Each assay was conducted by different technicians and on different days. These results permit estimation of the technician-to-technician and the within technician variability.

2.3 Optimized Assay Experiments Using Selected Test Substances

This set of experiments was designed to use the optimized assays to evaluate the response(s) of the microsomal preparations to detect an effect of different test substances. Test substances were selected to represent a wide range of inhibitory potency and mechanisms and sites of action. In addition, some test substances were selected that do not inhibit aromatase but do have endocrine disruptor activity. These test substances were selected in order to assess the specificity of the aromatase assay.

The test substances and their basis for being tested are:

- ♦ aminoglutethimide (non-steroidal aromatase inhibitor)
- ♦ 4-hydroxyandrostenedione (4-OH ASDN, potent steroidal aromatase inhibitor),
- ♦ chrysin (potent flavonoid)
- ♦ genistein (weak isoflavonoid)

- ketoconazole (weak imidazole anti-fungal)
- econazole (potent imidazole anti-fungal)
- atrazine (not an inhibitor, site of action is on aromatase gene expression)
- bis-(2-ethylhexyl)phthlate (not an inhibitor, site of action is on aromatase gene expression)
- nonylphenol (not an inhibitor, site of action is on ER/AR [Estrogen Receptor/Androgen Receptor])
- lindane (not an inhibitor, site of action is on StAR [Steroidogenic acute regulatory protein] and cholesterol metabolism)
- dibenz[*a,h*]anthracene (not a known inhibitor).

The optimized assays were used to determine IC_{50} (concentration at which there is 50% inhibition of the enzymatic reaction) values for those test substances for which an IC_{50} value exists in each of the microsomal preparations. For those test substances for which an IC_{50} value may not exist (i.e., non-inhibitors) the inhibition response curve was characterized across the range of test substance concentrations on a case-by-case basis. In addition, since each test substance was tested over a wide concentration range (10^{-9} to 10^{-3} M), the results were evaluated to determine what the IC_{50} might have been if only three concentrations of the test substance were tested. The results of this analysis were used to assess whether the assay could be conducted with more efficiency and less effort. These experiments are detailed in Section 3.7.

3.0 MATERIALS AND METHODS

3.1 Substrate Characterization

3.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione (ASDN). Non-radiolabeled and radiolabeled androstenedione were used. The non-radiolabeled androstenedione (Lot # 072K1134) was obtained from Sigma (St. Louis, MO) with a reported 99 % purity. The radiolabeled androstenedione, [1β-^{3}H]-androstenedione ([^{3}H]ASDN), was obtained from Perkin Elmer Life Science (Boston, MA). Two different lots of [^{3}H]ASDN were used in the experiments. Lot # 3467835 was used in all preoptimization and optimization experiments. Lot # 3474115 was used in the test substance phase of the study. Both lots of [^{3}H]ASDN had a reported specific activity of 25.3 Ci/mmol. The radiochemical purity and specific activity of the [^{3}H]ASDN were assessed as described below.

3.1.2 Radiochemical Purity

The radiochemical purity of the [^{3}H]ASDN was determined using high performance liquid chromatography (HPLC) and liquid scintillation counting. The HPLC system consisted 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 SB-C18 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 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). A reference standard of nonradiolabeled ASDN was analyzed by the same method and coelution of the nonradiolabeled and radiolabeled ASDN was confirmed.

3.1.3 Specific Activity Determination

Samples of known concentration (ca 0.5 to 4 or 22 ng ASDN/20 µL) of nonradiolabeled ASDN were analyzed by HPLC in duplicate and a standard curve containing 6-8 points was prepared relating peak height to ASDN concentration. Samples of [^{3}H]ASDN were analyzed by the same HPLC method and the fractions were collected and analyzed by LSS. The specific activity of the [^{3}H]ASDN was determined by the relationship between the height of the ASDN peak and the amount of radioactivity contained in the peak. Two lots of [^{3}H]ASDN were used on this study and the specific activity of each was determined.

3.2 Microsome Preparation

3.2.1 Human, Bovine, and Porcine Placentas

3.2.1.1 Source of the Placentas. Details of placenta procurement are included in Appendices 1-3. Briefly, human placenta was obtained from a local hospital and porcine and bovine placentas were obtained from North Carolina State University (NCSU) Field Laboratories.

3.2.1.2 Placental Microsome Preparation. Details of microsome preparation are contained in Appendices 1-3.

3.2.2 Human Recombinant Microsomes

3.2.2.1 Source of the Human Recombinant Microsomes. Human recombinant microsomes (Human CYP19 [Aromatase] Supersomes™) were obtained from Gentest™ (Woburn, MA; www.gentest.com). Two batches of microsomes were purchased. The first, Lot # 2, was used in the preoptimization phase of the study. Those results are presented in Appendix 1. The second batch, Lot #3, was used in all of the optimization experiments and also in the test substance phase of the study. The data sheet for Lot #3 is presented as Figure 1.

3.2.2.2 Human Recombinant Microsome Preparation. Preparation of the human recombinant microsomal preparation involved thawing the microsomes rapidly in a 37 ± 1°C water bath and then keeping them on ice until used. Microsomes were rehomogenized using a Potter Elvejhem homogenizer prior to use. Any remaining microsomes were flash frozen in liquid nitrogen and stored at -70 °C.

3.3 Protein Determination

The protein concentration of the human, bovine, and porcine placental microsome preparations, as well as the human recombinant microsomal preparations, was determined for each batch of microsomes prepared, and as necessary to determine the protein concentration of the microsomal preparations. 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 Bio-Rad (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. The absorbances are stable for about 1 h. Each sample (unknown and standards) was transferred to disposable polystyrene cuvettes and the absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed using the protein standards.

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Human CYP19 + P450 Reductase SUPERSOMES™

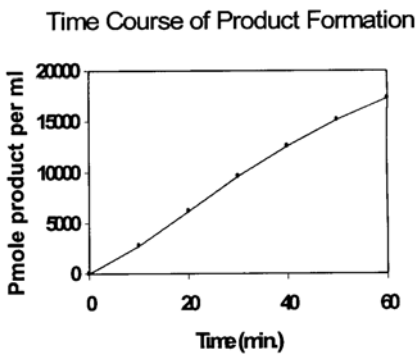
New Catalog Number....456260
Old Catalog Number.....P260
Lot Number.....3

Storage Conditions..STORE AT -80°C
Date Released .....2003 February
Best Used by.....2006 February

Package Contents.....0.5 nmole cytochrome P450 in 0.5 ml
Protein Content.....5.4 mg/ml in 100mM potassium phosphate (pH 7.4)
Cytochrome c Reductase Activity.....340 nmole/(min x mg protein)
Cytochrome P450 Content.....1000 pmol per ml
Aromatase Activity.....5.5 pmol product/(min x pmol P450)

This activity is catalyzed by human CYP19 which is expressed from human CYP19 cDNA using a baculovirus expression system. Baculovirus infected insect cells (BTI-TN-5B1-4) were used to prepare these microsomes. These microsomes also contain cDNA-expressed human P450 reductase. A microsome preparation using wild type virus (GENTEST Catalog No. P200 or P201) should be used as a control for native activities.

METHOD: A 0.25 ml reaction mixture containing 25 pmole P450, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.05 mM testosterone in 100 mM potassium phosphate (pH 7.4) was incubated at 37°C for 20 min. After incubation, the reaction was stopped by the addition of 125 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. 50 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and eluted isocratically at 45°C with a mobile phase of 60% water and 40% acetonitrile and at a flow rate of 1.5 ml per min. The product was detected by its absorbance at 200 nm and quantitated by comparing the absorbance to a standard curve of (beta)-estradiol.



ADVICE

- Thaw rapidly in a 37°C water bath. Keep on ice until use
- Aliquot to minimize freeze-thawing cycles. Less than 20% of the catalytic activity is lost after 6 freeze thaw cycles.
- Metabolite production is linear with respect to enzyme concentration up to at least 50 pmol P450 per ml.
- Metabolite production with testosterone is approximately linear for 40 minutes (see graph above).

THIS PRODUCT IS SUPPLIED FOR LABORATORY RESEARCH USE ONLY.

Figure 1. Human Recombinant CYP19 Microsomes Data Sheet

3.4 Cytochrome P450 Content

Cytochrome P450 content was determined during the pre-optimization phase in order to demonstrate that the human, bovine, and porcine placental microsome preparations, as well as the human recombinant microsomal preparation had cytochrome P450 present. Using the Carbon Monoxide (CO) spectrum assay of Omura and Sato (1964), each of the preparations was assayed as described below.

A sample of each microsomal preparation was diluted 1:20 in 0.1 M phosphate buffer (pH 7.4). The diluted sample was gently bubbled with carbon monoxide for approximately 15-20 s and then was divided between a pair of matched cuvettes. Next, a few grains of solid sodium dithionite was added to the sample cuvette with gentle mixing. The visible spectrum was then recorded from 400 to 500 nm using a split-beam spectrophotometer.

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

3.5 Cytochrome P450 Aromatase (CYP19) Activity

Aromatase activity was determined for the human, porcine and bovine placental microsome preparations and the human recombinant CYP19. A single experiment was conducted using only the substrate ([^{3}H]ASDN/ASDN) with each of the microsomal preparations. The assay was conducted as described in the following paragraph.

The [^{3}H]ASDN/ASDN substrate solution was prepared by combining solutions of [^{3}H]ASDN and ASDN. A 1 mg/mL solution of ASDN was prepared in ethanol. Serial dilutions of this solution were prepared in assay buffer to yield a solution containing ca. 1 µg ASDN/mL. The [^{3}H]ASDN stock was diluted 1:100 in assay buffer to yield a solution containing ca. 10 µCi/mL. The substrate solution was prepared by combining 275 µL of the 1 µg ASDN/mL solution, 100 µL of the 10 µCi [^{3}H]ASDN/mL solution and 625 µL buffer.

The assays were performed in 13x100 mm test tubes (two for each microsomal preparation) maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol was added to the tubes to serve as a co-solvent. The substrate, [^{3}H]ASDN (0.1 µCi, 50 nM), was added to the tubes. An NADPH-generating system comprised of NADP+ (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 unit) was added to each tube. The tubes were placed at 37 ± 1°C in the water bath for 5 min prior to initiation of the assay by the addition of the diluted microsomal suspension (~0.1 mg microsomal protein/mL).

The total volume was 2.0 mL, and the tubes were incubated for 30 min. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for about 5 s and placed on ice, then each tube was vortexed for about 20-25 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 min at a setting of 1000 rpm (which is approximately equivalent to 230*g*). The methylene chloride layer was removed to a vial and weighed; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time reserving and weighing the methylene chloride layer in a separate vial. The aqueous layers were transferred to vials, weighed, and duplicate aliquots (0.5 mL) were weighed into 20-mL liquid scintillation counting vials. Duplicate aliquots of each methylene chloride fraction were weighed into scintillation 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 the substrate solution was determined by analyzing 5 weighed aliquots by LSS. The substrate solution specific activity was determined by dividing the radiochemical content of the substrate solution (dpm/g) by the total concentration of ASDN in the solution (ASDN + [^{3}H]ASDN; nmol/g solution).

Analysis of the samples was performed using LSS. Radiolabel found in the aqueous fractions represents ^3H_2O formed, and that in the methylene chloride fractions represents unreacted substrate.

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 solution (expressed in dpm/nmol). The activity of the enzyme reaction was expressed in nmol (mg protein)$^{-1}$ min^{-1} and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 30 min.

3.6 Optimization of Experimental Design Factors and Conditions

3.6.1 Factorial Design Experiment

A factorial design experiment designed to determine optimal assay conditions was conducted for both the human placental and recombinant microsomes.

The original experimental design tested six different factors and five different levels of each factor. The experimental factors that were tested and the levels for each factor are summarized below in Table 1.

Table 1. Summary of Experimental Factors and Levels to be Optimized[a]

Experimental Factors	Units	Experimental Factor Levels				
		1	2	3	4	5
NADP+ (conc)	mM	0.1	0.5	1	2	4
Glucose-6-Phosphate (conc)	mM	0.1	1	2	3	4
Glucose-6-Phosphate Dehydrogenase (conc)	units	0.1	0.5	1	2	4
Androstenedione (substrate, conc)	nM	10	25	50	100	500
Protein (conc)	mg/mL	0.01	0.02	0.1	0.5	1
Incubation Time	min	10	15	30	60	120

[a] Was performed for each of the human placenta and human recombinant microsomes.

The assay was performed in the required number of 13x100 mm test tubes. An aliquot (100 µL) of propylene glycol was added to the tubes to serve as a co-solvent. The substrate, [^{3}H]ASDN (see Table 2 for concentration), was added to the tubes. An NADPH-generating system comprised of NADP+ (see Table 2), glucose-6-phosphate (see Table 2) and glucose-6-phosphate dehydrogenase (see Table 2) was added to each tube. All concentrations in Table 2 are final concentrations in the complete assay (2 mL volume).

The tubes were placed at 37 ± 1°C in the water bath for five minutes prior to the start of the assay by the addition of the diluted microsomal suspension (see Table 2). The total volume was 2.0 mL, and the tubes were incubated for the time described in Table 2. The incubations were stopped by the addition of methylene chloride (2.0 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 minutes at a setting of 1000 rpm. The methylene chloride layer was removed to a vial and weighed; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time reserving and weighing the methylene chloride layer in a separate vial. The aqueous layers were transferred to vials, weighed, and duplicate aliquots (0.5 mL) were weighed into 20-mL liquid scintillation counting vials. Duplicate aliquots of each methylene chloride fraction were weighed into scintillation vials. Methylene chloride samples were reserved and analyzed only for the first day of experimentation using the human placental microsomes. The results from that experiment showed good overall recovery of radiolabel and complete extraction of parent [^{3}H]ASDN over the course of the three methylene chloride extractions, so on all other experimental days the CH_2Cl_2 extracts were discarded. 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 as described above (see Section 3.5).

The 62 test runs (as described in Table 2) per microsomal preparation were conducted over the course of 3 days.

The aromatase activity for each run was calculated from the radioactivity in the aliquots, the protein concentration (average of the values determined over three experimental days) and the time of incubation using a validated Excel 97 spreadsheet. Statistical analysis of the aromatase activities under the various conditions was performed as described in Section 3.6.2.

Table 2. Factorial Design Experiments for Assay Optimization

Run No.	NADP Conc.	Glu-6-P Conc.	Glu-6-P D Conc	Substrate Conc.	Protein Conc.	Incub. Time
Part 1- Factorial Runs (32 Total Runs)						
1	0.5 mM	1 mM	0.5 units	25 nM	0.02 mg/mL	15 min
2				100 nM	0.5 mg/mL	
3			2 units	25 nM	0.5 mg/mL	
4				100 nM	0.02 mg/mL	
5		3 mM	0.5 units	25 nM	0.5 mg/mL	
6				100 nM	0.02 mg/mL	
7			2 units	25 nM		
8				100 nM	0.5 mg/mL	
9	2 mM	1 mM	0.5 units	25 nM		
10				100 nM	0.02 mg/mL	
11			2 units	25 nM		
12				100 nM	0.5 mg/mL	
13		3 mM	0.5 units	25 nM	0.02 mg/mL	
14				100 nM	0.5 mg/mL	
15			2 units	25 nM		
16				100 nM	0.02 mg/mL	
17	0.5 mM	1 mM	0.5 units	25 nM	0.5 mg/mL	60 min
18				100 nM	0.02 mg/mL	
19			2 units	25 nM	0.02 mg/mL	
20				100 nM	0.5 mg/mL	
21		3 mM	0.5 units	25 nM	0.02 mg/mL	
22				100 nM	0.5 mg/mL	
23			2 units	25 nM	0.5 mg/mL	
24				100 nM	0.02 mg/mL	
25	2 mM	1 mM	0.5 units	25 nM	0.02 mg/mL	
26				100 nM	0.5 mg/mL	

(continued)

Table 2. Factorial Design Experiments for Assay Optimization (continued)

Run No.	NADP Conc.	Glu-6-P Conc.	Glu-6-P D Conc	Substrate Conc.	Protein Conc.	Incub. Time
27	2 mM	1 mM	2 units	25 nM	0.5 mg/mL	60 min
28				100 nM	0.02 mg/mL	
29		3 mM	0.5 units	25 nM	0.5 mg/mL	
30				100 nM	0.02 mg/mL	
31			2 units	25 nM	0.02 mg/mL	
32				100 nM	0.5 mg/mL	
Part 2 – Center Point Run (1 Total Run)						
1	1 mM	2 mM	1 units	50 nM	0.1 mg/mL	30 min
Part 3 – Axial Point Runs (12 Total Runs)						
1	0.1 mM	2 mM	1 units	50 nM	0.1 mg/mL	30 min
2	4 mM					
3	1 mM	0.1 mM				
4		4 mM				
5		2 mM	0.1 units			
6			4 units			
7			1 units	10 nM		
8				500 nM		
9				50 nM	0.01 mg/mL	
10					1 mg/mL	
11					0.1 mg/mL	10 min
12						120 min
Part 4 - Replicate Runs (17 Total Runs)						
1	1 mM	2 mM	1 units	50 nM	0.1 mg/mL	30 min
2						
3						
4						
5						
6	0.1 mM					
7	4 mM					
8	1 mM	0.1 mM				
9		4 mM				
10		2 mM	0.1 units			
11			4 units			
12			1 units	10 nM		
13				500 nM		
14				50 nM	0.01 mg/mL	
15					1 mg/mL	
16					0.1 mg/mL	10 min
17						120 min

3.6.2 Analysis of Results

These experiments tested the effect(s) of six factors: NADP+, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, androstenedione, protein and incubation time. The factor settings used in this experiment are presented in Table 3.

Table 3. Experimental Factor Settings and Coded Values

Factor Identification	Units	Variable Name	Experimental Levels					Coded Experimental Levels				
			1	2	3	4	5	1	2	3	4	5
NADP+ (conc)	mM	NADP	0.1	0.5	1	2	4	-3.32	-1	0	+1	+2
Glucose-6-Phosphate (conc)	mM	G6P	0.1	1	2	3	4	-1.90	-1	0	+1	+2
Glucose-6-Phosphate Dehydrogenase (conc)	units	G6PDeH	0.1	0.5	1	2	4	-3.32	-1	0	+1	+2
Androstenedione (substrate) (conc)	nM	ASDN	10	25	50	100	500	-2.32	-1	0	+1	+3.32
Protein (conc)	mg/mL	Protein	0.01	0.02	0.1	0.5	1	-1.43	-1	0	+1	+1.43
Incubation Time	min	Inc time	10	15	30	60	120	-1.58	-1	0	+1	+2

3.6.2.1 Data. A SAS data set was constructed from the Excel data file and two fundamental types of dependent variables were used in the analyses: the original aromatase activities and the (natural) logarithm of the aromatase activities. Each observation included data identifying the levels of the pertinent factors.

3.6.2.2 Statistical Analysis Methods

3.6.2.2.1 <u>Human Placental and Human Recombinant Microsome Assay</u>. In order to assess the effects of each experimental factor on the aromatase activity, analysis of variance (ANOVA) models were fit to the data. All main effects and two-factor interactions (2fi) of the six factors were initially included in the models, along with day 2 and day 3 main effects and 2fi's to assess the effect of day to day variation. Tests for interactions were conducted and where they were not detected as statistically significant (p=0.10), a reduced model was employed that retained the main effects and only those 2fi's deemed to have significant effects. Once the final ANOVA model was determined, the data were fit using a response surface regression analysis to determine the maximum predicted value of aromatase activity and the experimental factor levels associated with the maximum. Additional details are provided in the Results section.

3.6.3 Additional Optimization Experiments

Additional experiments were conducted to ensure that the optimal conditions selected were in the linear range of the assay for protein concentration and time and would maintain initial rate conditions (with preferably no more than 10-15% of substrate consumed).

3.6.3.1 General Assay Method. Tubes containing buffer, substrate, propylene glycol, co-factor(s) and test substances (if applicable) in a total volume of 1 mL were placed at 37 ± 1°C in the water bath for five minutes prior to the start of the assay by the addition of the diluted microsomal suspension (1 mL). The total volume was 2.0 mL, and the tubes were incubated for the required time. The incubations were stopped by the addition of methylene chloride (2.0 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 minutes at a setting of 1000 rpm. The methylene chloride layer was removed and discarded; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time discarding the methylene chloride layer. The aqueous layers were transferred to vials and duplicate aliquots (0.5 mL) were transferred to 20-mL liquid scintillation counting vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) was added to each counting vial and shaken to mix the solution. The radiochemical content of each aliquot was determined as described above (see Section 3.5).

3.6.3.2 Aromatase Optimization Supplemental Studies; Experiment 1: Protocol Standardization. Experiments were conducted to determine the effect of protein concentration on aromatase activity in the human placental microsome assay. Three sets of experiments were conducted: Set 1, Set 2 and Set 3.

Set 1: The aromatase assay was conducted using human placental microsomes at final protein concentrations of 0.0125, 0.025 and 0.05 mg/mL. The assay mixtures also contained (in final concentrations, unless otherwise noted): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN, and an NADPH regenerating system consisting of 1.7 mM NADP, 2.8 mM glucose-6-phosphate and 1 unit glucose-6-phosphate dehydrogenase. The following reaction conditions were conducted in triplicate: Blank (boiled microsomes, substrate, NADPH regenerating system minus NADP), No NADP (microsomes, substrate, NADPH regenerating system minus NADP), Total Activity (complete system- microsomes, substrate, NADPH regenerating system), Inhibition (complete system plus 0.1 µM 4-OH-ASDN). The general assay method was as described in Section 3.6.3.1 above. Tubes were incubated for 15 min at 37 °C and then the reaction was stopped and extracted as described above.

The term "boiled microsomes" is defined as follows: microsomes that have been heated in a boiling water bath for 10 min. Boiled microsomes were chilled on ice prior to being reheated in a 37 ± 1 °C water bath before their inclusion in the reaction mixtures.

Set 2: This experiment was designed to investigate the source of high background activities that were sometimes seen in the "No NADP" tubes Set 1. The assay was conducted in triplicate at each of the protein concentrations defined above; other components were used at the concentrations described above unless otherwise noted. Three experimental conditions were investigated: Total Activity (complete system), No NADP (microsomes, substrate, NADPH regenerating system minus NADP) and No NADPH regenerating system (microsomes, substrate, no NADPH regenerating system). The general assay method was as described above.

Set 3: In this experiment the aromatase assay was conducted in triplicate at each of the protein concentrations defined above; other components were used at the concentrations described above unless otherwise noted. The following experimental conditions were used: Blank (boiled microsomes, substrate, NADPH regenerating system minus NADP), No NADP (microsomes, substrate, NADPH regenerating system minus NADP), Total Activity (complete system- microsomes, substrate, NADPH regenerating system), Inhibition (complete system plus 0.1 µM 4-OH-ASDN), No NADPH regenerating system (microsomes, substrate, no NADPH regenerating system) and NADPH (microsomes, substrate, 0.3 mM NADPH [instead of the regenerating system]). The general assay method was as described above.

3.6.3.3 Aromatase Optimization Supplemental Studies; Experiments 2 and 4: Linear Production of Product. These experiments were designed to demonstrate whether production of product was linear over time (to 30 min) and also to determine whether NADPH was limiting during the assay reaction time. Using the general assay method described in Section 3.6.3.1 above, the assay was conducted with 5 different incubation times (5, 10, 15, 20, 30 min). Each tube contained (final concentrations in a 2 mL reaction mixture): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN (ca. 0.1 µCi), 0.3 mM NADPH, microsomal protein (0.0125 mg/mL from human placenta [Exp. 2] or 0.005 mg/mL of recombinant [Exp. 4]) in a 0.1 M sodium phosphate buffer, pH 7.4, unless otherwise specified in the conditions below.

For each time point, the following conditions were used with all tubes in triplicate. The number of assay tubes was doubled (Total activity and Inhibition sets only) for the 30 min time point to use for NADPH experiment as outlined below. The number of assay tubes for the 15 min time point was doubled (No NADPH, Total Activity and Inhibition sets, only - 9 tubes) for Estrone/Estradiol Concentration analysis.

- Blank: (boiled microsomes, substrate but no NADPH)
- No NADPH: (microsomes, substrate but no NADPH)
- Total Activity: (microsomes, substrate, 0.3 mM NADPH)
- Inhibition: (microsomes, substrate, 0.3 mM NADPH, 0.1 µM 4-OH-androstenedione) The 4-OH androstenedione was added to the reaction mixture before the protein (enzyme) was added.

NADPH experiment: The second set of 30 min assay tubes (Set consisted of Total activity and Inhibition tubes, only), the NADPH concentration was supplemented by adding another aliquot of NADPH (0.3 mM final concentration) at the 15 min time point. The reaction was allowed to proceed another 15 min (e.g., a total reaction time of 30 min but supplemented at 15 min with additional NADPH).

Estrone/Estradiol Concentration: The extra set of No NADPH, Total Activity and Incubation tubes (15 min set) were frozen immediately after incubation and were assayed for assayed for estrone/estradiol in the laboratory of Dr. Susan Laws (U.S. EPA, RTP, NC). Estradiol was measured using RIA kits from Diagnostic Products, Inc and kits from Diagnostic Systems, Inc. were used to measure estrone.

3.6.3.4 Aromatase Optimization Supplemental Studies; Experiments 3 and 5: Inhibition Curve Using 4-OH ASDN. These experiments were designed to demonstrate the specificity of the assay through the generation of a competitive inhibition curve using 4-OH ASDN.

Using the general assay method described in Section 3.6.3.1 above, the assay was conducted with a 15 min incubation time. Inhibition curve samples contained 4-OH-ASDN at 6 concentrations spanning the range 5×10^{-6} to 1×10^{-9} M. Each tube contained (final concentrations in a 2 mL reaction mixture): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN (ca. 0.1 µCi), 0.3 mM NADPH, microsomal protein (0.0125 mg/mL from human placenta [Exp 3] or 0.004 mg/mL of recombinant [Exp 5]) in a 0.1 M sodium phosphate buffer, pH 7.4, unless otherwise specified in the conditions below.

The assay was run in triplicate for all conditions.

- Blank: (boiled microsomes, substrate but no NADPH)
- No NADPH: (microsomes, substrate but no NADPH)
- Total Activity: (microsomes, substrate, 0.3 mM NADPH)

- Inhibition: (microsomes, substrate, 0.3 mM NADPH, 5×10^{-6} to 1×10^{-9} M 4-OH-ASDN)

The 4-OH ASDN was added to the reaction mixture before the protein (enzyme) was added.

3.6.4 Variability Determination Using the Optimized Assay

After optimum conditions had been determined for the human placental microsomes and the human recombinant microsomes, each assay was conducted using the optimized factors to assess the variability of the results. Each assay was conducted independently by three technicians and at three separated times per technician. Triplicate runs were conducted by each technician on each day for a given microsomal preparation. The assays were run independently of one another. To better define the source of any technician to technician variances within a given day, one of the three technicians also ran the assay using the diluted microsome samples prepared by the other two technicians. The assays were conducted and samples were analyzed as described above in Section 3.6.3.1, except that optimized factor levels and conditions were used. Each assay used 100 nM [^{3}H]ASDN, 0.3 mM NADPH and a 15 min incubation time. Human placental protein final concentration was about 0.0125 mg/mL while the final protein concentration for the recombinant assay was 0.004 mg/mL.

3.7 Determination of the Response of the Optimized Assay to Selected Test Substances

This set of experiments was designed to use the optimized assays to evaluate the response(s) of the human placental microsomes and the human recombinant microsomes, to detect an effect of different test substances. Test substances were selected to represent a wide range of inhibitory potency and mechanisms and sites of action. In addition, some test substances were selected that do not inhibit aromatase but do have endocrine disruptor activity.

3.7.1 Experimental Design

The factor levels and conditions determined as optimal (based on the optimization experiments) were used to determine the effect of various test substances on aromatase activity. Each of 11 test substances, at up to 8 different concentrations (ranging from no less than 10^{-9} M to no higher than 10^{-3} M),were tested using each of the two microsomal preparations for which optimal conditions were determined, i.e. human placental microsomes and human recombinant microsomes. The vehicle controls were ethanol and DMSO, depending on the vehicle(s) used to

formulate the test substances. The aromatase activity in the absence of any test substance, was used as the benchmark (100 percent) activity.

3.7.1.1 Assay Method. The aromatase assay was performed as described below and as shown in the flow diagram (Figure 2). The assay was performed in the required number of 13x100 mm test tubes. An aliquot (100 µL) of propylene glycol was added to the tubes to serve as a co-solvent. Final protein, NADPH and substrate concentrations and incubation times are presented in Table 4. The substrate, buffer, test substance or vehicle and NADPH (where appropriate) were combined in the tubes. The tubes were placed in a 37 ± 1°C water bath for five minutes prior to the start of the assay by the addition of the diluted microsomal suspension. The total volume was 2.0 mL. The incubations were stopped after 15 min of incubation by the addition of methylene chloride (2.0 mL) vortex-mixing for 5 s and placing on ice. Each tube was vortex-mixed an additional 20-25 s to facilitate the extraction. The tubes were then centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The samples were extracted and assayed for radiochemical content as described in Section 3.6.3.1 above.

Table 4. Optimized Assay Conditions for Aromatase

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

Test substances were placed into three testing groups according to their solubility and expected inhibition character. Test substance solutions were prepared such that the appropriate final concentration could be achieved by the addition of 20 µL of the solution to the assay, thereby limiting the volume of solvent added to each tube to 1% of the total volume. Test substance group assignments, solvents and tested concentrations are presented in Table 5. On each day of assay, three or four test substances (from a single group) were assayed in singlet at each defined concentration. In addition, on each day of assay, a positive control (complete assay including 20 µL of vehicle, no test substance) and a negative control (assay including 20 µL of test substance vehicle but with no NADPH) quadruplicate set was run. Because Group 3 contained test substances with two different vehicles, the control sets were doubled (one set for each of the two solvents) during Group 3 testing. Each test substance group was assayed on four separate days for each of the human placental and human recombinant assays. All runs for a given microsomal preparation were conducted by a single technician.

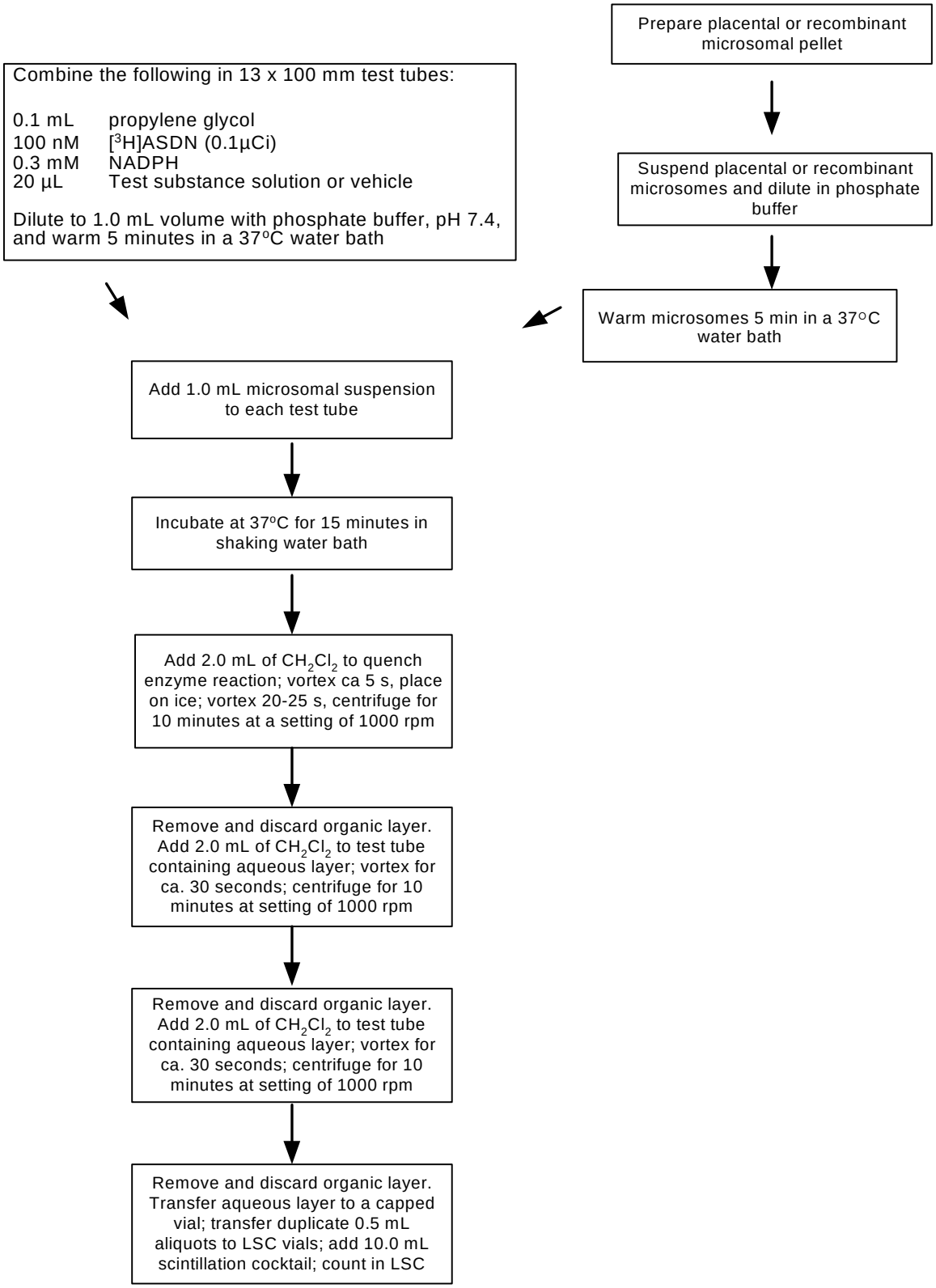


Figure 2. Aromatase Assay Flow Diagram

Table 5. Test Substance Groupings, Target Concentrations and Solvents

Test Group	Test Substance	Levels	Target Concentrations (M)	Solvent
1	econazole	8	10^{-6}; 10^{-7}; 2.5 and 5 x 10^{-7}; 10^{-8}; 2.5 and 5 x 10^{-8}; 10^{-9}	DMSO
	genistein	8	10^{-3}; 10^{-4}; 2.5 and 5 x 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}	DMSO
	atrazine	7	10^{-3} to 10^{-9}	DMSO
	bis-(2-ethylhexyl)phthlate	7	10^{-3} to 10^{-9}	DMSO
2	aminoglutethimide	8	10^{-3}; 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}; 10^{-7}; 10^{-8}	DMSO
	chrysin	8	10^{-3}; 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}; 10^{-7}; 10^{-8}	DMSO
	nonylphenol	7	10^{-3} to 10^{-9}	DMSO
	lindane	7	10^{-3} to 10^{-9}	DMSO
3	4-OH ASDN	8	10^{-6}; 10^{-7}; 2.5 and 5 x 10^{-7}; 10^{-8}; 2.5 and 5 x 10^{-8}; 10^{-9}	EtOH
	ketoconazole	8	0.8 x 10^{-3}; 10^{-4}; 2.5 and 5 x 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}	EtOH
	dibenz[*a,h*]anthracene	6	10^{-4} to 10^{-9}	DMSO

3.7.1.2 Calculation of IC_{50} and Slopes. The aromatase activities in the presence of inhibitors were converted to percent of control activities. These percent of control activities and their respective inhibitor concentrations were fitted to a non-linear regression equation using Prism (Version 3.02) software.

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

where:

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

Prism output includes estimated IC_{50} and slopes. Where the regression fit did not converge or where the greatest inhibition was no greater than 50% of control, no IC_{50} could be calculated. Statistical tests are described in Section 4.5.

4.0 RESULTS

4.1 Preoptimization Experiments

4.1.1 Substrate Characterization

Detailed results of the analyses of the nonradiolabeled ASDN (lot number 072K1134) and of Lot 3467835 of the [^{3}H]ASDN are presented in Appendix 1. Briefly, the [^{3}H]ASDN was found to be 98% radiochemically pure with an experimentally determined specific activity of 26.4 Ci/mmol and it coeluted with the nonradiolabeled substrate by HPLC. Since the measured specific activity was within 5% of that reported by the supplier, the supplier-reported specific activity of 25.3 Ci/mmol was used in all calculations.

[^{3}H]ASDN (lot number 3474115) was found to be 98% radiochemically pure by HPLC analysis. The specific activity of this material was determined on two occasions to be ca. 22.4 and 21.8 Ci/mmol, respectively, while the supplier-stated specific activity was 25.3 Ci/mmol. The experimentally determined values were ca. 89 and 86% of the supplier-stated specific activity. The supplier rechecked the specific activity and confirmed the originally stated value. Possible reasons for the discrepancy between the specific activity reported by the supplier and our experimentally determined specific activity include moisture in the nonradiolabeled ASDN that was used to prepare the standard curve. An inflated ASDN mass in the standard curve would result in apparently lower specific activity of the [^{3}H]ASDN. In fact, the experimentally determined (using the same standard curve as the second batch of [^{3}H]ASDN) specific activity of the first batch of [^{3}H]ASDN was only 23.3 Ci/mmol, 88% of the initially determined specific activity (26.4 Ci/mmol) for this batch. It appears, therefore, that the non-radiolabeled ASDN may have picked up a small amount of moisture over the period of the study.

Because the [^{3}H]ASDN makes up only about 3% of the mass of ASDN in the substrate solutions, the small discrepancy in the specific activity of [^{3}H]ASDN will have little effect on the final specific activity in the substrate solutions or the final aromatase activity calculations. Therefore, the manufacturer's stated specific activity was used in all calculations.

4.1.2 Initial Protein Concentration, P450 Content and Aromatase Activity Determination

Detailed results of this work are presented Appendices 1-3.

4.1.2.1 Porcine Placenta Microsomes. Six porcine placentas were obtained (two on one day and all the others on separate days) from a local research farm and microsomes were prepared from five of them (where two were obtained on a single day, only one was processed). See Appendix 2 for a more detailed description. A sample of each microsome preparation was thawed rapidly in a water bath and rehomogenized prior to assay for protein and cytochrome P450 content and aromatase activity. Only one of the five preparations had appreciable aromatase activity. The protein content for that preparation was determined to be ca. 28 mg/mL. The total protein yield for the preparation was calculated to be ca. 126 mg (ca. 4.5 mL microsomal suspension). P450 content of that preparation was determined to be ca. 0.053 nmol/mg protein. The aromatase activity of the porcine placental microsomes was ca. 3 pmol estrogen formed/mg protein/min; this value matches that found for the bovine placental microsome preparation (below). No literature value for porcine placenta microsomal aromatase activity was found.

4.1.2.2 Bovine Placenta Microsomes. A bovine placenta was obtained from a local research farm and microsomes were prepared. See Appendix 3 for a more detailed description. A sample of the microsomes was thawed rapidly in a 37 °C water bath and rehomogenized prior to assay for protein and P450 content and aromatase activity. The protein content of the bovine placental microsomes was determined to be ca. 45 mg/mL. The total protein yield for the preparation was calculated to be ca. 675 mg. This exceeds the 250 mg of protein criteria set in the protocol. P450 content of the bovine placental microsomes was determined to be ca. 0.031 nmol/mg protein. The aromatase activity of the bovine placental microsomes was ca. 3 pmol estrogen formed/mg protein/min; this is in good agreement with the 3.62 ± 0.78 pmol estrogen formed/mg protein/min reported by Tsumagari et al. (1993).

4.1.2.3 Human Placenta Microsomes. A human placenta from a 28 year old nonsmoker with a full term Caesarean-section delivery was obtained from a local hospital and microsomes were prepared. See Appendix 1 for a more detailed description. A sample of the microsomes was thawed rapidly in a water bath and rehomogenized prior to assay for protein and P450 content and aromatase activity. The protein content of the human placental microsomes was determined to be ca. 44 mg/mL. The total protein yield for the preparation was calculated to be ca. 900 mg. This exceeds the 250 mg of protein criteria set in the protocol. P450 content of the human placental microsomes was determined to be ca. 0.048 nmol/mg protein, which exceeds the criteria of 0.005 nmol P450/mg protein set in the protocol. The aromatase activity of the human placental microsomes was ca. 0.015 nmol estrogen formed/mg

protein/min; this exceeds the 5 pmol estrogen formed/mg protein/min acceptance criteria for this parameter.

4.1.2.4 Human Recombinant Microsomes. The data sheets for the two human recombinant CYP19 batches (Lots # 3 and 2) are presented in Figure 1 and Appendix 1, Figure 5, respectively. One tube of Lot 2 was thawed rapidly at 37 °C and the contents were rehomogenized and analyzed for protein and P450 content and aromatase activity. The protein content was found to be 3.5 mg/mL, compared with the 4.2 mg/mL stated on the data sheet. The P450 content was calculated to be 0.38 nmol/mg protein. This value is similar to the 0.24 nmol/mg value calculated from the data sheet information. This microsomal preparation had aromatase activity of 0.022 nmol estrogen formed/mg protein/min under the conditions of the assay as described above. The data sheet reported an aromatase activity value (1.38 nmol/mg protein/min) obtained using a different substrate at a significantly higher concentration. It is unclear whether the activities determined under such different conditions should be similar.

4.2 Optimization Experiments (Factorial Design)

4.2.1 Human Placental Microsome Assay

The human placental microsome aromatase activity data from the factorial design optimization experiment are presented in Table 6. The coded factor levels used in the analysis are presented in Table 3.

Table 6. Data Listing for Aromatase Human Placental Optimization Experiment

Part	Tube_ num	Run	Day_ fix	NADP _fix mM final	G6P _fix mM final	G6PDeH_ fix units final	ASDN _fix nM final	Protein_fix mg/mL final	Inc_time _fix min	Aromatase_activity nmol/mg/min	log_Aromatase
1 (factorial)	1	1	2	0.5	1.0	0.5	25	0.02	15	0.0360	-3.3248
	2	2	2	0.5	1.0	0.5	100	0.50	15	0.0087	-4.7427
	3	3	2	0.5	1.0	2.0	25	0.50	15	0.0021	-6.1870
	4	4	2	0.5	1.0	2.0	100	0.02	15	0.0613	-2.7917
	5	5	2	0.5	3.0	0.5	25	0.50	15	0.0020	-6.2088
	6	6	2	0.5	3.0	0.5	100	0.02	15	0.0575	-2.8560
	7	7	2	0.5	3.0	2.0	25	0.02	15	0.0369	-3.2987
	8	8	2	0.5	3.0	2.0	100	0.50	15	0.0086	-4.7610
	9	9	2	2.0	1.0	0.5	25	0.50	15	0.0020	-6.1916
	10	10	2	2.0	1.0	0.5	100	0.02	15	[a]	
	11	11	2	2.0	1.0	2.0	25	0.02	15	[a]	
	12	12	2	2.0	1.0	2.0	100	0.50	15	0.0085	-4.7700
	13	13	2	2.0	3.0	0.5	25	0.02	15	0.0348	-3.3591
	14	14	2	2.0	3.0	0.5	100	0.50	15	0.0086	-4.7610
	15	15	2	2.0	3.0	2.0	25	0.50	15	0.0020	-6.2170
	16	16	2	2.0	3.0	2.0	100	0.02	15	0.0557	-2.8875
	1	17	1	0.5	1.0	0.5	25	0.50	60	0.0006	-7.4300
	2	18	1	0.5	1.0	0.5	100	0.02	60	0.0361	-3.3213

(continued)

Table 6. Data Listing for Aromatase Human Placental Optimization Experiment (continued)

Part	Tube_ num	Run	Day_ fix	NADP _fix mM final	G6P _fix mM final	G6PDeH_ fix units final	ASDN _fix nM final	Protein_fix mg/mL final	Inc_time _fix min	Aromatase_activity nmol/mg/min	log_Aromatase
	3	19	1	0.5	1.0	2.0	25	0.02	60	0.0117	-4.4439
	4	20	1	0.5	1.0	2.0	100	0.50	60	0.0022	-6.1346
	5	21	1	0.5	3.0	0.5	25	0.02	60	0.0123	-4.3993
	6	22	1	0.5	3.0	0.5	100	0.50	60	0.0020	-6.1902
	7	23	1	0.5	3.0	2.0	25	0.50	60	0.0006	-7.4616
	8	24	1	0.5	3.0	2.0	100	0.02	60	0.0371	-3.2934
	9	25	1	2.0	1.0	0.5	25	0.02	60	0.0127	-4.3701
	10	26	1	2.0	1.0	0.5	100	0.50	60	0.0020	-6.2123
	11	27	1	2.0	1.0	2.0	25	0.50	60	0.0005	-7.6121
	12	28	1	2.0	1.0	2.0	100	0.02	60	0.0156	-4.1591
	13	29	1	2.0	3.0	0.5	25	0.50	60	0.0005	-7.5715
	14	30	1	2.0	3.0	0.5	100	0.02	60	0.0350	-3.3512
	15	31	1	2.0	3.0	2.0	25	0.02	60	0.0120	-4.4201
	16	32	1	2.0	3.0	2.0	100	0.50	60	0.0019	-6.2502
2 (centerpoint)	17	1	1	1.0	2.0	1.0	50	0.10	30	0.0103	-4.5794
3 (axials)	20	1	1	0.1	2.0	1.0	50	0.10	30	0.0100	-4.6039
	21	2	1	4.0	2.0	1.0	50	0.10	30	0.0090	-4.7068
	22	3	1	1.0	0.1	1.0	50	0.10	30	0.0100	-4.6058
	23	4	1	1.0	4.0	1.0	50	0.10	30	0.0100	-4.6038
	24	5	1	1.0	2.0	0.1	50	0.10	30	0.0100	-4.6031
	25	6	1	1.0	2.0	4.0	50	0.10	30	0.0102	-4.5883
	26	7	1	1.0	2.0	1.0	10	0.10	30	0.0020	-6.2196
	27	8	1	1.0	2.0	1.0	500	0.10	30	0.0597	-2.8180
	28	9	1	1.0	2.0	1.0	50	0.01	30	0.0329	-3.4147
	29	10	1	1.0	2.0	1.0	50	1.00	30	0.0010	-6.9118
	30	11	1	1.0	2.0	1.0	50	0.10	10	0.0295	-3.5223
	31	12	1	1.0	2.0	1.0	50	0.10	120	0.0025	-5.9760
4 (replicates)	18	1	1	1.0	2.0	1.0	50	0.10	30	0.0090	-4.7068
	19	2	1	1.0	2.0	1.0	50	0.10	30	0.0096	-4.6439
	17	3	2	1.0	2.0	1.0	50	0.10	30	0.0111	-4.5011
	18	4	2	1.0	2.0	1.0	50	0.10	30	0.0110	-4.5119
	19	5	2	1.0	2.0	1.0	50	0.10	30	0.0110	-4.5115
	20	6	2	0.1	2.0	1.0	50	0.10	30	0.0110	-4.5140
	21	7	2	4.0	2.0	1.0	50	0.10	30	0.0107	-4.5338
	22	8	2	1.0	0.1	1.0	50	0.10	30	0.0107	-4.5333
	23	9	2	1.0	4.0	1.0	50	0.10	30	0.0101	-4.5965
	24	10	2	1.0	2.0	0.1	50	0.10	30	0.0105	-4.5540
	25	11	2	1.0	2.0	4.0	50	0.10	30	0.0107	-4.5421
	26	12	2	1.0	2.0	1.0	10	0.10	30	0.0020	-6.1905
	27	13	2	1.0	2.0	1.0	500	0.10	30	0.0536	-2.9255
	28	14	2	1.0	2.0	1.0	50	0.01	30	0.0269	-3.6138
	29	15	2	1.0	2.0	1.0	50	1.00	30	0.0010	-6.8649
	30	16	2	1.0	2.0	1.0	50	0.10	10	0.0313	-3.4626
	31	17	2	1.0	2.0	1.0	50	0.10	120	0.0025	-5.9950
factorial	10	10	3	2.0	1.0	0.5	100	0.02	15	0.0511	-2.9732
	11	11	3	2.0	1.0	2.0	25	0.02	15	0.0458	-3.0831
centerpoint[b]	17	17	3	1.0	2.0	1.0	50	0.10	30	0.0102	-4.5900
replicate[b]	18	18	3	1.0	2.0	1.0	50	0.10	30	0.0102	-4.5902

[a]Error in assay conduct. No reportable data. Runs were repeated on Day 3.
[b]These tubes were run to allow for day-to-day comparisons.

To assess the effects of each experimental factor on aromatase activity, an ANOVA model was fit to the set of data. The ANOVA models containing all main effects, day effects and 2fi's were fit to the original aromatase activities and the logarithms of aromatase activities using PROC GLM. Upon examining the residuals from fitting the full ANOVA models, the

residuals for the original aromatase activities showed signs of heteroskedasticity. The residuals for the logarithms of aromatase activities appear homoskedastic and randomly distributed around 0, thereby satisfying the assumptions necessary for valid ANOVA modeling results. The logarithms of the aromatase activities were therefore chosen as the more appropriate dependent variables for the analysis of the human placental experiment data.

For the full ANOVA model using the logarithm of aromatase activity as the dependent variable, we examined the t-tests for the significance of each factor in the model. In the first round of model evaluation, we examined the t-test results for the 2fi's to see if any of those terms could be removed from the models. Any 2fi's with p-values greater than 0.1 were removed from the models. The reduced model was fit to the logarithms of aromatase activity again and the t-tests of the model terms were re-evaluated for significance. After 2 rounds of removing insignificant terms, we arrived at the final reduced model containing only significant 2fi's and first order factors.

To obtain the optimal combination of factors for each time point, the data were fit using a response surface regression model and the SAS procedure RSREG. The PROC GLM modeling results were used to determine which factors and 2fi's needed to remain in the models. Any factors which did not have any associated 2fi's in the final model were treated as covariates in the response surface regression model. The ANOVA results for the response surface regression are summarized in Table 7. The parameter estimates from the response surface regression model are presented in Table 8. Differentiation of the prediction equation is used to determine the location of the stationary point. In the case of the human placental data, the stationary point was not a point of maximal response. Under this circumstance the RIDGE technique in SAS is used to find a point of maximal response within the experimental region. Starting at the center of the experimental region, a hypersphere having a given radius is considered and the point on the hypersphere with the maximal response is determined. The radius is then incremented and the maximum on that hypersphere is determined. This process, when repeated, traces out a "ridge of maximal response." The iterations are terminated when one of the factors reaches the extremity of its range, as defined by the levels used in the experiment. We determined the factor combination that produced the maximum predicted value of logarithm of aromatase activity. The optimal factor results, presented in Table 9, show the factor levels that are associated with the maximum predicted value for the logarithm of aromatase activity.

In order to assess the effects of the day to day variation on the effects of the factors, the same analysis steps that were performed above were also repeated on the logarithms of aromatase activity but with the day effects removed from the models. The ANOVA results for the final reduced model without the day effects from PROC RSREG are shown in Table 10. The parameter estimates from the response surface regression model are presented in Table 11. Comparing the parameter estimates in Table 8 with those in Table 11 shows that all factors were

retained in the model that includes the day effects, however NADP and G6P dropped out of the model that excludes the day effects due to not having significant results in the variation of the aromatase activity. The optimal factor results, presented in Table 12, show the factor levels that are associated with the maximum predicted value for the logarithm of aromatase activity for the model excluding the day effects. The optimal factor values for G6PDeH, Protein and Inc_time are very similar between the two models. The optimal factor value for ASDN occurred at 101.2 for the model including the day effects while the optimal value for the model excluding the day effects occurred at 154.1.

Table 7. Human Placental Optimization ANOVA Results for PROC RSREG

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F Test	PValue
Day3	3	0.330	0.110	3.07	0.0388
NADP_code	7	0.154	0.022	0.62	0.7396
G6P_code	6	0.129	0.021	0.60	0.7288
G6PDeH_code	7	0.389	0.056	1.55	0.1789
ASDN_code	6	20.572	3.429	95.68	<.0001
Protein_code	6	65.709	10.952	305.61	<.0001

Table 8. Human Placental Optimization: Response Surface Regression Results

Parameter	DF	Beta Estimate	Standard Error	t Test	Pr > |t|
Intercept	1	-4.593	0.057	-81.10	<.0001
day3	1	0.003	0.145	0.02	0.9859
NADP_code	1	-0.029	0.028	-1.03	0.3082
G6P_code	1	0.007	0.029	0.24	0.8097
G6PDeH_code	1	-0.027	0.028	-0.96	0.3414
ASDN_code	1	0.573	0.026	22.04	<.0001
Protein_code	1	-1.294	0.031	-41.41	<.0001
NADP_code*day3	1	0.004	0.238	0.02	0.9858
NADP_code*NADP_code	1	-0.002	0.014	-0.13	0.8981
G6P_code*NADP_code	1	0.021	0.035	0.58	0.5633
G6P_code*G6P_code	1	0.012	0.027	0.46	0.6493

(continued)

Table 8. Human Placental Optimization: Response Surface Regression Results (continued)

Parameter	DF	Beta Estimate	Standard Error	t Test	Pr > |t|
G6PDeH_code*day3	1	0.549	0.181	3.03	0.0043
G6PDeH_code*NADP_code	1	-0.056	0.035	-1.60	0.1178
G6PDeH_code*G6P_code	1	0.057	0.035	1.61	0.1154
G6PDeH_code*G6PDeH_co	1	-0.001	0.014	-0.10	0.9233
ASDN_code*NADP_code	1	-0.003	0.035	-0.09	0.9258
ASDN_code*G6P_code	1	-0.002	0.035	-0.04	0.9653
ASDN_code*G6PDeH_code	1	-0.024	0.035	-0.67	0.5077
ASDN_code*ASDN_code	1	-0.021	0.013	-1.62	0.1137
Protein_code*NADP_code	1	0.013	0.035	0.37	0.7142
Protein_code*G6P_code	1	-0.024	0.035	-0.67	0.5083
Protein_code*G6PDeH_code	1	0.045	0.035	1.27	0.2118
Protein_code*ASDN_code	1	0.153	0.035	4.33	0.0001
Protein_code*Protein_code	1	-0.260	0.041	-6.28	<.0001
Inc_time_code	1	-0.605	0.029	-20.59	<.0001

Note: DF = degrees of freedom
Beta Estimate = regression coefficient, as applied to coded factors
Pr > |t| = significance level for t-test of hypothesis that the true Beta = 0

Table 9. Human Placental Optimal Factor Values

Factor	Factor Description	Coded Factor Value	Factor Value
NADP	NADP+ (mM)	-0.6615	0.6322
G6P	Glucose-6-Phosphate (mM)	0.0151	2.0151
G6PDeH	Glucose-6-Phosphate Dehydrogenase (units)	-0.7234	0.6057
ASDN	Androstenedione (substrate) (nM)	1.0166	101.1570
Protein	Protein (mg/mL)	-0.8959	0.0237
Inc_time	Incubation Time (min)	-1.5850	10.0000

Table 10. Human Placental Optimization: ANOVA Results for PROC RSREG When Day Effects are Removed from Model

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F Test	PValue
G6PDeH_code	5	0.084	0.017	0.61	0.6924
ASDN_code	5	20.218	4.044	147.03	<.0001
Protein_code	5	70.471	14.094	512.48	<.0001
Inc_time_code	5	17.090	3.418	124.28	<.0001

Table 11. Human Placental Optimization: Response Surface Regression Results for Day Effects Removed from Model

Parameter	DF	Beta Estimate	Standard Error	t Test	Pr > |t|
Intercept	1	-4.586	0.040	-113.51	<.0001
G6PDeH_code	1	-0.005	0.024	-0.22	0.8241
ASDN_code	1	0.554	0.022	25.17	<.0001
Protein_code	1	-1.294	0.026	-49.49	<.0001
Inc_time_code	1	-0.605	0.025	-24.12	<.0001
G6PDeH_code*G6PDeH_code	1	0.003	0.012	0.22	0.8296
ASDN_code*G6PDeH_code	1	-0.024	0.029	-0.82	0.4149
ASDN_code*ASDN_code	1	-0.019	0.011	-1.70	0.0947
Protein_code*G6PDeH_code	1	0.011	0.029	0.36	0.7207
Protein_code*ASDN_code	1	0.187	0.029	6.39	<.0001
Protein_code*Protein_code	1	-0.260	0.035	-7.48	<.0001
Inc_time_code*G6PDeH_code	1	-0.042	0.029	-1.44	0.1563
Inc_time_code*ASDN_code	1	0.046	0.029	1.57	0.1237
Inc_time_code*Protein_code	1	-0.120	0.029	-4.09	0.0002
Inc_time_code*Inc_time_code	1	-0.004	0.026	-0.14	0.8910

Note: DF = degrees of freedom
Beta Estimate = regression coefficient, as applied to coded factors
Pr > |t| = significance level for t-test of hypothesis that the true Beta = 0

Table 12. Human Placental Optimal Factor Values for Model with Day Effects Removed

Factor	Factor Description	Coded Factor Value	Factor Value
NADP	NADP+ (mM)	NS	NS
G6P	Glucose-6-Phosphate (mM)	NS	NS
G6PDeH	Glucose-6-Phosphate Dehydrogenase (units)	-0.6252	0.6483
ASDN	Androstenedione (substrate) (nM)	1.6243	154.1430
Protein	Protein (mg/mL)	-1.1206	0.0165
Inc_time	Incubation Time (min)	-1.5789	10.0423

Note: NS = Factor removed from model due to nonsignificant results

4.2.2 Human Recombinant Microsome Assay

The human recombinant microsome aromatase activity data from the factorial design optimization experiment are presented in Table 13. The coded factor values are presented in Table 3.

Table 13. Data Listing for Aromatase Human Recombinant Optimization Experiment

Part	Tube_ num	Run	Day_ fix	NADP _fix mM final	G6P _fix mM final	G6PDeH_ fix units final	ASDN_fix nM final	Protein_fix mg/mL final	Inc_time_fix min	Aromatase_ activity nmol/mg/min	log_ aromatase
1 (factorial)	1	1	2	0.5	1.0	0.5	25	0.02	15	0.0608	-2.7995
	2	2	2	0.5	1.0	0.5	100	0.50	15	0.0111	-4.4974
	3	3	2	0.5	1.0	2.0	25	0.50	15	0.0027	-5.9173
	4	4	2	0.5	1.0	2.0	100	0.02	15	0.2395	-1.4291
	5	5	2	0.5	3.0	0.5	25	0.50	15	0.0026	-5.9468
	6	6	2	0.5	3.0	0.5	100	0.02	15	0.2401	-1.4267
	7	7	2	0.5	3.0	2.0	25	0.02	15	0.0577	-2.8520
	8	8	2	0.5	3.0	2.0	100	0.50	15	0.0105	-4.5557
	9	9	2	2.0	1.0	0.5	25	0.50	15	0.0025	-5.9952
	10	10	2	2.0	1.0	0.5	100	0.02	15	0.2314	-1.4638
	11	11	2	2.0	1.0	2.0	25	0.02	15	0.0578	-2.8515
	12	12	2	2.0	1.0	2.0	100	0.50	15	0.0110	-4.5061
	13	13	2	2.0	3.0	0.5	25	0.02	15	0.0573	-2.8590
	14	14	2	2.0	3.0	0.5	100	0.50	15	0.0105	-4.5540
	15	15	2	2.0	3.0	2.0	25	0.50	15	0.0024	-6.0341
	16	16	2	2.0	3.0	2.0	100	0.02	15	0.2329	-1.4573
	1	17	1	0.5	1.0	0.5	25	0.50	60	0.0006	-7.3708
	2	18	1	0.5	1.0	0.5	100	0.02	60	0.0558	-2.8864
	3	19	1	0.5	1.0	2.0	25	0.02	60	0.0125	-4.3781
	4	20	1	0.5	1.0	2.0	100	0.50	60	0.0026	-5.9470
	5	21	1	0.5	3.0	0.5	25	0.02	60	0.0129	-4.3538
	6	22	1	0.5	3.0	0.5	100	0.50	60	0.0026	-5.9685
	7	23	1	0.5	3.0	2.0	25	0.50	60	0.0006	-7.3865
	8	24	1	0.5	3.0	2.0	100	0.02	60	0.0544	-2.9120
	9	25	1	2.0	1.0	0.5	25	0.02	60	0.0128	-4.3589
	10	26	1	2.0	1.0	0.5	100	0.50	60	0.0025	-5.9821
	11	27	1	2.0	1.0	2.0	25	0.50	60	0.0006	-7.4129
	12	28	1	2.0	1.0	2.0	100	0.02	60	0.0536	-2.9269
	13	29	1	2.0	3.0	0.5	25	0.50	60	0.0006	-7.4384
	14	30	1	2.0	3.0	0.5	100	0.02	60	0.0535	-2.9288
	15	31	1	2.0	3.0	2.0	25	0.02	60	0.0129	-4.3507
	16	32	1	2.0	3.0	2.0	100	0.50	60	0.0025	-5.9756
2 (centerpoint)	17	1	1	1.0	2.0	1.0	50	0.10	30	0.0111	-4.5035
3 (axials)	20	1	1	0.1	2.0	1.0	50	0.10	30	0.0112	-4.4943
	21	2	1	4.0	2.0	1.0	50	0.10	30	0.0102	-4.5855
	22	3	1	1.0	0.1	1.0	50	0.10	30	0.0111	-4.5025
	23	4	1	1.0	4.0	1.0	50	0.10	30	0.0108	-4.5299
	24	5	1	1.0	2.0	0.1	50	0.10	30	0.0108	-4.5303
	25	6	1	1.0	2.0	4.0	50	0.10	30	0.0110	-4.5130
	26	7	1	1.0	2.0	1.0	10	0.10	30	0.0022	-6.1019
	27	8	1	1.0	2.0	1.0	500	0.10	30	0.1102	-2.2054
	28	9	1	1.0	2.0	1.0	50	0.01	30	0.1050	-2.2540
	29	10	1	1.0	2.0	1.0	50	1.00	30	0.0013	-6.6769
	30	11	1	1.0	2.0	1.0	50	0.10	10	0.0330	-3.4127
	31	12	1	1.0	2.0	1.0	50	0.10	120	0.0029	-5.8503
4 (replicates)	18	1	1	1.0	2.0	1.0	50	0.10	30	0.0111	-4.4999
	19	2	1	1.0	2.0	1.0	50	0.10	30	0.0107	-4.5401
	17	3	2	1.0	2.0	1.0	50	0.10	30	0.0128	-4.3593
	18	4	2	1.0	2.0	1.0	50	0.10	30	0.0126	-4.3769
	19	5	2	1.0	2.0	1.0	50	0.10	30	0.0124	-4.3895
	20	6	2	0.1	2.0	1.0	50	0.10	30	0.0119	-4.4315

continued

Table 13. Data Listing for Aromatase Human Recombinant Optimization Experiment (continued)

Part	Tube_ num	Run	Day_ fix	NADP _fix mM final	G6P _fix mM final	G6PDeH_ fix units final	ASDN_fix nM final	Protein_fix mg/mL final	Inc_time_fix min	Aromatase_ activity nmol/mg/min	log_ aromatase
	21	7	2	4.0	2.0	1.0	50	0.10	30	0.0122	-4.4050
	22	8	2	1.0	0.1	1.0	50	0.10	30	0.0122	-4.4048
	23	9	2	1.0	4.0	1.0	50	0.10	30	0.0121	-4.4113
	24	10	2	1.0	2.0	0.1	50	0.10	30	0.0122	-4.4035
	25	11	2	1.0	2.0	4.0	50	0.10	30	0.0123	-4.4022
	26	12	2	1.0	2.0	1.0	10	0.10	30	0.0025	-5.9939
	27	13	2	1.0	2.0	1.0	500	0.10	30	0.1168	-2.1471
	28	14	2	1.0	2.0	1.0	50	0.01	30	[a]	
	29	15	2	1.0	2.0	1.0	50	1.00	30	[a]	
	30	16	2	1.0	2.0	1.0	50	0.10	10	[a]	
	31	17	2	1.0	2.0	1.0	50	0.10	120	[a]	
replicates	17[b]	3	3	1.0	2.0	1.0	50	0.10	30	0.0121	-4.4120
	18[b]	4	3	1.0	2.0	1.0	50	0.10	30	0.0124	-4.3912
	28	14	3	1.0	2.0	1.0	50	0.01	30	0.1165	-2.1500
	29	15	3	1.0	2.0	1.0	50	1.00	30	0.0013	-6.6196
	30	16	3	1.0	2.0	1.0	50	0.10	10	0.0362	-3.3193
	31	17	3	1.0	2.0	1.0	50	0.10	120	0.0033	-5.7037

[a]Error in assay conduct. No reportable data. Runs were repeated on Day 3.

[b]These tubes were run to allow for day-to-day comparisons.

The effects of each experimental factor on aromatase activity were assessed by fitting an ANOVA model as described in Section 4.2.1. The residuals for the logarithms of aromatase activities appeared homoskedastic and randomly distributed around 0, thereby satisfying the assumptions necessary for valid ANOVA modeling results. The logarithms of the aromatase activities were therefore chosen as the more appropriate dependent variables for the analysis of the human recombinant experiment data.

The t-tests for the significance of each factor and 2fi's in the model were examined as described in Section 4.2.1 for the full ANOVA model using the logarithm of aromatase activity as the dependent variable. In the first round of model evaluation, we examined the t-test results for the 2fi's to see if any of those terms could be removed from the models. Any 2fi's with p-values greater than 0.1 were removed from the models. The reduced model was fit to the logarithms of aromatase activity again and the t-tests of the model terms were re-evaluated for significance. After 2 rounds of removing insignificant terms, we arrived at the final reduced model containing only significant 2fi's and first order factors.

To obtain the optimal combination of factors for each time point, the data were fit using a response surface regression model and the SAS procedure RSREG. The PROC GLM modeling results were used to determine which factors and 2fi's needed to remain in the models. Any factors which did not have any associated 2fi's in the final model were treated as covariates in the response surface regression model. The ANOVA results for the response surface regression

are summarized in Table 14. The parameter estimates from the response surface regression model are presented in Table 15. Differentiation of the prediction equation is used to determine the location of the stationary point. In the case of the human recombinant data, the stationary point was not a point of maximal response. The RIDGE technique in SAS was used to find a point of maximal response within the experimental region as described in Section 4.2.1. The optimal factor results, presented in Table 16, show the factor levels that are associated with the maximum predicted value for the logarithm of aromatase activity.

In order to assess the effects of the day to day variation on the effects of the factors, the same analysis steps that were performed above were also repeated on the logarithms of aromatase activity but with the day effects removed from the models. The ANOVA results for the final reduced model without the day effects from PROC RSREG are shown in Table 17. The parameter estimates from the response surface regression model are presented in Table 18. Comparing the parameter estimates in Table 15 with those in Table 18 shows that fewer factors remained significant in the model when the day effects were removed. The optimal factor results, presented in Table 19, show the factor levels that are associated with the maximum predicted value for the logarithm of aromatase activity for the model excluding the day effects. Only two factors remained as significant in the model once the day effects were removed, but the location of the optimal values for those two factors are very close to the optimal values with the day effects included in the model.

Table 14. Human Recombinant Optimization ANOVA Results for PROC RSREG

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F Test	PValue
day2	5	0.1202	0.0240	34.14	<.0001
NADP_code	6	0.0151	0.0025	3.57	0.0061
ASDN_code	6	31.7224	5.2871	7,506.75	<.0001
Protein_code	6	95.5933	15.9322	22,621.10	<.0001
Inc_time_code	6	12.0661	2.0110	2,855.30	<.0001

Table 15. Human Recombinant Optimization Response Surface Regression Results

Parameter	DF	Beta Estimate	Standard Error	t Test	Pr > |t|
Intercept	1	-4.5084	0.0083	-544.27	<.0001
day2	1	0.1177	0.0109	10.76	<.0001
NADP_code	1	-0.0240	0.0062	-3.90	0.0003
ASDN_code	1	0.7079	0.0058	121.38	<.0001
Protein_code	1	-1.5535	0.0093	-167.54	<.0001
Inc_time_code	1	-0.6734	0.0070	-96.10	<.0001
NADP_code*day2	1	0.0205	0.0097	2.11	0.0412
NADP_code*NADP_code	1	-0.0051	0.0020	-2.63	0.0120
ASDN_code*day2	1	-0.0106	0.0093	-1.15	0.2582
ASDN_code*NADP_code	1	0.0039	0.0047	0.83	0.4130
ASDN_code*ASDN_code	1	-0.0031	0.0018	-1.74	0.0891
Protein_code*day2	1	0.0287	0.0208	1.38	0.1746
Protein_code*NADP_code	1	-0.0047	0.0047	-0.99	0.3259
Protein_code*ASDN_code	1	0.0045	0.0047	0.95	0.3463
Protein_code*Protein_code	1	0.0182	0.0071	2.58	0.0133
Inc_time_code*day2	1	-0.0021	0.0179	-0.12	0.9081
Inc_time_code*NADP_code	1	0.0142	0.0068	2.10	0.0422
Inc_time_code*ASDN_code	1	-0.0004	0.0066	-0.06	0.9513
Inc_time_code*Protein_code	1	0.0294	0.0114	2.58	0.0135
Inc_time_code*Inc_time_code	1	0.0086	0.0051	1.70	0.0967
day3	1	0.1006	0.0129	7.83	<.0001
G6P_code	1	-0.0073	0.0039	-1.90	0.0649

Note: DF = degrees of freedom
Beta Estimate = regression coefficient, as applied to coded factors
Pr > |t| = significance level for t-test of hypothesis that the true Beta = 0

Table 16. Human Recombinant Optimal Factor Values with Day Effects Included in Model

Factor	Factor Description	Coded Factor Value	Factor Value
NADP	NADP+ (mM)	-0.6623	0.6319
G6P	Glucose-6-Phosphate (mM)	NS	NS
G6PDeH	Glucose-6-Phosphate Dehydrogenase (nM)	NS	NS
ASDN	Androstenedione (substrate) (nM)	1.1150	108.2950
Protein	Protein (mg/mL)	-1.4293	0.0100
Inc_time	Incubation Time (min)	-0.4504	21.9556

Note: NS = Factor removed from model due to nonsignificant results

Table 17. Human Recombinant Optimization: ANOVA Results for PROC RSEG when Day Effects are Removed from Model

Factor	Degrees of Freedom	Sum of Squares	Mean Square	F Test	PValue
Protein_code	3	95.5911	31.8637	10087.00	<.0001
Inc_time_code	3	23.0013	7.6671	2427.15	<.0001

Table 18. Human Recombinant Optimization: Response Surface Regression Results for Day Effects Removed from Model

Parameter	DF	Beta Estimate	Standard Error	t Test	Pr > |t|
Intercept	1	-4.4584	0.0114	-392.52	<.0001
Protein_code	1	-1.5421	0.0089	-173.95	<.0001
Inc_time_code	1	-0.7172	0.0085	-84.38	<.0001
Protein_code*Protein_code	1	0.0150	0.0117	1.28	0.2044
Inc_time_code*Protein_code	1	0.0151	0.0099	1.51	0.1353
Inc_time_code*Inc_time_code	1	0.0153	0.0086	1.79	0.0793
ASDN_code	1	0.7005	0.0070	100.25	<.0001

Note: DF = degrees of freedom
Beta Estimate = regression coefficient, as applied to coded factors
Pr > |t| = significance level for t-test of hypothesis that the true Beta = 0

Table 19. Human Recombinant Optimal Factor Values for Model with Day Effects Removed

Factor	Factor Description	Coded Factor Value	Factor Value
NADP	NADP+ (mM)	NS	NS
G6P	Glucose-6-Phosphate (mM)	NS	NS
G6PDeH	Glucose-6-Phosphate Dehydrogenase (units)	NS	NS
ASDN	Androstenedione (substrate) (nM)	NS	NS
Protein	Protein (mg/mL)	-1.4283	0.0100
Inc_time	Incubation Time (min)	-0.4681	21.6874

Note: NS = Factor removed from model due to nonsignificant results

4.3 Aromatase Optimization Supplemental Studies

4.3.1 Experiment 1

Complete results for this experiment are presented in Appendix 5. Summary product formation rates, percent substrate conversion to product and percent inhibition are presented in Table 20. Only the assay using 0.0125 mg/mL protein had a percent substrate conversion less than the target of 10-15%. Average aromatase activity data are presented in Table 21. In each case, the radioactivity in the Blank tubes was subtracted from that in the assay tubes prior to

calculation of aromatase activity. The calculated activity in the No NADP tubes of Sets 2 and 3 was much lower than that found for Set 1. A change in pipetting order was initiated between Set 1 and Set 2 that was intended to minimize the possibility of NADP carryover from tube to tube. This change may explain the variance in the activities between the Set 1 and Set 2 'No NADP' tubes; the high apparent activity in the Set 1 'No NADP' tubes may be due to their inadvertent contamination with NADP. Only about 0.1% of the radioactivity in the reaction mixture remained in the aqueous phase of the 'Blank' and 'No system' tubes after extraction. This is evidence that the extraction of ^3H_2O was complete. The use of 0.3 mM NADPH instead of the regenerating system gave aromatase activities similar to those found in the tubes containing the regenerating system. At a protein concentration of 0.0125 mg/mL, the aqueous portion contained approximately 15,000 DPM/mL after extraction. If the activity were inhibited 90% by a test substance, the aqueous portion would be expected to contain about 1500 DPM/mL. This is still an easily quantifiable level of radioactivity. Therefore, a protein level of 0.0125 mg/mL was selected for use in the next studies.

Table 20. Product Formation Rates, Substrate and Inhibition Percentages

Protein Concentration (mg/mL)	Experiment ID	Product formation rate (nmol/min)	% conversion of substrate to product	% inhibition
Complete system				
0.05	Set 1	0.0052	38.29	NA
	Set 2	0.0060	45.05	
	Set 3	0.0060	44.85	
	Mean (SD)	0.0058 (0.0004)	42.73 (3.38)	
0.025	Set 1	0.0025	18.43	
	Set 2	0.0029	21.50	
	Set 3	0.0028	20.82	
	Mean (SD)	0.0027 (0.0002)	20.25 (1.44)	
0.0125	Set 1	0.0012	9.12	
	Set 2	0.0014	10.29	
	Set 3	0.0013	10.08	
	Mean (SD)	0.0013 (0.0001)	9.83 (0.56)	
Inhibited system				
0.05	Set 1	0.0012	9.25	76.0
	Set 3	0.0014	10.30	77.2
	Mean (SD)	0.0013 (0.0001)	9.78 (0.61)	
0.025	Set 1	0.0006	4.46	76.2
	Set 3	0.0006	4.85	77.1
	Mean (SD)	0.0006 (0.0000)	4.66 (0.23)	
0.0125	Set 1	0.0003	2.19	77.2
	Set 3	0.0003	2.43	76.6
	Mean (SD)	0.0003 (0.0000)	2.33 (0.16)	

NA = not applicable

Table 21. Average Aromatase Activity (nmol/mg/min)

Conditions	Set 1	Set 2	Set 3	Mean (SD)
0.05 mg/mL protein				
Complete	0.0518	0.0602	0.0603	0.0574 (0.0043)
Inhibited	0.0124		0.0137	0.0131 (0.0008)
- NADP	0.0411	0.0030	0.0023	0.0155 (0.0193)
- System			0.0000	0.0000 (0.0000)
+ NADPH			0.0572	0.0572 (0.0005)
0.025 mg/mL protein				
Complete	0.0497	0.0573	0.0559	0.0543 (0.0036)
Inhibited	0.0118		0.0128	0.0123 (0.0006)
- NADP	0.0383	0.0127	0.0009	0.0173 (0.0166)
- System			0.0001	0.0001 (0.0000)
+ NADPH			0.0541	0.0541 (0.0001)
0.0125 mg/mL protein				
Complete	0.0488	0.0546	0.0538	0.0524 (0.0028)
Inhibited	0.0111		0.0126	0.0119 (0.0009)
- NADP	0.0382	0.0101	0.0055	0.0179 (0.0155)
- System			0.0001	0.0001 (0.0001)
+ NADPH			0.0516	0.0516 (0.0008)

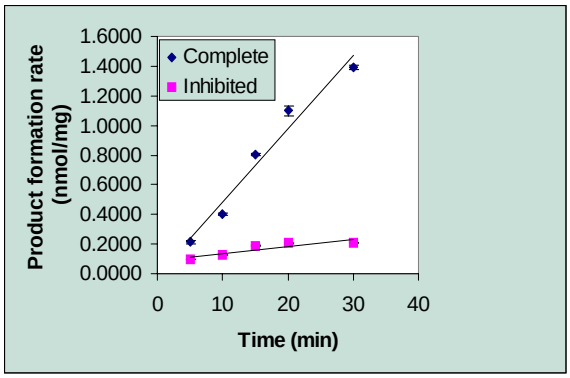
4.3.2 Aromatase Optimization Supplemental Studies: Experiments 2 and 4: Linearity of Product Production

For this work, the protein concentration was fixed at 0.0125 mg/mL (human placental microsomes, Experiment 2) or 0.005 mg/mL (human recombinant microsomes, Experiment 4), the [^{3}H]ASDN concentration was 100 nM and NADPH concentration was 0.3 mM. Some tubes also contained 100 nM 4-OH ASDN as an inhibitor. The response of the assay to various incubation times (5-30 min) was explored.

The results for Experiment 2 and 4 are presented in Table 22 and Figures 3 and 4. The aromatase activities measured at each time point in the presence and the absence of the inhibitor are presented in Figure 4. In both microsome preparations, product formation increased linearly with time in both the presence and absence of inhibitor. The activity was inhibited by 100 nM 4-OH ASDN (both microsome sources). The extent of inhibition increased with time for this suicide inhibitor.

Whether or not NADPH became limiting during the course of the reaction was tested by the addition of a second aliquot (0.3 mM final concentration) of NADPH midway through a 30 min incubation period of some runs. Table 23 shows a comparison of the activities measured in the NADPH-supplemented and -unsupplemented cases. There was no increase in aromatase activity in either the presence or absence of inhibitor in the supplemented versus the unsupplemented case. Therefore, a concentration of 0.3 mM NADPH was determined to be sufficient to support full aromatase activity over at least a 30 min reaction time.

Human Placental



Recombinant

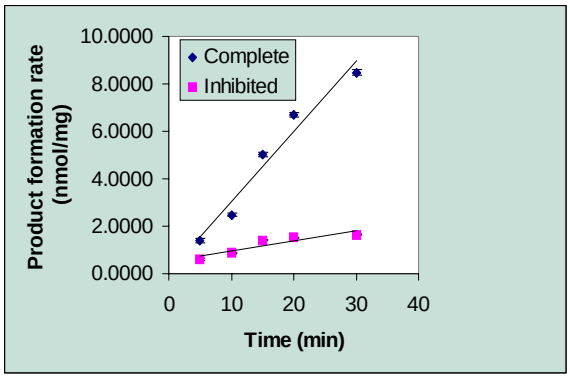
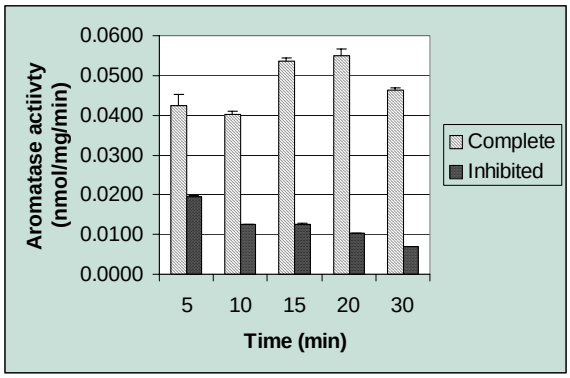


Figure 3. Linearity of Product Formation with Time

Human Placental



Recombinant

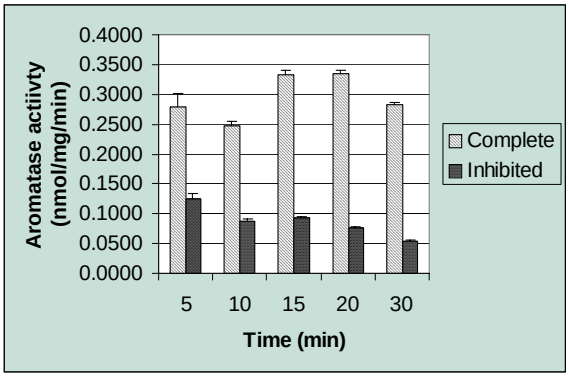


Figure 4: Aromatase Activity at Various Incubation Times

Table 22. Human Placental Assay: Response of Activity to Incubation Time

Time (min)	Product formation rate (nmol/mg)				Aromatase activity (nmol/mg/min)				% substrate conversion (complete assay)	% Inhibition
	Total activity		Inhibited		Total activity		Inhibited			
	average	SD	average	SD	average	SD	average	SD		
	Human Placental microsomes									
5	0.2124	0.0130	0.0981	0.0008	0.0425	0.0026	0.0196	0.0002	2.73	53.8
10	0.4025	0.0090	0.1245	0.0005	0.0403	0.0009	0.0124	0.0000	5.11	69.1
15	0.8039	0.0108	0.1890	0.0053	0.0536	0.0007	0.0126	0.0004	10.16	76.5
20	1.0987	0.0345	0.2054	0.0029	0.0549	0.0017	0.0103	0.0001	13.84	81.3
30	1.3922	0.0138	0.2085	0.0020	0.0464	0.0005	0.0070	0.0001	17.53	85.0
	Recombinant microsomes									
5	1.3972	0.1108	0.6196	0.0505	0.2794	0.0222	0.1239	0.0101	6.90	55.7
10	2.4712	0.0733	0.8665	0.0373	0.2471	0.0073	0.0866	0.0037	12.16	64.9
15	5.0069	0.0999	1.4029	0.0234	0.3338	0.0067	0.0935	0.0016	24.56	72.0
20	6.7141	0.0906	1.5180	0.0331	0.3357	0.0045	0.0759	0.0017	32.91	77.4
30	8.4794	0.1021	1.6452	0.0130	0.2826	0.0034	0.0548	0.0004	41.55	80.6

Table 23. Comparison of Aromatase Activity with and without NADPH Supplementation

	Aromatase activity (nmol/mg/min)			
	Total activity		Inhibited	
	average	SD	average	SD
Human Placental microsomes				
Unsupplemented	0.0464	0.0005	0.0070	0.0001
Supplemented	0.0445	0.0007	0.0071	0.0001
Recombinant microsomes				
Unsupplemented	0.2826	0.0034	0.0548	0.0004
Supplemented	0.2779	0.062	0.0543	0.0000

4.3.3 Aromatase Optimization Supplemental Studies: Experiments 3 and 5: Inhibition Curve Using 4-OH ASDN

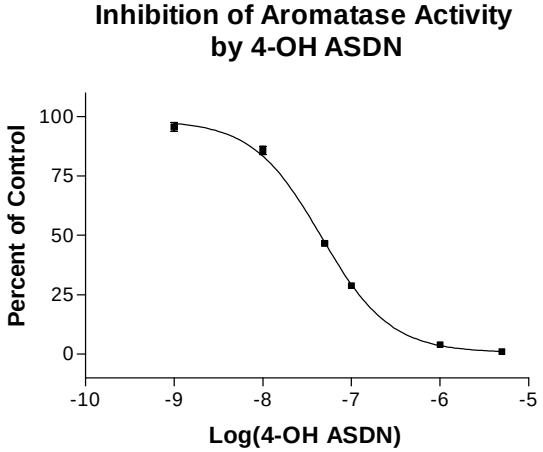
In this task, the assay conditions were set at 100 nM [^{3}H]ASDN, 0.3 mM NADPH, 0.0125 mg/mL human placental microsomal protein or 0.004 mg/mL human recombinant microsomal protein and a 15 min incubation time. The effect of varying concentrations of the inhibitor 4-OH ASDN on aromatase activity was determined. The aromatase activities in the presence of inhibitor were converted to percent of control activities. These percent of control activities and their respective inhibitor concentrations were fitted to a non-linear regression equation using Prism (Version 3.02) software.

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

where:

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

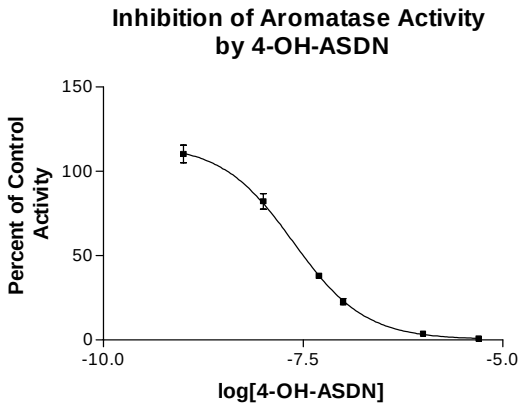
The response curves and IC_{50} data are presented in Figures 5 and 6 for the human placental and human recombinant assays, respectively. The calculated IC_{50} for the human placental microsomes, 46 nM, falls within the 30-50 nM range reported in the protocol. The calculated IC_{50} for the human recombinant microsomes, 24 nM, is near the reported range. The aromatase activities measured over the course of the Supplemental Studies are presented in Table 24 and Figure 7. Day to day variation within the human placental assay was very low. Somewhat higher variation was found in the recombinant assay.



Equation 1	
Best-fit values	
BOTTOM	0.6545
TOP	98.49
LOGEC50	-7.340
HILLSLOPE	-1.122
EC50	4.5720e-008
Std. Error	
BOTTOM	0.2144
TOP	1.596
LOGEC50	0.02030
HILLSLOPE	0.04674
95% Confidence Intervals	
BOTTOM	0.1946 to 1.114
TOP	95.06 to 101.9
LOGEC50	-7.383 to -7.296
HILLSLOPE	-1.222 to -1.022
EC50	4.1360e-008 to 5.0540e-008
Goodness of Fit	
Degrees of Freedom	14
R^2 (unweighted)	0.9972
Weighted Sum of Squares (1/Y)	1.051
Absolute Sum of Squares	68.45
Sy.x	2.211
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

Figure 5: Determination of IC_{50} for 4-OH ASDN in the Human Placental Aromatase Assay[a]

[a]EC_{50} equals IC_{50}. IC_{50} expressed in M.



Equation 1	
Best-fit values	
BOTTOM	0.2224
TOP	115.7
LOGEC50	-7.622
HILLSLOPE	-0.9707
EC50	2.3880e-008
Std. Error	
BOTTOM	0.2409
TOP	2.783
LOGEC50	0.03317
HILLSLOPE	0.04416
95% Confidence Intervals	
BOTTOM	-0.2944 to 0.7392
TOP	109.8 to 121.7
LOGEC50	-7.693 to -7.551
HILLSLOPE	-1.065 to -0.8760
EC50	2.0270e-008 to 2.8130e-008
Goodness of Fit	
Degrees of Freedom	14
R^2 (unweighted)	0.9963
Weighted Sum of Squares (1/Y)	1.637
Absolute Sum of Squares	110.3
Sy.x	2.807
Data	
Number of X values	6
Number of Y replicates	3
Total number of values	18
Number of missing values	0

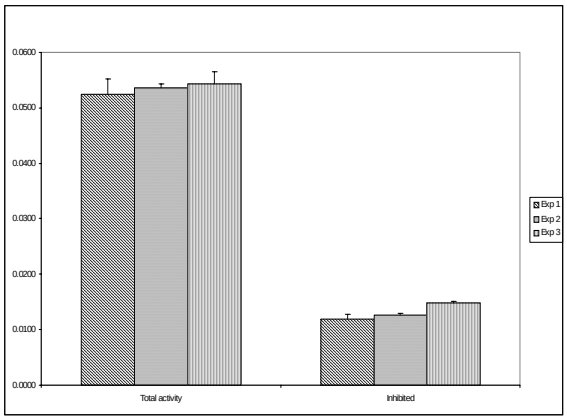
Figure 6: Determination of IC_{50} for 4-OH ASDN in the Human Recombinant Aromatase Assay[a]

[a]EC_{50} equals IC_{50}. IC_{50} expressed in M.

Table 24. Aromatase Activity for Aromatase Supplemental Studies: Experiments 1-5

Experiment	Aromatase activity (nmol/mg/min)			
	Total activity		Inhibited	
	average	SD	average	SD
	Human Placental			
1	0.0524	0.0028	0.0119	0.0009
2	0.0536	0.0007	0.0126	0.0004
3	0.0543	0.0022	0.0148	0.0003
	Recombinant			
4	0.3338	0.0067	0.0935	0.0016
5	0.4429	0.0027	0.0865	0.0066

Human Placental Microsomes



Recombinant Microsomes

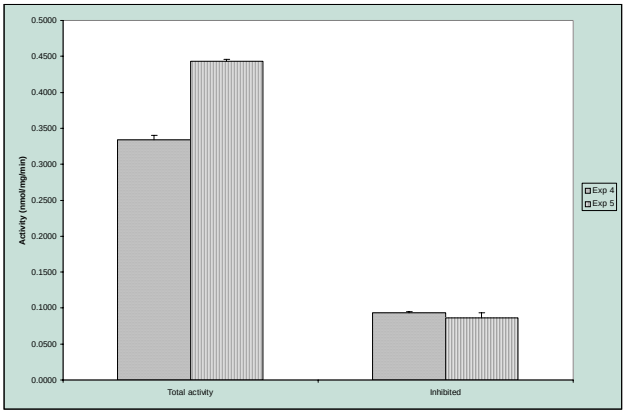


Figure 7. Day to Day Aromatase Activity (nmol/mg/min) in the Supplemental Studies

The optimized assay conditions for the human placental and human recombinant aromatase assay are summarized in Table 25 below. These conditions were used in the subsequent variance assessment and the test substance phases of the project.

Table 25. Optimized Assay Conditions for Aromatase

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

4.4 Variance Testing of the Optimized Assay

The optimized conditions for the human placental and recombinant assays were used in the variability assessment of the assays. Three technicians conducted each assay independently on three separate days. In addition, Technician 3 ran the assay using the diluted microsome samples prepared by Technicians 1 and 2 each day and using all other co-factors prepared by Technician 3. The activity results are presented in Tables 26 and 27 for the placental and recombinant assays, respectively. There is little variation in activities determined within a technician on a given day. Variability between technicians was marked but not significant (Tables 28 and 30). Day to day variation was statistically significant ($p<0.1$, Table 29 and 31). Possible sources of this variation include 1) variation in protein concentration in final dilution of microsomes, 2) errors in substrate preparation and 3) technician technique. Because the protein concentration in the final microsome dilution (that is actually used in the assay) is below the bounds of the protein standard curve, protein concentration was assayed 1 or 2 dilution steps upstream of the final dilution and the protein concentration in the final dilution was then calculated using the appropriate dilution factors. The activities determined by Technician 2 on Day 5 (both placental and recombinant - a single assay day) were very different than those determined by that technician on other days and from those determined by the other technicians on Day 5. Also, the activities determined by Technician 3 on Day 5 using the protein dilution prepared by Technician 2, but with other co-factors prepared by Technician 3 are more similar to the mean of the activities from the other days than to those measured by Technician 2 on Day 5. Since even minimal amounts of NADPH will provide enough reducing equivalents to drive the assay, it is most likely that the low activities measured by Technician 2 on Day 5 are related to a substrate preparation error. The substrate solution was prepared by combining a solution of non-radiolabeled ASDN with a solution of [^{3}H]ASDN to prepare a final solution with the appropriate ASDN concentration and specific activity. Improper mixing or dilution of the non-radiolabeled ASDN could have led to a final substrate solution with a specific activity that was different from that used in the calculations (which was based on ASDN mass and LSS analysis of the substrate solution to determine radiochemical content). This type of error could be eliminated by the preparation of a stock [^{3}H]ASDN of a suitable specific activity for use directly in the substrate solution. Variability between technicians using the same microsome dilution on a given day was usually less than 10%, so it appears that technician technique is uniform and that most of the variation seen between technicians and days may be related to variation in final protein concentrations.

Table 26. Aromatase Activity Measured in the Placenta Assay

Technician	Day	Replicate	Activity (nmol/mg/min)	Technician 3 determined Activity (nmol/mg/min)[a]	Mean and SD[b]
1	3	1	0.0342	0.0411	
		2	0.0351	0.0422	
		3	0.0337	0.0422	
		Mean	0.0343	0.0418	0.0381
		SD	0.0007	0.0006	0.0041
1	4	1	0.0376	0.0467	
		2	0.0392	0.0452	
		3	0.0473	0.0439	
		Mean	0.0414	0.0453	0.0433
		SD	0.0052	0.0014	0.0040
1	5	1	0.0565	0.0515	
		2	0.0529	0.0520	
		3	0.0560	0.0480	
		Mean	0.0551	0.0505	0.0528
		SD	0.0020	0.0022	0.0031
2	3	1	0.0750	0.0673	
		2	0.0723	0.0672	
		3	0.0714	0.0633	
		Mean	0.0729	0.0660	0.0694
		SD	0.0018	0.0023	0.0042
2	4	1	0.0600	0.0534	
		2	0.0598	0.0536	
		3	0.0581	0.0471	
		Mean	0.0593	0.0514	0.0553
		SD	0.0010	0.0036	0.0050
2	5	1	0.0181	0.0370	
		2	0.0185	0.0373	
		3	0.0178	0.0354	
		Mean	0.0181	0.0366	0.0273
		SD	0.0003	0.0010	0.0101
3	3	1	0.0846		
		2	0.0896		
		3	0.0845		
		Mean	0.0862		
		SD	0.0029		
3	4	1	0.0800		
		2	0.0826		
		3	0.0818		
		Mean	0.0815		
		SD	0.0013		
3	5	1	0.0643		
		2	0.0650		
		3	0.0664		
		Mean	0.0652		
		SD	0.0011		

[a]Activity determined by Technician 3 using the same protein samples as the subject Technician
[b]Mean and SD across activity determinations by Technician 3 and subject Technician on a given day.

Table 27. Aromatase Activity Measured in the Recombinant Assay

Technician	Day	Replicate	Activity (nmol/mg/min)	Technician 3 determined Activity (nmol/mg/min)[a]	Mean and SD[b]
1	3	1	0.2623	0.2824	
		2	0.2499	0.2888	
		3	0.2498	0.2827	
		Mean	0.2540	0.2846	0.2693
		SD	0.0072	0.0036	0.0176
1	4	1	0.2124	0.2266	
		2	0.2094	0.2369	
		3	0.2118	0.2347	
		Mean	0.2112	0.2327	0.2220
		SD	0.0016	0.0054	0.0123
1	5	1	0.3260	0.3135	
		2	0.2888	0.3134	
		3	0.3110	0.3136	
		Mean	0.3086	0.3135	0.3111
		SD	0.0187	0.0001	0.0122
2	3	1	0.3792	0.3086	
		2	0.3698	0.3078	
		3	0.3461	0.2742	
		Mean	0.3650	0.2969	0.3309
		SD	0.0170	0.0196	0.0408
2	4	1	0.2996	0.2588	
		2	0.2950	0.2647	
		3	0.3196	0.2667	
		Mean	0.3048	0.2634	0.2841
		SD	0.0131	0.0041	0.0242
2	5	1	0.0837	0.2143	
		2	0.0868	0.2229	
		3	0.0914	0.1992	
		Mean	0.0873	0.2121	0.1497
		SD	0.0038	0.0120	0.0688
3	3	1	0.2759		
		2	0.2757		
		3	0.2746		
		Mean	0.2754		
		SD	0.0007		
3	4	1	0.4364		
		2	0.4257		
		3	0.4182		
		Mean	0.4268		
		SD	0.0091		
3	5	1	0.4096		
		2	0.3972		
		3	0.3897		
		Mean	0.3988		
		SD	0.0100		

[a]Activity determined by Technician 3 using the same protein samples as the subject Technician
[b]Mean and SD across activity determinations by Technician 3 and subject Technician on a given day.

Table 28. Tests for Technician Variability

Type	Effect	Num DF	Den DF	F Value	Pr > F
Placental	Technician	2	6	2.81	0.1378
Recombinant	Technician	2	6	1.24	0.3530

Table 29. Tests for Day-to-Day Variation within Technicians

Type	Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr > |Z|
Placental	Day	Technician	0.000347	0.000201	1.72	0.0424
Placental	Residual		5.247E-6	1.749E-6	3.00	0.0013
Recombinant	Day	Technician	0.01003	0.005815	1.73	0.0422
Recombinant	Residual		0.000118	0.000039	3.00	0.0013

Table 30. Tests for Technician Variability for Differences Between Techs Using Same Solutions

Type	Effect	Num DF	Den DF	F Value	Pr > F
Placental	Technician	1	4	0.01	0.9142
Recombinant	Technician	1	4	0.05	0.8301

Table 31. Tests for Day-to-Day Variation Within Technicians for Differences Between Techs Using Same Solutions

Type	Cov Parm	Subject	Estimate	Standard Error	Z Value	Pr > |Z|
Placental	Day	Technician	0.000128	0.000093	1.37	0.0846
Placental	Residual		0.000011	4.454E-6	2.45	0.0072
Recombinant	Day	Technician	0.005503	0.003925	1.40	0.0805
Recombinant	Residual		0.000145	0.000059	2.45	0.0072

4.5 Determination of the Response of the Optimized Assay to Selected Test Substances

The aromatase assay was conducted using human placental microsomes and recombinant microsomes. Aromatase activity was measured in the presence of various concentrations of 11 different test substances in order to determine the response of the assay to these known inhibitors and non-inhibitors. Test substance groupings (Table 5) were made based on solubility

and whether the chemicals were expected to be inhibitors. Some inhibitors and some non-inhibitors were included in each group. On a given day, a group of test substances was run in singlet over the entire test range (as defined in the protocol). Each group of test substances was run on each of four different days. See Table 32 for a key to Group/Day testing order. On each day of testing a positive control (full assay with no test substance) and a negative control (assay minus NADPH) were run in quadruplicate with each quadruplicate set split so that half were conducted at the beginning of the test substance set and half at the end. Group 3 consisted of some chemicals that were soluble in EtOH and some that were soluble in DMSO. For this group only, a doubled set of positive and negative controls (quadruplicate each with DMSO and EtOH) were run. All placental assays were conducted by a single technician and all recombinant assays were conducted by a different technician. The assays were conducted on concurrent days so that, for example, Group 1, Day 1 of the placental and recombinant assays were conducted on the same day. The technicians shared a complete set of test substance dilutions on each day. Each technician prepared the ASDN substrate solution each day using a common stock (prepared fresh each day) of ASDN and [^{3}H]ASDN.

Table 32. Key to Test Substance Groups Conducted on Each Day

Assay Day	Group/Day
1	Group 1/ Day 1
2	Group 2/ Day 1
3	Group 1/ Day 2
4	Group 2/ Day 2
5	Group 3/Day 1
6	Group 1/ Day 3
7	Group 3/ Day 2
8	Group 2/Day 3
9	Group 3/ Day 3
10	Group 1/ Day 4
11	Group 2/ Day 4
12	Group 3/ Day 4

4.5.1 Control Analysis

Control activities were calculated and comparisons were made as described in protocol Section 4.4.3.1. The average beginning (and end) positive control activity was calculated for each Group/Day/Protein type. These average positive control data are presented in Figures 8 and 9 for the placental and recombinant assay, respectively. Positive and negative control mean

activities and standard deviations for both positive and negative controls are presented in Table 33.

From Figure 8, it is clear that the Group 1/Day 2 and Group 2/Day 1 positive control activities are significantly higher than the mean activity of the remaining sets. HPLC analysis of the entire set of substrate solutions used in the placental assay indicates a markedly higher ratio of the [^{3}H]ASDN peak height to that of the non-radiolabeled ASDN in those substrate solutions used for the Group 1/Day 2 and Group 2/Day 1 assays than was found in the substrate solutions used for all other Group/Day combinations. Indeed, Group 1/ Day 2 and Group 2/Day 1 substrate solutions had a higher specific activity (approximately 47 and 54 µCi/µg, respectively) than that used (1.503 and 1.684 µCi/µg, respectively) in the activity calculations. The specific activity used in aromatase activity calculations was based on the mass of ASDN dissolved in a given volume and the measured (by LSS) [^{3}H]ASDN in the final substrate solution. If the ASDN solution was incompletely mixed at some point in the dilution procedure, less mass of ASDN may have been added to the final substrate solution than would be expected. Since the aromatase activity is calculated based on the substrate specific activity, use of an erroneously low specific activity (µCi/nmol) would lead to high apparent aromatase activity. This appears to be the cause of the high positive control activities found for the placental Group 1/Day 2 and Group 2/Day 1 positive control activities.

Figure 9 indicates a large variance in the recombinant positive control data. No substrate solutions were available from this set for HPLC analysis of specific activity. It is likely that the high control activities found for Group 1/Day 2 and Group 2/Day 1 result from a substrate solution preparation error as described above, since the same ASDN dilutions were used in the preparation of both the recombinant and placental substrate solutions. It appears that the positive control activities did converge to a fairly consistent value by the sixth assay day (Group 1/Day 3, see Table 32). It appears, therefore, that it is important for the technician conducting the assays to have adequate time to practice the assay prior to conduct of the definitive assay. It may be that this phenomenon is not apparent in the placental assay because that technician had already gained experience with the assay prior to start of the test substance series.

Results of ANOVA for the control data are presented in Table 34. Whereas some of the negative control activities showed statistically significant differences between portion or in group and portion interactions, these differences are probably not of any practical importance since all of the negative control activities were essentially zero. Significant variances were detected for all sources in the positive control data. These variances may not have had a significant effect on calculation of IC_{50} values since all data are analyzed as percent of control.

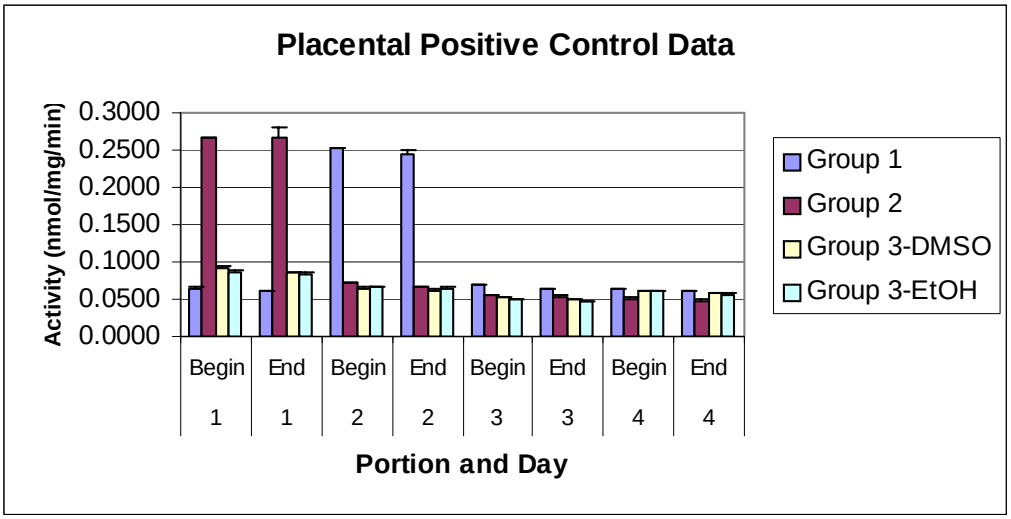


Figure 8. Mean Placental Positive Control Activities by Day and Portion

n = 2 for each bar

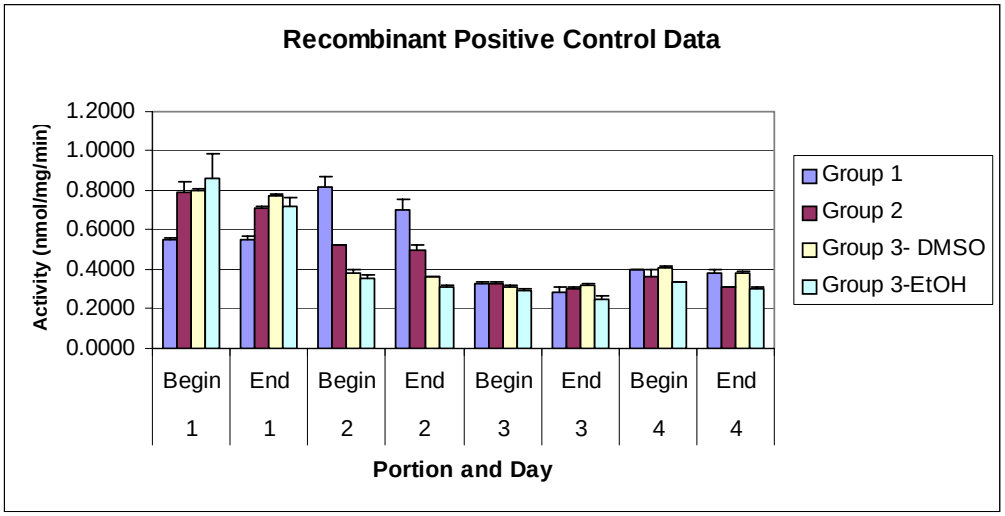


Figure 9. Mean Recombinant Positive Control Activities by Day and Portion

n = 2 for each bar

Table 33. Mean and Standard Deviations of Control Activities (nmol/mg/min)

Microsome Type	Control Type	Group	Portion of Batch	Batch 1		Batch 2		Batch 3		Batch 4	
				Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
Placental	Negative	1	Begin	-0.0013	0.0000	-0.0004	0.0006	0.0000	0.0000	0.0000	0.0000
Placental	Negative	1	End	0.0013	0.0008	0.0004	0.0001	0.0000	0.0000	0.0000	0.0001
Placental	Negative	2	Begin	-0.0026	0.0018	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Placental	Negative	2	End	0.0026	0.0076	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000
Placental	Negative	3-DMSO	Begin	-0.0003	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Placental	Negative	3-DMSO	End	0.0003	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
Placental	Negative	3-EtOH	Begin	-0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
Placental	Negative	3-EtOH	End	0.0002	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000	0.0000
Placental	Positive	1	Begin	0.0647	0.0021	0.2520	0.0016	0.0695	0.0001	0.0629	0.0009
Placental	Positive	1	End	0.0598	0.0020	0.2444	0.0063	0.0646	0.0003	0.0610	0.0015
Placental	Positive	2	Begin	0.2655	0.0005	0.0715	0.0016	0.0549	0.0005	0.0504	0.0010
Placental	Positive	2	End	0.2657	0.0142	0.0666	0.0012	0.0538	0.0005	0.0474	0.0016
Placental	Positive	3-DMSO	Begin	0.0917	0.0028	0.0647	0.0022	0.0519	0.0003	0.0606	0.0002
Placental	Positive	3-DMSO	End	0.0862	0.0011	0.0615	0.0021	0.0493	0.0008	0.0577	0.0010
Placental	Positive	3-EtOH	Begin	0.0863	0.0015	0.0670	0.0009	0.0499	0.0014	0.0610	0.0008
Placental	Positive	3-EtOH	End	0.0843	0.0005	0.0645	0.0029	0.0471	0.0006	0.0566	0.0009
Recombinant	Negative	1	Begin	0.0000	0.0003	0.0000	0.0001	0.0000	0.0001	0.0000	0.0001
Recombinant	Negative	1	End	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001	0.0000	0.0002
Recombinant	Negative	2	Begin	-0.0001	0.0001	0.0000	0.0000	-0.0001	0.0000	0.0000	0.0000
Recombinant	Negative	2	End	0.0001	0.0001	0.0000	0.0000	0.0001	0.0002	0.0000	0.0001
Recombinant	Negative	3-DMSO	Begin	0.0000	0.0001	0.0000	0.0000	0.0001	0.0000	0.0002	0.0003
Recombinant	Negative	3-DMSO	End	0.0000	0.0001	0.0000	0.0002	-0.0001	0.0000	-0.0002	0.0001
Recombinant	Negative	3-EtOH	Begin	0.0001	0.0001	0.0000	0.0001	0.0001	0.0001	0.0001	0.0000
Recombinant	Negative	3-EtOH	End	-0.0001	0.0002	0.0000	0.0001	-0.0001	0.0001	-0.0001	0.0001
Recombinant	Positive	1	Begin	0.5545	0.0023	0.8213	0.0478	0.3260	0.0116	0.3985	0.0040
Recombinant	Positive	1	End	0.5483	0.0181	0.7002	0.0547	0.2878	0.0213	0.3781	0.0259
Recombinant	Positive	2	Begin	0.7927	0.0510	0.5220	0.0028	0.3298	0.0050	0.3609	0.0393
Recombinant	Positive	2	End	0.7146	0.0019	0.4973	0.0249	0.3013	0.0131	0.3092	0.0001
Recombinant	Positive	3-DMSO	Begin	0.8042	0.0033	0.3805	0.0185	0.3077	0.0141	0.4126	0.0053
Recombinant	Positive	3-DMSO	End	0.7704	0.0141	0.3627	0.0033	0.3174	0.0084	0.3822	0.0066
Recombinant	Positive	3-EtOH	Begin	0.8588	0.1277	0.3546	0.0207	0.2925	0.0055	0.3346	0.0051
Recombinant	Positive	3-EtOH	End	0.7218	0.0467	0.3102	0.0121	0.2455	0.0180	0.2992	0.0154

Table 34. ANOVA Results for Control Data

Microsome Type	Control Type	Source	DF	Type I SS	Mean Square	F Value	Pr > F
Placental	Negative	Group	3	8.634E-36	2.878E-36	0.00	1.0000
Placental	Negative	Batch(Group)	12	3.242E-35	2.702E-36	0.00	1.0000
Placental	Negative	Portion	1	5.868E-06	5.868E-06	2.96	0.0925
Placental	Negative	Group*Portion	3	4.030E-06	1.343E-06	0.68	0.5708
Placental	Positive	Group	3	5.147E-04	1.716E-04	84.89	<.0001
Placental	Positive	Batch(Group)	10	7.091E-03	7.091E-04	350.86	<.0001
Placental	Positive	Portion	1	1.541E-04	1.541E-04	76.26	<.0001
Placental	Positive	Group*Portion	3	2.255E-06	7.516E-07	0.37	0.7738
Recombinant	Negative	Group	3	2.660E-37	8.865E-38	0.00	1.0000
Recombinant	Negative	Batch(Group)	12	1.652E-36	1.376E-37	0.00	1.0000
Recombinant	Negative	Portion	1	8.330E-09	8.330E-09	0.59	0.4481
Recombinant	Negative	Group*Portion	3	1.222E-07	4.072E-08	2.86	0.0474
Recombinant	Positive	Group	3	4.673E-02	1.558E-02	13.67	<.0001
Recombinant	Positive	Batch(Group)	12	2.250E+00	1.875E-01	164.56	<.0001
Recombinant	Positive	Portion	1	3.107E-02	3.107E-02	27.26	<.0001
Recombinant	Positive	Group*Portion	3	4.644E-03	1.548E-03	1.36	0.2680

4.5.2 Test Substance Response Curves

Aromatase activities in the presence of the various test substances were converted to percent of full activity (positive control). The aromatase activity and percent of full activity data are presented in Tables 35 and 36 for the placental and recombinant assay, respectively. In some instances, the test chemicals were not soluble at the stated concentrations in the assay mixture. Aromatase activities measured in those cases (noted in Tables 35 and 36) were excluded from the set of data from which response curves were generated. The data was fitted to the model presented in Section 4.3.3. The resulting response curves are presented in Figures 10 and 11 for the placental and recombinant assay, respectively.

Table 35. Placental Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals

Chemical	Level	Concentration (M)	Log (concentration)	Day 1		Day 2		Day 3		Day 4	
				Activity	% Full activity	Activity	% Full activity	Activity	% Full activity	Activity	% Full activity
Econazole	1	1.00E-06	-6.00	-0.0012	-1.88	-0.0018	-0.73	0.0004	0.56	0.0001	0.18
Econazole	2	5.00E-07	-6.30	-0.0012	-1.88	-0.0017	-0.69	0.0005	0.68	0.0002	0.35
Econazole	3	2.50E-07	-6.60	-0.0008	-1.34	-0.0015	-0.61	0.0008	1.18	0.0005	0.80
Econazole	4	1.00E-07	-7.00	-0.0001	-0.24	-0.0005	-0.21	0.0018	2.73	0.0014	2.04
Econazole	5	5.00E-08	-7.30	0.0010	1.61	0.0007	0.30	0.0034	5.10	0.0031	4.60
Econazole	6	2.50E-08	-7.60	0.0032	5.17	0.0038	1.52	0.0063	9.33	0.0050	7.53
Econazole	7	1.00E-08	-8.00	0.0089	14.23	0.0115	4.65	0.0134	19.98	0.0116	17.37
Econazole	8	1.00E-09	-9.00	0.0422	67.83	0.0941	37.91	0.0506	75.49	0.0456	68.08
Genistein	1	1.00E-03*	-3.00	0.0414	66.55	0.0887	35.75	0.0508	75.85	0.0394	58.76
Genistein	2	5.00E-04*	-3.30	0.0431	69.18	0.1123	45.26	0.0565	84.33	0.0467	69.66
Genistein	3	2.50E-04	-3.60	0.0471	75.64	0.1323	53.30	0.0569	84.94	0.0500	74.62
Genistein	4	1.00E-04	-4.00	0.0564	90.65	0.1987	80.08	0.0605	90.29	0.0551	82.20
Genistein	5	5.00E-05	-4.30	0.0591	94.95	0.2305	92.90	0.0685	102.27	0.0618	92.26
Genistein	6	2.50E-05	-4.60	0.0589	94.55	0.2463	99.25	0.0705	105.14	0.0609	90.85
Genistein	7	1.00E-05	-5.00	0.0603	96.81	0.2502	100.83	0.0682	101.75	0.0624	93.07
Genistein	8	1.00E-06	-6.00	0.0606	97.29	0.2475	99.73	0.0674	100.50	0.0596	88.96
Atrazine	1	1.00E-03*	-3.00	0.0560	89.92	0.1550	62.47	0.0632	94.23	0.0555	82.81
Atrazine	2	1.00E-04	-4.00	0.0607	97.51	0.2214	89.21	0.0674	100.56	0.0642	95.76
Atrazine	3	1.00E-05	-5.00	0.0624	100.20	0.2527	101.83	0.0676	100.85	0.0610	90.98
Atrazine	4	1.00E-06	-6.00	0.0621	99.77	0.2548	102.68	0.0656	97.83	0.0640	95.49
Atrazine	5	1.00E-07	-7.00	0.0617	99.03	0.2539	102.33	0.0693	103.42	0.0635	94.69
Atrazine	6	1.00E-08	-8.00	0.0621	99.74	0.2491	100.38	0.0682	101.71	0.0616	91.97
Atrazine	7	1.00E-09	-9.00	0.0613	98.41	0.2501	100.78	0.0673	100.37	0.0611	91.16
bis(2-ethylhexyl)phthalate	1	1.00E-03	-3.00	0.0528	84.87	0.2248	90.59	0.0638	95.26	0.0596	88.93
bis(2-ethylhexyl)phthalate	2	1.00E-04	-4.00	0.0598	95.98	0.2478	99.85	0.0665	99.28	0.0608	90.74
bis(2-ethylhexyl)phthalate	3	1.00E-05	-5.00	0.0165	26.47	0.2536	102.21	0.0674	100.56	0.0639	95.38
bis(2-ethylhexyl)phthalate	4	1.00E-06	-6.00	0.0640	102.83	0.2610	105.15	0.0673	100.46	0.0631	94.09
bis(2-ethylhexyl)phthalate	5	1.00E-07	-7.00	0.0633	101.67	0.2474	99.68	0.0674	100.56	0.0625	93.25
bis(2-ethylhexyl)phthalate	6	1.00E-08	-8.00	0.0631	101.38	0.2596	104.61	0.0651	97.16	0.0607	90.61
bis(2-ethylhexyl)phthalate	7	1.00E-09	-9.00	0.0614	98.58	0.2426	97.77	0.0639	95.27	0.0598	89.18

(continued)

Table 35. Placental Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals (continued)

Chemical	Level	Concentration (M)	Log (concentration)	Day 1		Day 2		Day 3		Day 4	
				Activity	% Full activity	Activity	% Full activity	Activity	% Full activity	Activity	% Full activity
Aminoglutethimide	1	1.00E-03	-3.00	-0.0094	-3.55	0.0005	0.75	0.0004	0.68	0.0003	0.65
Aminoglutethimide	2	1.00E-04	-4.00	-0.0036	-1.37	0.0048	6.98	0.0038	7.07	0.0033	6.79
Aminoglutethimide	3	5.00E-05	-4.30	0.0012	0.45	0.0087	12.63	0.0069	12.65	0.0066	13.43
Aminoglutethimide	4	2.50E-05	-4.60	0.0104	3.91	0.0179	25.97	0.0125	23.07	0.0110	22.57
Aminoglutethimide	5	1.00E-05	-5.00	0.0417	15.68	0.0304	44.04	0.0234	43.07	0.0229	46.88
Aminoglutethimide	6	1.00E-06	-6.00	0.1817	68.39	0.0596	86.33	0.0477	87.84	0.0461	94.26
Aminoglutethimide	7	1.00E-07	-7.00	0.2568	96.67	0.0693	100.39	0.0548	100.86	0.0514	105.12
Aminoglutethimide	8	1.00E-08	-8.00	0.2640	99.39	0.0700	101.29	0.0557	102.43	0.0491	100.44
Chrysin	1	1.00E-03*	-3.00	0.0231	8.71	0.0223	32.22	0.0181	33.25	0.0160	32.64
Chrysin	2	1.00E-04*	-4.00	0.0256	9.62	0.0239	34.53	0.0195	35.91	0.0178	36.45
Chrysin	3	5.00E-05	-4.30	0.0167	6.30	0.0142	20.60	0.0078	14.34	0.0126	25.87
Chrysin	4	2.50E-05	-4.60	0.0075	2.84	0.0133	19.30	0.0104	19.22	0.0088	17.99
Chrysin	5	1.00E-05	-5.00	0.0292	11.01	0.0254	36.71	0.0208	38.29	0.0179	36.67
Chrysin	6	1.00E-06	-6.00	0.1660	62.50	0.0593	85.90	0.0499	91.83	0.0418	85.42
Chrysin	7	1.00E-07	-7.00	0.2548	95.91	0.0667	96.56	0.0565	103.99	0.0477	97.56
Chrysin	8	1.00E-08	-8.00	0.2717	102.28	0.0677	97.97	0.0553	101.79	0.0482	98.66
Nonylphenol	1	1.00E-03*	-3.00	-0.0098	-3.68	0.0000	0.06	0.0000	0.09	-0.0001	-0.15
Nonylphenol	2	1.00E-04*	-4.00	-0.0001	-0.02	0.0022	3.15	0.0020	3.62	0.0018	3.59
Nonylphenol	3	1.00E-05	-5.00	0.1742	65.59	0.0594	85.99	0.0465	85.53	0.0429	87.82
Nonylphenol	4	1.00E-06	-6.00	0.2572	96.83	0.0694	100.42	0.0578	106.43	0.0485	99.29
Nonylphenol	5	1.00E-07	-7.00	0.2671	100.55	0.0669	96.93	0.0551	101.42	0.0473	96.72
Nonylphenol	6	1.00E-08	-8.00	0.2803	105.53	0.0689	99.78	0.0580	106.76	0.0479	97.92
Nonylphenol	7	1.00E-09	-9.00	0.2616	98.47	0.0690	99.88	0.0535	98.41	0.0479	97.92
Lindane	1	1.00E-03*	-3.00	0.2126	80.02	0.0611	88.50	0.0504	92.75	0.0378	77.37
Lindane	2	1.00E-04	-4.00	0.2127	80.09	0.0662	95.82	0.0515	94.73	0.0442	90.41
Lindane	3	1.00E-05	-5.00	0.2577	97.02	0.0655	94.87	0.0549	101.00	0.0478	97.76
Lindane	4	1.00E-06	-6.00	0.2688	101.19	0.0666	96.47	0.0574	105.59	0.0472	96.61
Lindane	5	1.00E-07	-7.00	0.2673	100.65	0.0656	94.91	0.0563	103.58	0.0448	91.73
Lindane	6	1.00E-08	-8.00	0.2719	102.34	0.0669	96.85	0.0565	103.92	0.0445	91.13
Lindane	7	1.00E-09	-9.00	0.2622	98.73	0.0672	97.36	0.0549	100.96	0.0477	97.55
Dibenz[*a,h*]anthracene	1	1.00E-04	-4.00	0.0810	91.10	0.0614	97.27	0.0492	97.21	0.0585	99.02
Dibenz[*a,h*]anthracene	2	1.00E-05	-5.00	0.0902	101.36	0.0646	102.41	0.0511	101.02	0.0581	98.22
Dibenz[*a,h*]anthracene	3	1.00E-06	-6.00	0.0873	98.12	0.0646	102.42	0.0499	98.50	0.0604	102.10
Dibenz[*a,h*]anthracene	4	1.00E-07	-7.00	0.0874	98.20	0.0643	101.96	0.0509	100.62	0.0596	100.85
Dibenz[*a,h*]anthracene	5	1.00E-08	-8.00	0.0891	100.14	0.0644	102.04	0.0507	100.09	0.0596	100.77
Dibenz[*a,h*]anthracene	6	1.00E-09	-9.00	0.0865	97.23	0.0635	100.70	0.0498	98.37	0.0580	98.08

(continued)

Table 35. Placental Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals (continued)

Chemical	Level	Concentration (M)	Log (concentration)	Day 1		Day 2		Day 3		Day 4	
				Activity	% Full activity	Activity	% Full activity	Activity	% Full activity	Activity	% Full activity
Ketoconazole	1	8.00E-04	-3.10	-0.0001	-0.09	0.0001	0.19	0.0000	0.07	0.0002	0.34
Ketoconazole	2	5.00E-04	-3.30	0.0016	1.93	0.0004	0.65	0.0002	0.33	0.0003	0.58
Ketoconazole	3	2.50E-04	-3.60	0.0019	2.21	0.0010	1.46	0.0005	1.04	0.0009	1.55
Ketoconazole	4	1.00E-04	-4.00	0.0081	9.48	0.0064	9.69	0.0042	8.56	0.0049	8.35
Ketoconazole	5	5.00E-05	-4.30	0.0143	16.80	0.0116	17.69	0.0078	16.06	0.0111	18.81
Ketoconazole	6	2.50E-05	-4.60	0.0269	31.50	0.0211	32.08	0.0146	30.05	0.0176	29.84
Ketoconazole	7	1.00E-05	-5.00	0.0461	54.05	0.0347	52.80	0.0254	52.29	0.0292	49.69
Ketoconazole	8	1.00E-06	-6.00	0.0789	92.50	0.0575	87.48	0.0454	93.64	0.0524	89.06
4-OH androstenedione	1	1.00E-06	-6.00	0.0030	3.54	0.0025	3.74	0.0020	4.19	0.0026	4.36
4-OH androstenedione	2	5.00E-07	-6.30	0.0058	6.77	0.0044	6.70	0.0038	7.75	0.0044	7.40
4-OH androstenedione	3	2.50E-07	-6.60	0.0116	13.60	0.0081	12.40	0.0072	14.80	0.0086	14.67
4-OH androstenedione	4	1.00E-07	-7.00	0.0237	27.79	0.0176	26.75	0.0148	30.42	0.0171	29.15
4-OH androstenedione	5	5.00E-08	-7.30	0.0399	46.78	0.0297	45.14	0.0242	49.86	0.0286	48.69
4-OH androstenedione	6	2.50E-08	-7.60	0.0332	38.96	0.0411	62.44	0.0343	70.72	0.0404	68.76
4-OH androstenedione	7	1.00E-08	-8.00	0.0729	85.44	0.0527	80.13	0.0432	88.97	0.0492	83.62
4-OH androstenedione	8	1.00E-09	-9.00	0.0846	99.23	0.0584	88.80	0.0499	102.75	0.0567	96.43

* Data were not used because of test substance insolubility in the assay mixture at this concentration.

Table 36. Recombinant Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals

Chemical	Level	Concentration (M)	Log (concentration)	Day 1		Day 2		Day 3		Day 4	
				Activity	% Full activity	Activity	% Full activity	Activity	% Full activity	Activity	% Full activity
Econazole	1	1.00E-06	-6.00	0.0014	0.26	0.0014	0.19	0.0009	0.31	0.0011	0.28
Econazole	2	5.00E-07	-6.30	0.0023	0.41	0.0016	0.21	0.0016	0.53	0.0015	0.38
Econazole	3	2.50E-07	-6.60	0.0048	0.87	0.0042	0.55	0.0028	0.93	0.0033	0.84
Econazole	4	1.00E-07	-7.00	0.0138	2.50	0.0102	1.34	0.0071	2.30	0.0069	1.77
Econazole	5	5.00E-08	-7.30	0.0215	3.90	0.0204	2.68	0.0122	3.96	0.0141	3.62
Econazole	6	2.50E-08	-7.60	0.0435	7.89	0.0327	4.29	0.0306	9.98	0.0256	6.58
Econazole	7	1.00E-08	-8.00	0.1031	18.70	0.1004	13.20	0.0606	19.74	0.0631	16.24
Econazole	8	1.00E-09	-9.00	0.3940	71.46	0.5893	77.47	0.2512	81.84	0.2851	73.42
Genistein	1	1.00E-03*	-3.00	0.2423	43.95	0.3424	45.01	0.1620	52.80	0.1619	41.71
Genistein	2	5.00E-04*	-3.30	0.2983	54.11	0.5291	69.55	0.1903	62.00	0.1957	50.40
Genistein	3	2.50E-04	-3.60	0.4047	73.40	0.6409	84.24	0.2510	81.80	0.2804	72.21
Genistein	4	1.00E-04	-4.00	0.4875	88.42	0.6792	89.28	0.3004	97.90	0.3024	77.87
Genistein	5	5.00E-05	-4.30	0.4836	87.70	0.7720	101.48	0.3131	102.01	0.3738	96.27
Genistein	6	2.50E-05	-4.60	0.5436	98.58	0.8465	111.28	0.3085	100.51	0.3837	98.82
Genistein	7	1.00E-05	-5.00	0.5334	96.74	0.6406	84.20	0.3288	107.15	0.3880	99.92
Genistein	8	1.00E-06	-6.00	0.6050	109.72	0.7188	94.48	0.3043	99.15	0.4139	106.59
Atrazine	1	1.00E-03*	-3.00	0.4423	80.21	0.5360	70.45	0.2144	69.87	0.2763	71.16
Atrazine	2	1.00E-04	-4.00	0.5167	93.70	0.6788	89.23	0.2966	96.66	0.3895	100.31
Atrazine	3	1.00E-05	-5.00	0.5813	105.43	0.7655	100.63	0.3183	103.72	0.3758	96.77
Atrazine	4	1.00E-06	-6.00	0.5600	101.55	0.6320	83.08	0.2962	96.51	0.3814	98.23
Atrazine	5	1.00E-07	-7.00	0.5418	98.27	0.7069	92.92	0.3078	100.29	0.3732	96.11
Atrazine	6	1.00E-08	-8.00	0.5565	100.93	0.7243	95.22	0.3005	97.93	0.3979	102.48
Atrazine	7	1.00E-09	-9.00	0.5264	95.46	0.8338	109.60	0.2857	93.08	0.4115	105.98
bis(2-ethylhexyl)phthalate	1	1.00E-03	-3.00	0.4542	82.37	0.5829	76.62	0.2935	95.64	0.3822	98.43
bis(2-ethylhexyl)phthalate	2	1.00E-04	-4.00	0.5740	104.10	0.8447	111.05	0.3060	99.69	0.4055	104.44
bis(2-ethylhexyl)phthalate	3	1.00E-05	-5.00	0.5415	98.21	0.6084	79.97	0.3159	102.94	0.4330	111.51
bis(2-ethylhexyl)phthalate	4	1.00E-06	-6.00	0.5550	100.65	0.7357	96.71	0.3005	97.92	0.3738	96.25
bis(2-ethylhexyl)phthalate	5	1.00E-07	-7.00	0.5320	96.48	0.6432	84.54	0.2720	88.63	0.3672	94.56
bis(2-ethylhexyl)phthalate	6	1.00E-08	-8.00	0.5348	96.98	0.6658	87.52	0.2926	95.34	0.3479	89.60
bis(2-ethylhexyl)phthalate	7	1.00E-09	-9.00	0.5448	98.80	0.6621	87.04	0.2788	90.85	0.3584	92.31

(continued)

Table 36. Recombinant Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals (continued)

Chemical	Level	Concentration (M)	Log (concentration)	Day 1		Day 2		Day 3		Day 4	
				Activity	% Full activity	Activity	% Full activity	Activity	% Full activity	Activity	% Full activity
Aminoglutethimide	1	1.00E-03	-3.00	0.0026	0.34	0.0024	0.47	0.0017	0.55	0.0021	0.63
Aminoglutethimide	2	1.00E-04	-4.00	0.0297	3.94	0.0273	5.35	0.0161	5.10	0.0208	6.20
Aminoglutethimide	3	5.00E-05	-4.30	0.0548	7.28	0.0454	8.91	0.0330	10.46	0.0361	10.79
Aminoglutethimide	4	2.50E-05	-4.60	0.1061	14.08	0.0960	18.85	0.0652	20.67	0.0560	16.71
Aminoglutethimide	5	1.00E-05	-5.00	0.2515	33.38	0.1700	33.35	0.1075	34.07	0.1071	31.97
Aminoglutethimide	6	1.00E-06	-6.00	0.6070	80.54	0.4007	78.62	0.2779	88.08	0.2830	84.48
Aminoglutethimide	7	1.00E-07	-7.00	0.7206	95.61	0.5079	99.67	0.3239	102.67	0.3304	98.60
Aminoglutethimide	8	1.00E-08	-8.00	0.7253	96.24	0.5162	101.28	0.3202	101.47	0.3495	104.32
Chrysin	1	1.00E-03*	-3.00	0.1507	20.00	0.1286	25.23	0.0790	25.04	0.0803	23.98
Chrysin	2	1.00E-04*	-4.00	0.0655	8.69	0.0663	13.01	0.0646	20.47	0.0477	14.25
Chrysin	3	5.00E-05	-4.30	0.0600	7.97	0.0600	11.77	0.0504	15.97	0.0483	14.40
Chrysin	4	2.50E-05	-4.60	0.0776	10.30	0.0646	12.67	0.0480	15.22	0.0490	14.62
Chrysin	5	1.00E-05	-5.00	0.1975	26.21	0.1368	26.85	0.0997	31.61	0.0955	28.49
Chrysin	6	1.00E-06	-6.00	0.6060	80.41	0.3886	76.26	0.2467	78.19	0.2900	86.57
Chrysin	7	1.00E-07	-7.00	0.8014	106.34	0.5112	100.30	0.3176	100.65	0.3345	99.83
Chrysin	8	1.00E-08	-8.00	0.7909	104.95	0.5036	98.82	0.3303	104.69	0.3401	101.52
Nonylphenol	1	1.00E-03*	-3.00	0.0001	0.01	0.0003	0.06	0.0001	0.03	0.0000	0.01
Nonylphenol	2	1.00E-04*	-4.00	0.0044	0.58	0.0048	0.95	0.0025	0.78	0.0023	0.69
Nonylphenol	3	1.00E-05	-5.00	0.5447	72.27	0.3648	71.57	0.2642	83.72	0.2297	68.57
Nonylphenol	4	1.00E-06	-6.00	0.7642	101.41	0.5206	102.14	0.3299	104.55	0.3305	98.66
Nonylphenol	5	1.00E-07	-7.00	0.7246	96.15	0.5256	103.14	0.3090	97.92	0.3424	102.20
Nonylphenol	6	1.00E-08	-8.00	0.7361	97.67	0.5047	99.04	0.3130	99.21	0.3256	97.18
Nonylphenol	7	1.00E-09	-9.00	0.7326	97.21	0.4807	94.33	0.2988	94.70	0.3403	101.57
Lindane	1	1.00E-03*	-3.00	0.5671	75.24	0.3961	77.73	0.2403	76.17	0.2659	79.37
Lindane	2	1.00E-04	-4.00	0.6349	84.25	0.4666	91.55	0.2622	83.10	0.2994	89.37
Lindane	3	1.00E-05	-5.00	0.6856	90.98	0.4820	94.57	0.2905	92.08	0.3078	91.87
Lindane	4	1.00E-06	-6.00	0.7034	93.33	0.4827	94.71	0.3045	96.51	0.3225	96.25
Lindane	5	1.00E-07	-7.00	0.7234	95.99	0.4934	96.81	0.2960	93.82	0.3361	100.31
Lindane	6	1.00E-08	-8.00	0.7397	98.15	0.5001	98.13	0.3156	100.01	0.3040	90.73
Lindane	7	1.00E-09	-9.00	0.7294	96.78	0.5065	99.38	0.3226	102.25	0.3057	91.23
Dibenz[*a,h*]anthracene	1	1.00E-04	-4.00	0.6839	86.86	0.3218	86.60	0.2915	93.28	0.3482	87.61
Dibenz[*a,h*]anthracene	2	1.00E-05	-5.00	0.8088	102.73	0.3633	97.77	0.3191	102.11	0.3957	99.58
Dibenz[*a,h*]anthracene	3	1.00E-06	-6.00	0.7737	98.26	0.3441	92.60	0.3773	120.73	0.4113	103.50
Dibenz[*a,h*]anthracene	4	1.00E-07	-7.00	0.9105	115.65	0.3681	99.05	0.3308	105.84	0.4353	109.53
Dibenz[*a,h*]anthracene	5	1.00E-08	-8.00	0.9131	115.97	0.3547	95.46	0.3150	100.80	0.4178	105.14
Dibenz[*a,h*]anthracene	6	1.00E-09	-9.00	0.8045	102.19	0.3430	92.31	0.2928	93.70	0.3416	85.95

(continued)

Table 36. Recombinant Aromatase Activity (nmol/mg/min) in the Presence of Various Test Chemicals (continued)

Chemical	Level	Concentration (M)	Log (concentration)	Day 1		Day 2		Day 3		Day 4	
				Activity	% Full activity	Activity	% Full activity	Activity	% Full activity	Activity	% Full activity
Ketoconazole	1	8.00E-04	-3.10	0.0006	0.08	0.0003	0.10	-0.0001	-0.03	0.0002	0.08
Ketoconazole	2	5.00E-04	-3.30	0.0042	0.53	0.0026	0.79	0.0002	0.06	0.0018	0.58
Ketoconazole	3	2.50E-04	-3.60	0.0083	1.05	0.0025	0.74	0.0010	0.37	0.0019	0.60
Ketoconazole	4	1.00E-04	-4.00	0.0280	3.54	0.0219	6.59	0.0147	5.47	0.0180	5.67
Ketoconazole	5	5.00E-05	-4.30	0.0655	8.29	0.0435	13.07	0.0342	12.72	0.0367	11.57
Ketoconazole	6	2.50E-05	-4.60	0.0958	12.12	0.0772	23.24	0.0600	22.31	0.0805	25.40
Ketoconazole	7	1.00E-05	-5.00	0.2439	30.86	0.1433	43.12	0.1194	44.40	0.1308	41.28
Ketoconazole	8	1.00E-06	-6.00	0.6739	85.27	0.2644	79.55	0.2390	88.84	0.2955	93.25
4-OH androstenedione	1	1.00E-06	-6.00	0.0251	3.18	0.0298	8.97	0.0201	7.46	0.0397	12.54
4-OH androstenedione	2	5.00E-07	-6.30	0.0465	5.89	0.0443	13.32	0.0362	13.46	0.0518	16.36
4-OH androstenedione	3	2.50E-07	-6.60	0.0789	9.98	0.0648	19.49	0.0597	22.21	0.1010	31.87
4-OH androstenedione	4	1.00E-07	-7.00	0.1413	17.88	0.1246	37.50	0.0964	35.84	0.1627	51.35
4-OH androstenedione	5	5.00E-08	-7.30	0.2300	29.10	0.1587	47.75	0.1454	54.04	0.2178	68.71
4-OH androstenedione	6	2.50E-08	-7.60	0.3845	48.65	0.1962	59.02	0.1914	71.17	0.2467	77.85
4-OH androstenedione	7	1.00E-08	-8.00	0.5280	66.81	0.2541	76.43	0.2550	94.81	0.2800	88.35
4-OH androstenedione	8	1.00E-09	-9.00	0.6729	85.15	0.2739	82.39	0.2970	110.40	0.3036	95.79

* Data were not used because of test substance insolubility in the assay mixture at this concentration.

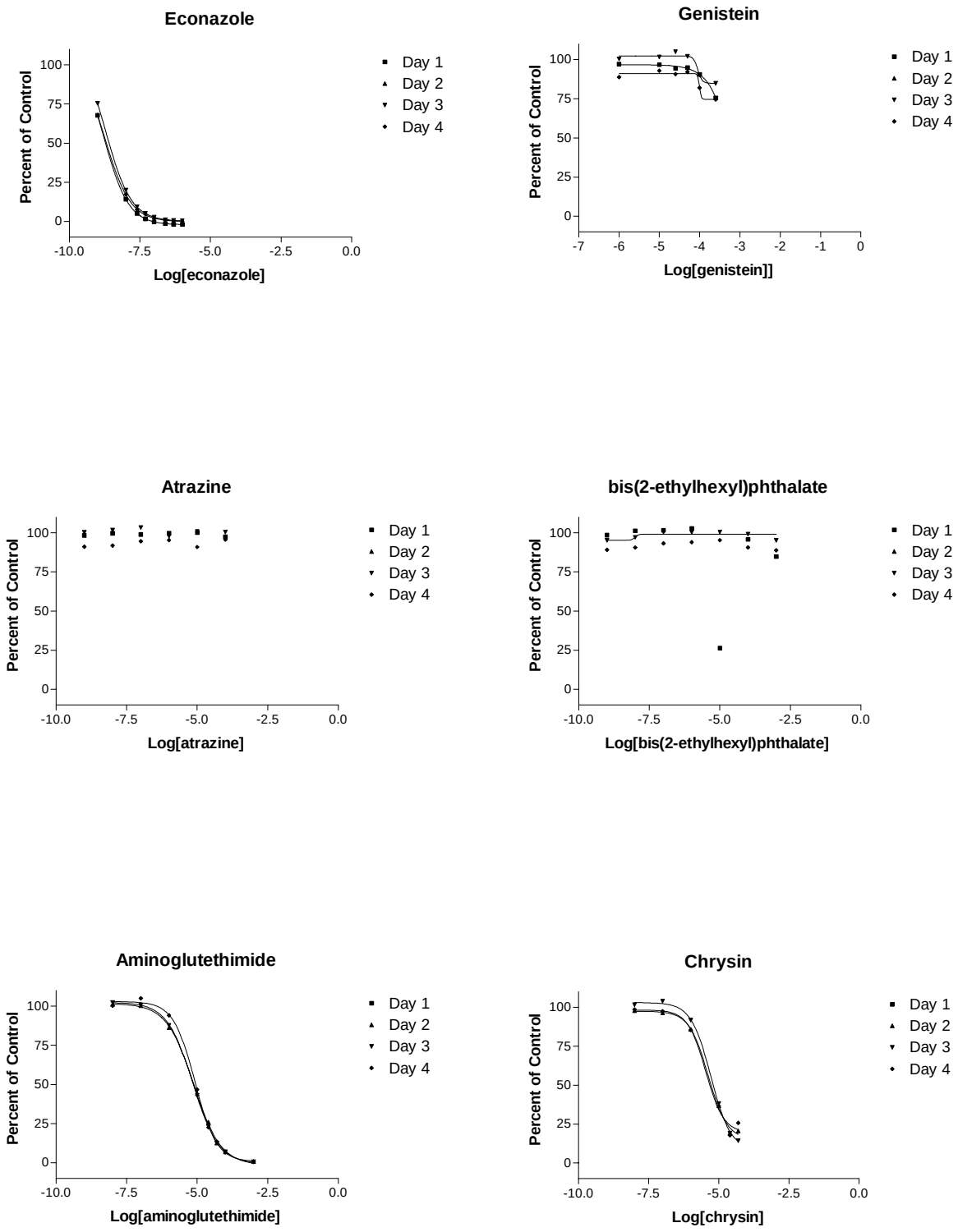


Figure 10. Human Placental Aromatase Assay Inhibition Response Curves

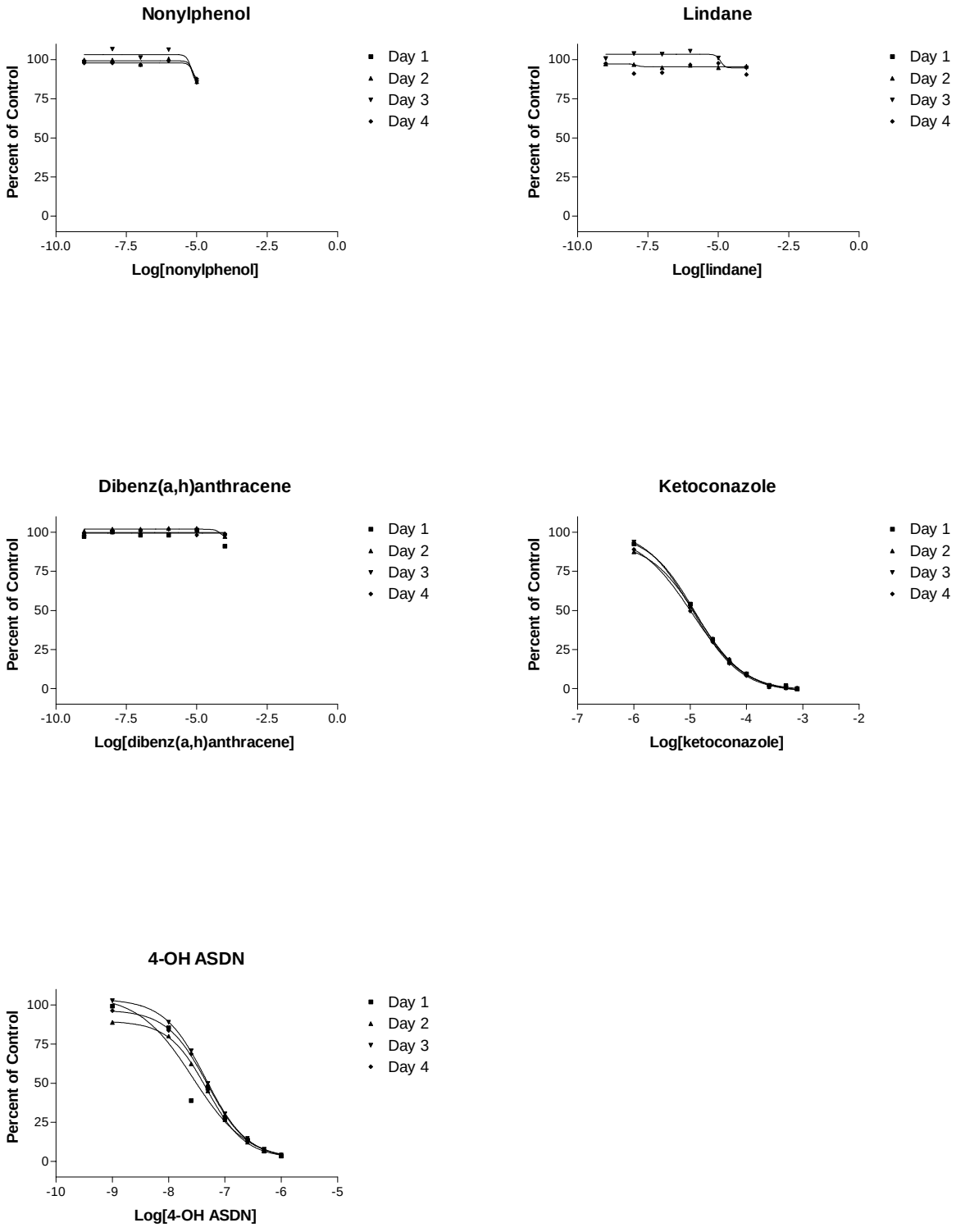


Figure 10. Human Placental Aromatase Assay Inhibition Response Curves (continued)

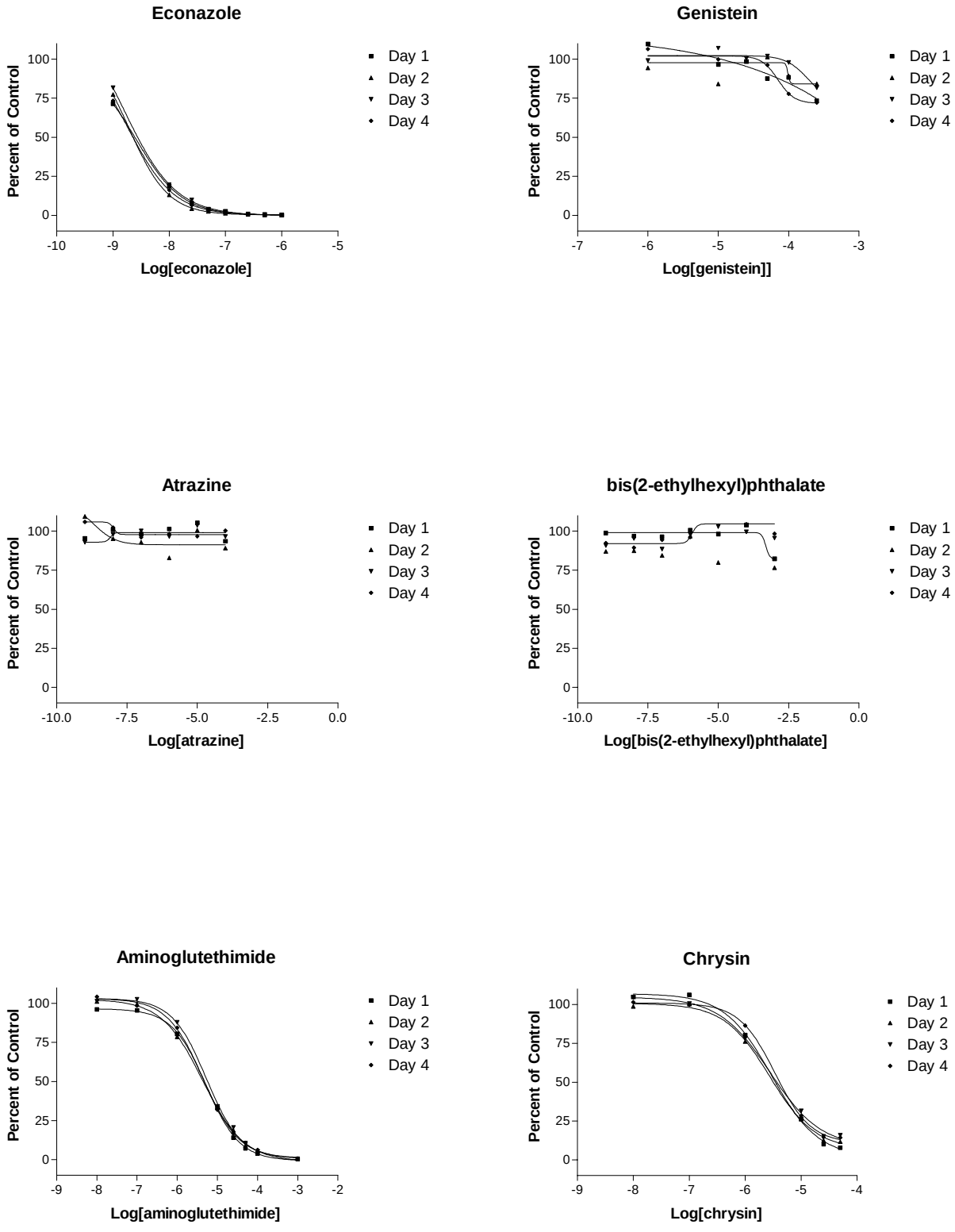


Figure 11. Human Recombinant Aromatase Assay Inhibition Response Curves

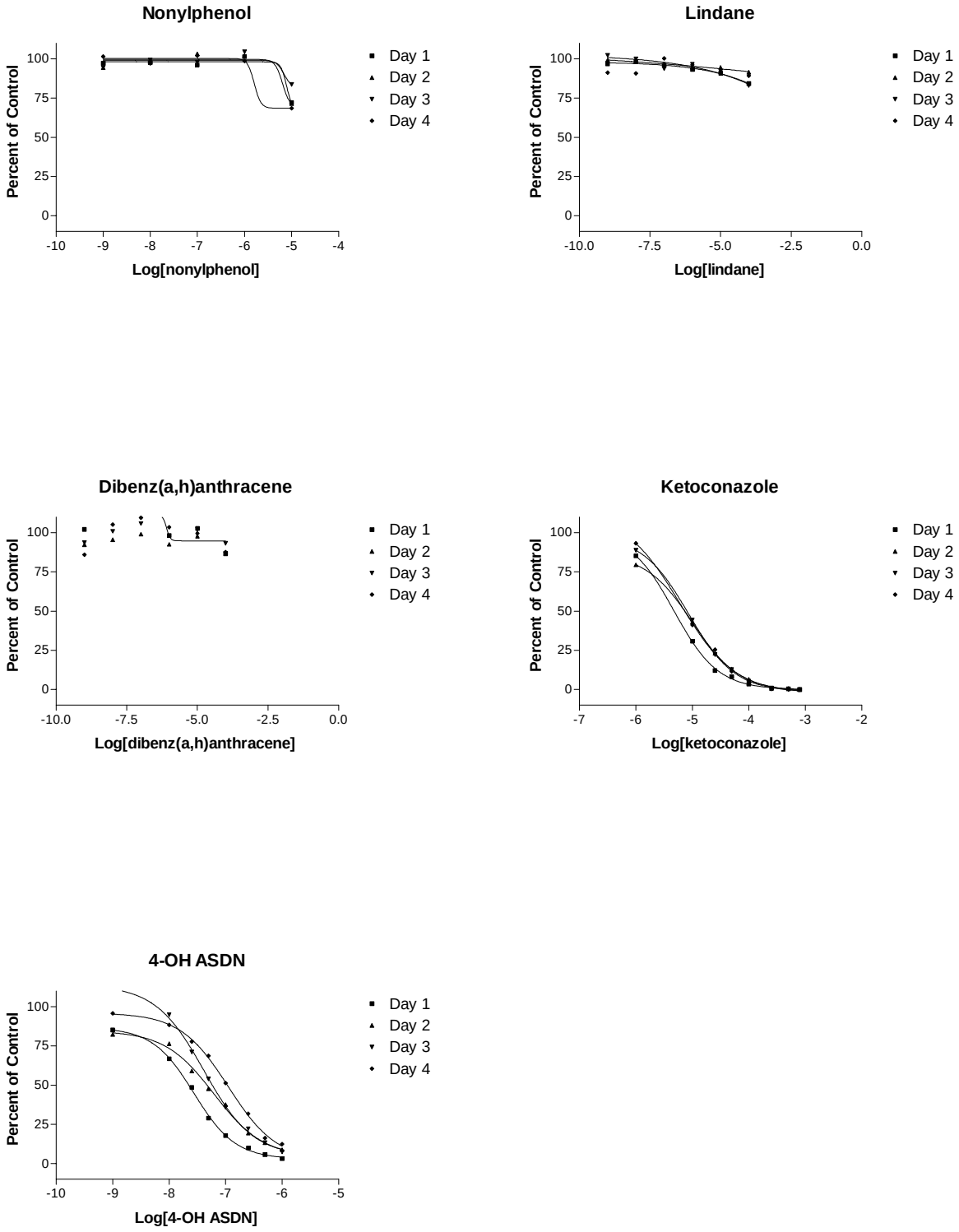


Figure 11. Human Recombinant Aromatase Assay Inhibition Response Curves (continued)

The response curves (Figures 10 and 11 for placental and recombinant, respectively) indicate that genistein, atrazine, bis(2-ethylhexyl)phthalate, nonylphenol, lindane and dibenz*(a,h)*anthracene do not significantly inhibit aromatase activity over the range of concentrations tested. Therefore, no meaningful IC_{50} values could be calculated for those compounds. Econazole, aminoglutethimide, chrysin, ketoconazole and 4-OH ASDN did inhibit aromatase activity over the concentration ranges tested, and IC_{50} and slope estimates for these compounds are presented in Tables 37 and 38, respectively.

For those cases where there was a diagnosed problem with the substrate solutions used, econazole (Group 1/Day 2) and aminoglutethimide and chrysin (Group 2, Day 1) mean IC_{50} values and slopes were calculated with the appropriate data excluded (Tables 37 and 38). When those data were excluded, no statistically significant ($p < 0.1$) day-to-day compared to within day variance was found in the Log(IC_{50}) or slopes for the placental assay. Day–to-day variance of Log(IC_{50}) for 4-OH ASDN and ketoconazole was significantly greater than within day variance for the recombinant assay. Summary statistics for Log(IC_{50}), slope and their standard errors are presented in Table 39 and the results of the t-tests comparing those factors between the recombinant and placental assays are presented in Table 40. Only Log(IC_{50}) varied significantly ($p<0.1$) between assays and then only for aminoglutethimide, chrysin and ketoconazole. Generally, similar IC_{50} values were obtained in both the placental and recombinant assays for each test chemical. The experimentally determined IC_{50} values fall within the literature ranges cited in the protocol for all test chemicals except for econazole and ketoconazole where the measured IC_{50} values were both lower than the literature value ranges cited in the protocol. However, White et al. (1999) reported an IC_{50} for ketoconazole of 6 µM and noted that literature values vary widely (7-60 µM). The response curve for genistein did not allow for estimation of a meaningful IC_{50} although genistein has been identified in the literature as an inhibitor of aromatase with a reported IC_{50} of 30-100 µM.. White et al. (1999) found genistein did not inhibit aromatase >20% even at concentrations up to 1 mM. Genistein was not soluble in the reaction mixture at the two highest concentrations tested (1 and 0.5 mM) so the activity data measured at those two concentrations was excluded from the response curve model. Even when that data was included, the response curve parameters are highly variant so a good estimate of IC_{50} was not possible (data not shown).

4.5.3 Test Substance Response Curves from a Reduced Number of Test Concentrations

Comparisons based on data from a reduced number of test concentrations are prescribed in protocol section 4.4.5. Data input into the curve fitting program Prism were limited to three concentrations: 10^{-7}, 10^{-6} and 10^{-5} M. In all cases, this resulted in too few points for curve-fitting and no IC_{50} or other parameters were calculable.

Table 37. IC_{50} Estimates by Chemical and Microsome Type[a]

Chemical	Microsome Type	Day1	Day 2	Day 3	Day 4	Geo Mean	Reduced Geo Mean
Econazole	Placental	1.632E-09	3.778E-10*	1.922E-09	1.820E-09	1.212E-09	1.787E-09
	Recombinant	2.604E-09	1.910E-09	1.575E-09	1.914E-09	1.968E-09	
Aminoglutethimide	Placental	2.268E-06*	7.535E-06	7.032E-06	8.348E-06	5.628E-06	7.619E-06
	Recombinant	5.157E-06	4.309E-06	5.334E-06	4.405E-06	4.780E-06	
Chrysin	Placental	1.414E-06*	4.193E-06	5.259E-06	3.455E-06	3.222E-06	4.239E-06
	Recombinant	2.957E-06	2.754E-06	2.783E-06	3.415E-06	2.966E-06	
Ketoconazole	Placental	1.215E-05	1.352E-05	1.101E-05	1.071E-05	1.180E-05	
	Recombinant	4.749E-06	1.013E-05	8.561E-06	5.977E-06	7.044E-06	
4-OH ASDN	Placental	2.701E-08	4.798E-08	4.568E-08	4.872E-08	4.121E-08	
	Recombinant	2.760E-08	6.235E-08	4.186E-08	1.119E-07	5.328E-08	

[a]IC_{50} expressed in mol/L

* These data were excluded from the calculation of the reduced geometric mean

Table 38. Slope Estimates by Chemical and Microsome Type

Chemical	Microsome Type	Day 1	Day 2	Day 3	Day 4	Mean	Std Dev	Mean[a]	Std Dev[a]
Econazole	Placental	-0.9789	-0.9737*	-0.9553	-0.9592	-0.9668	0.0113	-0.9645	0.0127
	Recombinant	-1.073	-1.234	-0.9441	-1.069	-1.080	0.1188		
Aminoglutethimide	Placental	-1.013*	-0.9013	-0.9443	-1.133	-0.9979	0.1011	-0.9929	0.1232
	Recombinant	-1.027	-0.8488	-1.017	-0.9681	-0.9652	0.0818		
Chrysin	Placental	-1.211*	-1.291	-1.234	-1.362	-1.275	0.0673	-1.296	0.0641
	Recombinant	-1.037	-1.072	-0.954	-1.355	-1.105	0.1742		
Ketoconazole	Placental	-1.048	-1.011	-1.014	-0.9065	-0.9949	0.0613		
	Recombinant	-1.115	-1.044	-1.057	-0.8903	-1.027	0.0959		
4-OH ASDN	Placental	-0.8971	-1.271	-1.178	-1.208	-1.139	0.1656		
	Recombinant	-1.185	-0.9928	-1.006	-1.018	-1.050	0.0903		

[a] Mean and standard deviation of reduced set of data (excluding Day 2 for Econazole and Day 1 for Aminoglutethimide and Chrysin)

* These data were excluded from the calculation of the reduced mean and standard deviations.

Table 39. Summary Statistics for Log (IC_{50}), Slope and their Standard Errors

Chemical	Variable	Type	N	Mean	Std Error	Lower Limit of Mean	Upper Limit of Mean
Econazole	LogIC50	Placental	3	-8.748	0.021	-8.838	-8.658
Econazole	LogIC50	Recombinant	4	-8.706	0.045	-8.850	-8.562
Econazole	LogIC50	Diff (1-2)	_	-0.042	0.056	-0.186	0.103
Econazole	SE_LogIC50	Placental	3	0.043	0.021	-0.045	0.131
Econazole	SE_LogIC50	Recombinant	4	0.096	0.034	-0.012	0.204
Econazole	SE_LogIC50	Diff (1-2)	_	-0.053	0.044	-0.165	0.060
Econazole	Slope	Placental	3	-0.964	0.007	-0.996	-0.933
Econazole	Slope	Recombinant	4	-1.080	0.059	-1.269	-0.891
Econazole	Slope	Diff (1-2)	_	0.116	0.071	-0.066	0.297
Econazole	SE_Slope	Placental	3	0.027	0.013	-0.029	0.083
Econazole	SE_Slope	Recombinant	4	0.072	0.020	0.009	0.135
Econazole	SE_Slope	Diff (1-2)	_	-0.045	0.026	-0.111	0.021
Aminoglutethimide	LogIC50	Placental	3	-5.118	0.022	-5.211	-5.025
Aminoglutethimide	LogIC50	Recombinant	4	-5.321	0.024	-5.395	-5.246
Aminoglutethimide	LogIC50	Diff (1-2)	_	0.202	0.033	0.117	0.288
Aminoglutethimide	SE_LogIC50	Placental	3	0.025	0.007	-0.004	0.055
Aminoglutethimide	SE_LogIC50	Recombinant	4	0.032	0.003	0.022	0.042
Aminoglutethimide	SE_LogIC50	Diff (1-2)	_	-0.006	0.007	-0.024	0.011
Aminoglutethimide	Slope	Placental	3	-0.993	0.071	-1.299	-0.687
Aminoglutethimide	Slope	Recombinant	4	-0.965	0.041	-1.095	-0.835
Aminoglutethimide	Slope	Diff (1-2)	_	-0.028	0.077	-0.225	0.170
Aminoglutethimide	SE_Slope	Placental	3	0.055	0.022	-0.038	0.148
Aminoglutethimide	SE_Slope	Recombinant	4	0.053	0.008	0.029	0.077
Aminoglutethimide	SE_Slope	Diff (1-2)	_	0.003	0.020	-0.049	0.054
Chrysin	LogIC50	Placental	3	-5.373	0.053	-5.600	-5.146
Chrysin	LogIC50	Recombinant	4	-5.528	0.022	-5.596	-5.459
Chrysin	LogIC50	Diff (1-2)	_	0.155	0.051	0.024	0.286
Chrysin	SE_LogIC50	Placental	3	0.099	0.037	-0.061	0.260
Chrysin	SE_LogIC50	Recombinant	4	0.076	0.012	0.039	0.113
Chrysin	SE_LogIC50	Diff (1-2)	_	0.023	0.034	-0.064	0.111
Chrysin	Slope	Placental	3	-1.296	0.037	-1.455	-1.136
Chrysin	Slope	Recombinant	4	-1.105	0.087	-1.382	-0.827
Chrysin	Slope	Diff (1-2)	_	-0.191	0.108	-0.468	0.085
Chrysin	SE_Slope	Placental	3	0.254	0.093	-0.147	0.656
Chrysin	SE_Slope	Recombinant	4	0.144	0.012	0.106	0.183
Chrysin	SE_Slope	Diff (1-2)		0.110	0.079	-0.094	0.314

(continued)

Table 39. Summary Statistics for Log (IC_{50}), Slope and their Standard Errors (continued)

Chemical	Variable	Type	N	Mean	Std Error	Lower Limit of Mean	Upper Limit of Mean
4-OH androstenedione	LogIC50	Placental	4	-7.385	0.061	-7.581	-7.189
4-OH androstenedione	LogIC50	Recombinant	4	-7.273	0.129	-7.685	-6.861
4-OH androstenedione	LogIC50	Diff (1-2)	_	-0.112	0.143	-0.462	0.239
4-OH androstenedione	SE_LogIC50	Placental	4	0.063	0.050	-0.097	0.223
4-OH androstenedione	SE_LogIC50	Recombinant	4	0.043	0.009	0.016	0.070
4-OH androstenedione	SE_LogIC50	Diff (1-2)	_	0.020	0.051	-0.105	0.144
4-OH androstenedione	Slope	Placental	4	-1.139	0.083	-1.402	-0.875
4-OH androstenedione	Slope	Recombinant	4	-1.050	0.045	-1.194	-0.907
4-OH androstenedione	Slope	Diff (1-2)	_	-0.088	0.094	-0.319	0.143
4-OH androstenedione	SE_Slope	Placental	4	0.151	0.108	-0.192	0.494
4-OH androstenedione	SE_Slope	Recombinant	4	0.104	0.015	0.057	0.151
4-OH androstenedione	SE_Slope	Diff (1-2)	_	0.047	0.109	-0.219	0.313
Ketoconazole	LogIC50	Placental	4	-4.928	0.023	-5.001	-4.855
Ketoconazole	LogIC50	Recombinant	4	-5.152	0.074	-5.389	-4.915
Ketoconazole	LogIC50	Diff (1-2)	_	0.224	0.078	0.033	0.415
Ketoconazole	SE_LogIC50	Placental	4	0.025	0.004	0.011	0.039
Ketoconazole	SE_LogIC50	Recombinant	4	0.054	0.020	-0.008	0.117
Ketoconazole	SE_LogIC50	Diff (1-2)	_	-0.029	0.020	-0.079	0.020
Ketoconazole	Slope	Placental	4	-0.995	0.031	-1.092	-0.897
Ketoconazole	Slope	Recombinant	4	-1.027	0.048	-1.179	-0.874
Ketoconazole	Slope	Diff (1-2)	_	0.032	0.057	-0.108	0.171
Ketoconazole	SE_Slope	Placental	4	0.055	0.005	0.039	0.070
Ketoconazole	SE_Slope	Recombinant	4	0.085	0.021	0.019	0.152
Ketoconazole	SE_Slope	Diff (1-2)		-0.030	0.021	-0.083	0.022

Table 40. Heterogeneous Variance 2-Sample T-Test Results

Chemical	Variable	DF	t Value	Pr > |t|
Econazole	LogIC50	4.14	-0.84	0.4467
Econazole	SE_LogIC50	4.66	-1.33	0.2457
Econazole	Slope	3.09	1.93	0.1464
Econazole	SE_Slope	4.81	-1.90	0.1182
Aminoglutethimide	LogIC50	4.93	6.33	0.0015
Aminoglutethimide	SE_LogIC50	2.88	-0.85	0.4589
Aminoglutethimide	Slope	3.30	-0.34	0.7566
Aminoglutethimide	SE_Slope	2.49	0.11	0.9188
Chrysin	LogIC50	2.67	2.72	0.0821
Chrysin	SE_LogIC50	2.40	0.60	0.6007
Chrysin	Slope	3.99	-2.02	0.1137
Chrysin	SE_Slope	2.07	1.17	0.3592
4-OH androstenedione	LogIC50	4.29	-0.78	0.4769
4-OH androstenedione	SE_LogIC50	3.17	0.39	0.7231
4-OH androstenedione	Slope	4.64	-0.93	0.3963
4-OH androstenedione	SE_Slope	3.11	0.43	0.6929
Ketoconazole	LogIC50	3.57	2.88	0.0519
Ketoconazole	SE_LogIC50	3.30	-1.45	0.2361
Ketoconazole	Slope	5.10	0.56	0.6011
Ketoconazole	SE_Slope	3.33	-1.42	0.2429

5.0 DISCUSSION

5.1 Preoptimization Experiments

5.1.1 Substrate Characterization

Two batches of the radiolabeled substrate,[^{3}H]ASDN, were obtained for use in this study. Both batches were shown by HPLC to coelute with nonradiolabeled ASDN and to be ≥98% radiochemically pure. While not formally addressed in this project, [^{3}H]ASDN appeared to have good stability in both ethanol and aqueous solutions. Specific activity of the first batch of [^{3}H]ASDN was determined to be within 5% of that reported (25.3 Ci/mmol) by the manufacturer, while that of the second was only ca. 86-89% of the reported specific activity. Because the radiolabeled substrate comprises only ca. 3% of the mass of ASDN in the substrate solutions, small variations in stock specific activity have little effect on the final specific activity of the substrate solution. Therefore, the reported specific activity was used in all calculations.

5.1.2 Microsome Preparation

Microsomes were prepared from bovine, porcine and human placentas, and these tissue types vary greatly in morphology and mass.

Bovine placenta is cotyledonous in structure. The cotyledons were dissected from the placental membrane and microsomes were prepared. Sufficient microsomal protein with acceptable aromatase activity for the conduct of the study was obtained from a single bovine placenta.

Porcine placenta is of the diffuse type. In this type of placenta, there is a very thin layer of velvety tissue containing microcotyledons inside the placental membrane. This layer of tissue must be carefully scraped from the membrane using a razor blade or other appropriate tool. All the while, it is important to keep the tissue chilled on ice to preserve aromatase activity. Of the five porcine placentas that were processed into microsomes, only one had appreciable aromatase activity (near that found in the bovine placental microsomes) and that placenta yielded only about half of the microsomal protein that was projected to be required for the study.

Procurement of bovine and porcine placentas was problematic. Births are rarely attended by the farmers unless there is a particularly difficult delivery, and this makes the collection of fresh placentas (< 0.5 h between delivery and icing-down) impractical. There may not be a ready year-round supply of placental tissue because of the seasonality of livestock breeding.

Time between collection and work-up must be kept short (usually <1 h) to preserve maximum aromatase activity so it is important that farms be located near the laboratories where the microsome preparation is to occur. Because of these issues, further investigation into aromatase activity in bovine and porcine placental microsomes past the pre-optimization phase was cancelled by the Sponsor.

Human placenta is of the discoid type. A single human placenta was obtained from local hospital following a full-term Caesarean delivery. Microsomes were prepared and were found to have ample aromatase activity and protein content for use in the experiments. The human placenta was by far the easiest of the three placentas to process as it was only necessary to dissect the membranes away from the soft tissue prior to processing the soft tissue into microsomes. Of course, effective infection-control practices need to be followed when working with human tissue.

Human recombinant microsomes (prepared from Baculovirus-infected insect cells) were purchased from a commercial supplier and were found to have sufficient P450 content and aromatase activity for use in these studies. There is no need for special infection-control practices when working with the recombinant protein.

5.2 Optimization of Experimental Design Factors and Conditions

The results of the factorial design experiment for the human placental microsome aromatase assay indicated that the optimal conditions include incubation concentrations of 154 nM ASDN, 0.0165 mg/mL microsomal protein and an incubation time of 10 min. The co-factors NADP and glucose 6-phosphate were dropped from the model because of non-significant results (i.e., there was no dependance over a broad range of concentrations) while glucose 6-phosphate dehydrogenase proved to optimize activity at a concentration of ca. 0.6 U/incubation. In the case of the recombinant microsomes, the optimal conditions were determined to be 108 nM ASDN, 0.01 mg/mL protein and an incubation time of 22 min. All of the NADPH regenerating system components were dropped from the model because of insignificant results (i.e., a lack of dependence of activity across a broad range of concentrations). These optimal conditions were those that would yield the highest possible rate of aromatase activity (V_{max}). However, there was concern that V_{max} conditions may be far removed from the range 1/3 to 3 K_m, where current best practices recommend determination of inhibitory constants (Bjornsson, T. D., et al. 2003). An alternate analysis of the optimization data conducted by Dr. Paul Feder (Battelle) suggested additional criteria to be tested (Appendix 4) and predicted K_m. Additional optimization experiments were undertaken to establish the experimental validity of these recommendations and to obtain a more appropriate set of optimal conditions for the determination of inhibitory constants.

The assay was examined for linearity with respect to protein concentration and incubation time under the constraint of ca. 10-15% consumption of substrate (to ensure initial rate conditions). A substrate concentration of 100 nM was chosen based on literature reports of K_m for the assay and from Dr. Feder's predictions based on the factorial design experiment data. The final assay conditions selected for use are presented in Table 41.

Table 41. Optimized Conditions for Aromatase Assay

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

The assay was tested for day-to-day and technician-to-technician variance.

5.3 Determination of the Response of the Optimized Assay to Selected Test Substances

The aromatase assay was conducted using human placental and human recombinant microsomes over four days each with 11 test chemicals to determine the response of the assay to a range of known inhibitors and non-inhibitors. In each case, the assay correctly identified reported aromatase inhibitors and non-inhibitors. Generally, similar IC_{50} values were obtained in both the placental and recombinant assays for each test chemical. The experimentally determined IC_{50} values fall within the literature ranges cited in the protocol for all test chemicals except for econazole and ketoconazole where the measured IC_{50} values were both lower than the literature value ranges cited in the protocol. In the case of ketoconazole, the experimentally determined IC_{50} were 12 and 7 µM for the placental and recombinant microsomes, respectively, which is in contrast to the >65 µM reported for the literature in the protocol. However, White et al. (1999) report IC_{50} for ketoconazole of 6 µM and noted that literature values vary widely (7-60 µM). For econazole, measured IC_{50} was 1.2 and 1.9 nM for the placental and recombinant microsomes, respectively, compared with 30-50 nM reported for the literature. Genistein was identified in the study protocol as a weak isoflavonoid aromatase inhibitor but did not have a significant effect on aromatase activity in the current study. White et al. (1999) found genistein did not inhibit aromatase >20% even at concentrations up to 1 mM. Selection of the proper range of test substance concentrations for testing is crucial to obtaining meaningful results. However, because of the wide range of IC_{50} values for the various inhibitors, predetermination of the correct range will not always be possible. Initial testing at 10^{-9} to 10^{-3} M should give

adequate data for initial assessment, and can guide modifications to the testing concentration range where necessary to obtain definitive IC_{50}s. It is important to include enough concentrations (suggest 7-8) in the test set for proper curve fitting.

6.0 CONCLUSIONS

The aromatase assay was optimized in human placental and human recombinant microsomes. The recombinant protein showed a higher aromatase activity (nmol/mg/min) than the placental protein, likely due to the direct linking of the necessary reductase to the recombinant protein. The assay correctly identified both aromatase inhibitors and non-inhibitors. Both assays were equally sensitive to the effects of known aromatase inhibitors and non-inhibitors.

7.0 REFERENCES

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Appendix A-1

Letter Report 05-05-03

Preoptimization Experiment Results Related to Substrate Characterization and Human Placental and Human Recombinant Microsomes

Letter Report 05 05 03

Pre-Validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

EDSP WA 2-24

1.0 Introduction

The pre-optimization experiments were 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 placental microsomes. In addition, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and the human recombinant microsomes) will be analyzed for protein concentration, cytochrome P450 (P450) content, and aromatase activity. The P450 content measurement provides assurance that the enzyme is present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. Finally, a single aromatase activity assay run 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 definitive optimization experiments.

This report includes the results of the pre-optimization experiments related to substrate characterization and human recombinant and human placental microsomes.

2.0 Materials and Methods

2.1 Chemicals

Non-radiolabeled 4-androstene-3,17-dione (ASDN) was received through Battelle from Sigma (St. Louis, MO). [1β-^{3}H(N)]Androst-4-ene-3,17-dione ([^{3}H]ASDN) was obtained from Perkin Elmer Life Science, Boston, MA. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glycerol, niacinamide, dithiothreitol and bovine serum albumin (BSA) were purchased from Sigma. Sodium phosphate monobasic, sodium phosphate dibasic, sucrose and propylene glycol were from JT Baker (Phillipsburg, NJ). Human recombinant CYP19, coexpressed with P450 reductase, (Human CYP19 + P450 Reductase SUPERSOMES™) was purchased from BD Gentest (Woburn, MA). Ultima Gold scintillation cocktail was purchased from Packard Instruments (Meriden, CT). DC Protein assay kit was purchased from Biorad (Hercules, CA) .

2.2 HPLC System

The HPLC system consisted 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) with a 250 µL glass scintillant cell. Data was collected using Waters Millennium[32] Client/Server Chromatography Data System Software, Version 4.0.

2.3 Substrate Characterization

The nonradiolabeled ASDN was dissolved in ethanol (0.01 mg/mL) and analyzed by HPLC using a Zorbax SB-C18 column (4.6 x 250 mm). The mobile phase was 55:15:30 (v:v:v) distilled, deionized water: tetrahydrofuran: methanol with a flow rate of 1 mL/min. The eluant was monitored by UV absorbance at 240 nm. Under these conditions, the ASDN had a retention time of ca. 15 min.

The purity of the [^{3}H]androstenedione ([^{3}H]ASDN) was determined by HPLC using the conditions described above, with the addition of monitoring eluant with the 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). The retention time of [^{3}H]ASDN was ca. 15 min.

2.4 Specific Activity Determination

A sample containing 1 mg ASDN/mL ethanol was prepared. Dilutions containing 0.54 to 4.28 ng ASDN/20 µL were prepared in distilled, deionized water and were analyzed by HPLC in duplicate using the conditions described above. A standard curve was constructed relating peak height to ASDN concentration. Samples of [^{3}H]ASDN were analyzed by HPLC using the same conditions and fractions were collected and assayed for radiochemical content by LSS. The specific activity of the [^{3}H]ASDN was determined by the relationship between the height of the ASDN peak and the amount of radioactivity contained in the peak.

2.5 Placental Microsome Preparation

A human placenta was received from a local hospital and placed on ice within 10 min of delivery. The tissue was placed on a ice-chilled board and the soft tissue was dissected away from the membrane. The soft tissue was placed in ice-cold buffer (2:1 tissue weight:buffer; 0.25 M sucrose, 0.05 M sodium phosphate (pH 7.0), 0.04 M niacinamide), minced with scissors and then was homogenized in portions using a Polytron homogenizer. The homogenate was transferred to centrifuge tubes and centrifuged at a setting of 10,000*g* for 30 min at 4 °C in an

IEC B-22M centrifuge. The supernatant was transferred to ultracentrifuge tubes and was centrifuged at a setting of 35,000 rpm (which is equivalent to approximately 100,000*g*) in a refrigerated Beckman L5-50B Ultracentrifuge for 1 h to obtain the crude microsomal pellet. The supernatant was decanted and discarded and the microsomal pellet was resuspended in a chilled buffer containing 0.1 M sodium phosphate buffer, pH 7.4. The sample was centrifuged again at a setting of 35,000 rpm in the Beckman L5-50B for 1 h to wash the microsomes. This washing procedure was repeated one additional time. The twice-washed microsomal pellet was resuspended in chilled 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol and 0.05 M dithiothreitol. The microsomal suspension (total volume ca. 20.2 mL) was divided among 30 vials, was flash frozen in liquid nitrogen and was stored at ca. -70 °C.

2.6 Protein Determination

The protein concentration of the human placental microsome preparation, and the human recombinant microsomal preparation, was determined. A 6-point standard curve was prepared using BSA, ranging from 0.13 to 1.5 mg protein/mL. Protein was determined by using a DC Protein Assay kit. 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. The absorbances are stable for about 1 h. Each sample (unknowns and standards) was transferred to disposable polystyrene cuvettes and the visible absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed from the absorbance of the protein standards.

2.7 P450 Content

P450 content was determined for the human placental microsome preparation and the human recombinant microsomal preparation. Using the carbon monoxide (CO) spectrum assay of Omura and Sato (1964), a single experiment using each of the preparations was conducted as described below.

A sample of each microsomal preparation was diluted 1:20 in 0.1 M phosphate buffer (pH 7.4). The diluted sample was gently bubbled with carbon monoxide for approximately 15-20 s and then was divided between a pair of matched cuvettes (1 mL/cuvette). Next, a few grains of solid sodium dithionite was added to the sample cuvette with gentle mixing. The visible spectrum was then recorded from 400 to 500 nm using a split-beam spectrophotometer.

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

2.8 Aromatase Activity

Aromatase activity was determined for the human placental microsome preparation and the humna recombinant CYP19. A single experiment was conducted using only the substrate ([^{3}H]ASDN/ASDN) with each of the microsomal preparations. The assay was conducted as described in the following paragraph.

The [^{3}H]ASDN/ASDN substrate solution was prepared by combining solutions of [^{3}H]ASDN and ASDN. A 1 mg/mL solution of ASDN was prepared in ethanol. Serial dilutions of this solution were prepared in assay buffer to yield a solution containing ca. 1 μg ASDN/mL. The [^{3}H]ASDN stock was diluted 1:100 in assay buffer to yield a solution containing ca. 10 μCi/mL. The substrate solution was prepared by combining 275 μL of the 1 μg ASDN/mL solution, 100 μL of the 10 μCi [^{3}H]ASDN/mL solution and 625 μL buffer.

The assays were performed in 13x100 mm test tubes (two for each microsomal preparation) maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 μL) of propylene glycol was added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 μCi, 50 nM), was added to the tubes. An NADPH-generating system comprised of NADP+ (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) was added to each tube. The tubes were placed at 37 ± 1°C in the water bath for 5 min prior to initiation of the assay by the addition of the diluted microsomal suspension (~0.1 mg microsomal protein/mL). The total volume was 2.0 mL, and the tubes were incubated for 30 min. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for about 30 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 min at a setting of 1000 rpm (which is approximately equivalent to 230*g*). The methylene chloride layer was removed to a vial and weighed; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time reserving and weighing the methylene chloride layer in a separate vial. The aqueous layers was transferred to vials, weighed, and duplicate aliquots (0.5 mL) were weighed into 20-mL liquid scintillation counting vials. Duplicate aliquots of each methylene chloride fraction were weighed into scintillation 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 the substrate solution was determined by analyzing 5 weighed aliquots by LSS. The substrate solution specific activity was determined by dividing

the radiochemical content of the substrate solution (dpm/g) by the total concentration of ASDN in the solution (ASDN + [^{3}H]ASDN; nmol/g solution).

Analysis of the samples was performed using LSS as described in SOP METAB-610. Radiolabel found in the aqueous fractions represents ^3H_2O formed, and that in the methylene chloride fractions represents unreacted substrate.

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 solution (expressed in dpm/nmol). The activity of the enzyme reaction was expressed in nmol (mg protein)$^{-1}$ min^{-1} and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 30 min.

3.0 Results and Discussion

3.1 Substrate Analysis

4-Androstene-3,17-dione (ASDN), lot number 072K1134, had a stated purity of 99% (Figure 1). The [^{3}H]ASDN coeluted with the nonradiolabeled ASDN on HPLC (Figure 2). Information provided by the supplier of [^{3}H]ASDN regarding its purity and specific activity is presented in Figure 3. The radiochemical purity of the [^{3}H]ASDN was determined by HPLC at RTI to be 98% (Figure 4). Samples of known concentration of ASDN were analyzed by HPLC, and a standard curve relating peak height to concentration of ASDN was generated. A sample of [^{3}H]ASDN was analyzed by HPLC in triplicate and eluant fractions were collected and assayed for radiochemical content by LSS. The specific activity of the [^{3}H]ASDN stock was determined by dividing the dpm in the peak fractions by the amount of ASDN in the peak (calculated using the peak height and the standard curve parameters) . The data are presented in Table 1. The calculated specific activity is 26.4 Ci/mmol. This figure is within 5% of the specific activity value (25.3 Ci/mmol) provided by the supplier, therefore, 25.3 Ci/mmol will be used as the specific activity of the stock [^{3}H]ASDN for this study. The data presented confirm that the ASDN and [^{3}H]ASDN are suitable for use in these studies.

3.2 Human Recombinant CYP19

The data sheet for the Human Recombinant CYP19 is presented in Figure 5. One tube of this product was thawed rapidly at 37 °C and the contents were rehomogenized and analyzed for protein and P450 content and aromatase activity. The protein content was found to be 3.5 mg/mL, compared with the 4.2 mg/mL stated on the data sheet. The P450 content was calculated to be 0.38 nmol/mg protein. This value is similar to the 0.24 nmol/mg value

calculated from the data sheet information. This microsomal preparation had aromatase activity of 0.022 nmol estrogen formed/mg protein/min under the conditions of the assay as described above. The data sheet reported an aromatase activity value (1.38 nmol/mg protein/min) obtained using a different substrate at a significantly higher concentration. It is unclear whether the activities determined under such different conditions should be similar.

3.3 Human Placental Microsomes

A human placenta from a 28 year old nonsmoker with a full term Caesarean-section delivery was obtained from local hospital and microsomes were prepared. A sample of the microsomes was thawed rapidly in a water bath and rehomogenized prior to assay for protein and P450 content and aromatase activity. The protein content of the human placental microsomes was determined to be ca. 44 mg/mL. The total protein yield for the preparation was calculated to be ca. 900 mg. This exceeds the 250 mg of protein criteria set in the protocol. P450 content of the human placental microsomes was determined to be ca. 0.048 nmol/mg protein, which exceeds the criteria of 0.005 nmol P450/mg protein set in the protocol. The aromatase activity of the human placental microsomes was ca. 0.015 nmol estrogen formed/mg protein/min; this exceeds the 5 pmol estrogen formed/mg protein/min acceptance criteria for this parameter.

4.0 Conclusion

The ASDN and [^{3}H]ASDN substrates are of sufficient purity for use in these studies. The specific activity stated by the supplier of the [^{3}H]ASDN was confirmed experimentally. Both the human recombinant CYP19 and the human placental microsomes had sufficient protein and P450 content for the conduct of these studies. The aromatase activity for the two microsome preparations was similar and sufficient to proceed with the optimization phase of the study.

5.0 References

Omura, T.; Sato, R. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 1964**, 239**, 2370-2378.

10691-7 SAB 1/16/08

Sigma-Aldrich Certificate of Analysis

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {072K1134} RESULTS
Product Name	4-Androstene-3,17-dione	
Product Number	A9630	
CAS Number	63058	
Formula	$C_{19}H_{26}O_2$	
Formula Weight	286.4	
APPEARANCE	WHITE TO OFF-WHITE POWDER	OFF-WHITE POWDER
SOLUBILITY	CLEAR, COLORLESS TO FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF CHLOROFORM	CLEAR COLORLESS
ULTRAVIOLET/VISIBLE SPECTRUM	EMM = 15.9 TO 16.5 AT LAMBDA MAX 239 TO 240 NM IN ETHANOL	EMM = 16.3 AT LAMBDA MAX 239 NM
PURITY BY HPLC	MINIMUM 98%	99%
SHELF LIFE SOP QC-12-006	5 YEARS	AUGUST 2007
QC ACCEPTANCE DATE		AUGUST 2002

David Feldker, Manager
Analytical Services

Figure 1. Data Sheet for ASDN

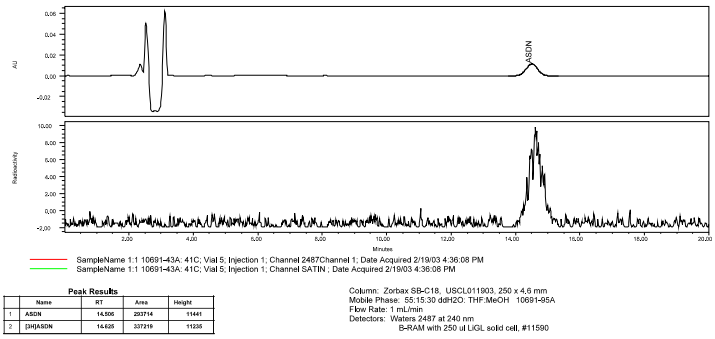


Figure 2. HPLC Radiochromatoram of ASDN and [^{3}H]ASDN

10691-5-500 4/30/02

PerkinElmer life sciences

PerkinElmer Life Sciences, Inc.
549 Albany Street
Boston, MA 02118

Products You Trust NEN Waller

^{3}H Technical Data Certificate of Analysis

Caution: For Laboratory Use. A research chemical for research purposes only.

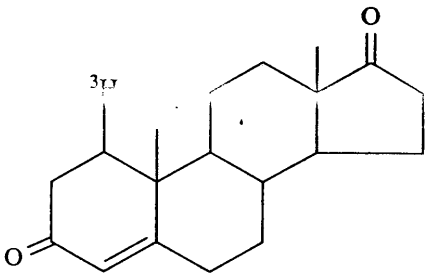
NET-926 ANDROST-4-ENE-3, 17-DIONE, [1β-^{3}H(N)]-

Catalog Number: NET926

3467835 sub 2/20/03

Lot Number: 3467835

Specific Activity: 25.300000 Ci/mmol
936.100000 GBq/mmol



M. W. 286.4

PACKAGING: 1.0 mCi/ml (37 MBq/ml) in Ethanol.

STABILITY AND STORAGE RECOMMENDATIONS:

When androst-4-ene-3, 17-dione, [1β-^{3}H(N)]- is stored at -20°C in its original solvent and at its original concentration, the rate of decomposition is approximately 1% for 6 months from date of purification. Lot to lot variation may occur and it is advisable to check purity prior to use.

SPECIFIC ACTIVITY RANGE: 15-30 Ci/mmol (0.55-1.11 TBq/mmol)

RADIOCHEMICAL PURITY: This product initially found to be greater than 97% when determined by the following methods:

1. High pressure liquid chromatography on a Zorbax ODS column using the following mobile phase:

   water : tetrahydrofuran : methanol (40:15:45)

2. Paper chromatography on Whatman No. 1 treated with 30% formamide in acetone using the following solvent system:

   hexane saturated with formamide.

3. Thin layer chromatography on Silica Gel using the following solvent system:

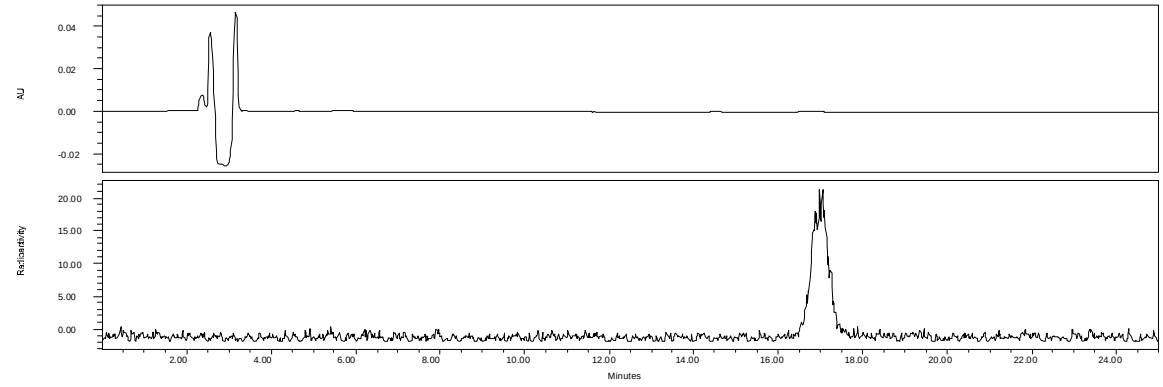
   toluene : ethyl acetate, (2:1).

QUALITY CONTROL: The radiochemical purity of androst-4-ene-3, 17-dione, [1β-^{3}H(N)]-is checked at appropriate intervals using the first listed chromatography method. Current purity data is available upon request.

PREPARATIVE PROCEDURE: Androst-4-ene-3, 17-dione, [1β-^{3}H(N)]- is prepared by treatment of androst-4-ene-3, 17-dione, [1β,2β-^{3}H(N)]- with potassium hydroxide under appropriate conditions (Ref.) Purification is by HPLC.

99197-0401

Figure 3. Data Sheet for [^{3}H]ASDN



Peak Results

	Name	RT	Area	Height
1	[3H]androstenedione	16.971	681945	21997
2	Androstenedione	17.000		

Column: Zorbax SB-C18, USCL011903, 250 x 4.6 mm
Mobile Phase: 55:15:30 ddH2O: THF:MeOH 10691-42B
Flow Rate: 1 mL/min
Detectors: Waters 2487 at 240 nm
B-RAM with 250 ul LiGL solid cell, #11590

Figure 4. HPLC Purity analysis of [^{3}H]ASDN

10691-19 SUP 1/15/13

A BD Biosciences Company
GENTEST
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www.gentest.com
BD Biosciences
Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen
BD

Human CYP19 + P450 Reductase SUPERSOMES™

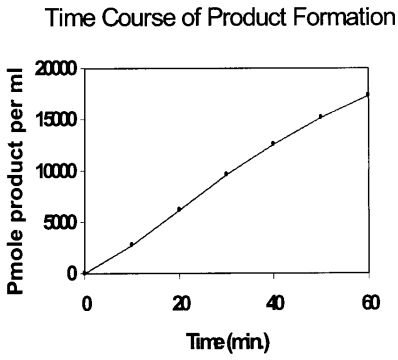
New Catalog Number....456260
Old Catalog Number.....P260
Lot Number.....2

Storage Conditions..STORE AT -80°C
Date Released .....2000 August
Best Used by.....2003 August

Package Contents.....0.5 nmole cytochrome P450 in 0.5 ml
Protein Content.....4.2 mg/ml in 100mM potassium phosphate (pH 7.4)
Cytochrome c Reductase Activity.....460 nmole/(min x mg protein)
Cytochrome P450 Content.....1000 pmol per ml
Aromatase Activity.....5.8 pmol product/(min x pmol P450)

This activity is catalyzed by human CYP19 which is expressed from human CYP19 cDNA using a baculovirus expression system. Baculovirus infected insect cells (BTI-TN-5B1-4) were used to prepare these microsomes. These microsomes also contain cDNA-expressed human P450 reductase. A microsome preparation using wild type virus (GENTEST Catalog No. P200 or P201) should be used as a control for native activities.

METHOD: A 0.25 ml reaction mixture containing 25 pmole P450, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.05 mM testosterone in 100 mM potassium phosphate (pH 7.4) was incubated at 37°C for 20 min. After incubation, the reaction was stopped by the addition of 125 ul acetonitrile and centrifuged (10,000 x g) for 3 minutes. 50 ul of the supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and eluted isocratically at 45°C with a mobile phase of 60% water and 40% acetonitrile and at a flow rate of 1.5 ml per min. The product was detected by its absorbance at 200 nm and quantitated by comparing the absorbance to a standard curve of (beta)-estradiol.



ADVICE

- Thaw rapidly in a 37°C water bath. Keep on ice until use
- Aliquot to minimize freeze-thawing cycles. Less than 20% of the catalytic activity is lost after 6 freeze thaw cycles.
- Metabolite production is linear with respect to enzyme concentration up to at least 50 pmol P450 per ml.
- Metabolite production with testosterone is approximately linear for 40 minutes (see graph above).

THIS PRODUCT IS SUPPLIED FOR LABORATORY RESEARCH USE ONLY.

Figure 5. Data Sheet for Recombinant Human CYP19

Table 1. Determination of Specific Activity of [^{3}H] ASDN Stock

HPLC Run #	ng ASDN in peak	DPM in peak	DPM/ ng ASDN	Ci/mmol	Average Ci/mmol
1	2.008	408691	203531	26.3	26.4
2	2.000	411805	205903	26.6	
3	1.994	406758	203991	26.3	

Appendix A-2

Letter Report - Porcine Phase 1 05 15 03
Preoptimization Experiment Results Related to Porcine Placental Microsomes

Letter Report–Porcine Phase 1 05 15 03

Pre-Validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

EDSP WA 2-24

1.0 Introduction

The pre-optimization experiments were designed to assess the chemical and biological properties of the critical components that are used in the aromatase assay. These experiments included characterizing the radiolabeled substrate and preparation of placental microsomes. In addition, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and the human recombinant microsomes) were analyzed for protein concentration, cytochrome P450 (P450) content, and aromatase activity. The P450 content measurement confirms that the enzyme is present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. Finally, a single aromatase activity assay determination using each type of microsomal preparation was included as a pre-optimization experiment in order to determine whether the preparations were of sufficient activity to conduct the definitive optimization experiments.

This report includes the results of the pre-optimization experiments related to porcine placental microsomes.

2.0 Materials and Methods

2.1 Chemicals

Non-radiolabeled 4-androstene-3,17-dione (ASDN) was received through Battelle from Sigma (St. Louis, MO). [1β-^{3}H(N)]Androst-4-ene-3,17-dione ([^{3}H]ASDN) was obtained from Perkin Elmer Life Science, Boston, MA. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glycerol, niacinamide, dithiothreitol and bovine serum albumin (BSA) were purchased from Sigma. Sodium phosphate monobasic, sodium phosphate dibasic, sucrose and propylene glycol were from JT Baker (Phillipsburg, NC). Ultima Gold scintillation cocktail was purchased from Packard Instruments (Meriden, CT). DC Protein assay kit was purchased from Biorad (Hercules, CA) .

2.2 Placental Microsome Preparation

All porcine placentas were received from NCSU's University Field Laboratories Swine Educational Unit. Two placentas were obtained on the first day of collection and thereafter, only one placenta was obtained on a given day. With the exception of the first two placentas collected, which were at room temperature for an undetermined length of time before being placed on ice, each placenta was chilled (either in a cold room or on ice) within 5 minutes of delivery by the sow. Of the first two placentas collected, only one was processed into microsomes. Each placenta was transported on ice to RTI within about 1 h after collection. The tissue was placed on a ice-chilled board and the velvety tissue was scraped away from the membrane using a razor blade. The collected tissue was transferred into beakers containing ice-cold buffer (ca. 2:1, w/v; tissue :buffer; 0.25 M sucrose, 0.05 M sodium phosphate (pH 7.0), 0.04 M niacinamide). The tissue was minced with scissors where necessary and then was homogenized in portions using either a Potter Elvejhem homogenizer alone, or a Polytron homogenizer followed by a Potter Elvejhem homogenizer. The homogenate was transferred to centrifuge tubes and centrifuged at a setting of 10,000*g* for 30 min at 4 °C in an IEC B-22M centrifuge. The supernatant was transferred to ultracentrifuge tubes and was centrifuged at a setting of 35,000 rpm (which is equivalent to approximately 100,000*g*) in a refrigerated Beckman L5-50B Ultracentrifuge for 1 h to obtain the crude microsomal pellet. The supernatant was decanted and discarded and the microsomal pellet was resuspended in a chilled buffer containing 0.1 M sodium phosphate buffer, pH 7.4. The sample was centrifuged again at a setting of 35,000 rpm in the Beckman L5-50B for 1 h to wash the microsomes. This washing procedure was repeated one additional time. The twice-washed microsomal pellet was resuspended in chilled 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol and either 0.05 M or 0.05 mM dithiothreitol. The microsomal suspensions were divided into ca. 0.2-0.5 mL portions, flash frozen in liquid nitrogen, and stored at ca. -70 °C.

2.3 Protein Determination

The protein concentration of the porcine placental microsome preparations were determined. A 6-point standard curve was prepared using BSA, ranging from 0.13 to 1.5 mg protein/mL on each day of analysis. Protein concentration was determined by using a DC Protein Assay kit. 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. The absorbances are stable for about 1 h. Each sample (unknowns and standards) was transferred to disposable polystyrene cuvettes and the visible absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed from the absorbance of the protein standards.

2.4 Cytochrome P450 Content

Cytochrome P450 content was determined for the porcine placental microsome preparations. Using the carbon monoxide (CO) spectral assay of Omura and Sato (1964), a single experiment for each preparation was conducted as described below.

A sample of each microsomal preparation was diluted 1:20 (on one occasion a 1:10 dilution was assayed because of low protein content in the microsomal preparation) in 0.1 M phosphate buffer (pH 7.4). The diluted samples were gently bubbled with carbon monoxide for approximately 10 s and then each sample was divided between a pair of matched cuvettes (1 mL/cuvette). Next, a few grains of solid sodium dithionite was added to the sample cuvette with gentle mixing by inversion. The difference spectrum was then recorded from 400 to 500 nm using an Aminco split-beam spectrophotometer.

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

2.5 Aromatase Activity

Aromatase activity was determined for each porcine placental microsome preparation. A single experiment (for each preparation) was conducted using only the substrate ([^{3}H]ASDN/ASDN). The assay was conducted as described in the following paragraph.

The [^{3}H]ASDN/ASDN substrate solution was prepared by combining solutions of [^{3}H]ASDN and ASDN. A 1 mg/mL solution of ASDN was prepared in ethanol. Serial dilutions of this solution were prepared in assay buffer to yield a solution containing ca. 1 μg ASDN/mL. The [^{3}H]ASDN stock was diluted 1:100 in assay buffer to yield a solution containing ca. 10 μCi/mL. The substrate solution was prepared by combining 275 μL of the 1 μg ASDN/mL solution, 100 μL of the 10 μCi [^{3}H]ASDN/mL solution and 625 μL buffer.

The assay was performed in duplicate in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 μL) of propylene glycol was added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 μCi, 50 nM), was added to the tubes. An NADPH-generating system comprised of NADP (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) was added to each tube. The tubes were placed at 37 ± 1°C in the water bath for 5 min prior to initiation of the assay by the addition of the diluted microsomal suspension (~0.1 mg microsomal protein/mL). The total volume was 2.0 mL, and the tubes were incubated for 30 min. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for about 30 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 min at a setting

of 1000 rpm (which is approximately equivalent to 230*g*). The methylene chloride layer was removed to a vial and weighed; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time reserving and weighing the methylene chloride layer in a separate vial. The aqueous layers were transferred to vials, weighed, and duplicate aliquots (0.5 mL) were weighed into 20-mL liquid scintillation counting vials. Duplicate aliquots of each methylene chloride fraction were weighed into scintillation 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 the substrate solution was determined by analyzing 5 weighed aliquots by LSS. The substrate solution specific activity was determined by dividing the radiochemical content of the substrate solution (dpm/g) by the total concentration of ASDN in the solution (ASDN + [^{3}H]ASDN; nmol/g solution).

Analysis of the samples was performed using LSS as described in SOP METAB-610. Radiolabel found in the aqueous fractions represents ^3H_2O formed, and that in the methylene chloride fractions represents unreacted substrate.

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 solution (expressed in dpm/nmol). The activity of the enzyme reaction was expressed in nmol (mg protein)$^{-1}$ min^{-1} and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 30 min.

3.0 Results and Discussion

Six porcine placentas were obtained (two on one day and all the others on separate days) from a local research farm and microsomes were prepared from five of them (where two were obtained on a single day, only one was processed). A sample of each microsome preparation was thawed rapidly in a water bath and rehomogenized prior to assay for protein and cytochrome P450 content and aromatase activity. Only one of the five preparations had appreciable aromatase activity. The protein content for that preparation was determined to be ca. 28 mg/mL. The total protein yield for the preparation was calculated to be ca. 126 mg (ca. 4.5 mL microsomal suspension). P450 content of that preparation was determined to be ca. 0.053 nmol/mg protein. The aromatase activity of the porcine placental microsomes was ca. 3 pmol estrogen formed/mg protein/min; this value matches that found earlier for the bovine placental microsome preparation. No literature value for porcine placenta microsomal aromatase activity was found.

4.0 Conclusion

Isolation of microsomes with good aromatase activity from porcine placentas was problematic at best. Collection of the thin layer of tissue was difficult and time-consuming. Prompt chilling and work-up of the tissue is reported to be essential in the preservation of good aromatase activity (B. Brueggemeier, personal communication). The first processed placenta, which remained at room temperature for an undetermined length of time prior to work-up was not expected to contain active enzyme because of the poor collection conditions and, in fact, did not. Of the four placentas that were collected and chilled promptly after delivery by the sow, only one yielded microsomes with appreciable aromatase activity. Of those four preparations, two were stored in a final resuspension buffer containing 0.05 M dithiothreitol and two in a buffer containing 0.05 mM of that preservative. One of the preparations stored in 0.05 M dithithreitol-containing buffer had good aromatase activity and the other did not, while none of those stored in the buffer with the lower dithiothreitol concentration had measurable aromatase activity. There does not appear to be a clear relationship between dithiothreitol concentration in the resuspension buffer and aromatase activity. No experimental variables were identified that led to this low activity. In fact, with the exception of minor changes to the homogenization procedure, all placentas were processed by the same method. In some cases a Polytron homogenizer was used for initial homogenization prior to final homogenization with a Potter Elvejhem homogenizer, but this change was not expected to affect final aromatase activity. Additional placental microsomes with sufficient aromatase activity would be needed for the conduct of the other phases of this project. The unavailability of literature values for aromatase activity in porcine placental microsomes makes it difficult to determine whether the aromatase activity found here is in the 'normal' range.

5.0 References

Omura, T.; Sato, R. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* 1964**, 239**, 2370-2378.

Appendix A-3

Letter Report - Bovine Phase 1
Preoptimization Experiment Results Related to Bovine Placental Microsomes

Letter Report–Bovine Phase 1

Pre-Validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

EDSP WA 2-24

1.0 Introduction

The pre-optimization experiments were designed to assess the chemical and biological properties of the critical components that are used in the aromatase assay. These experiments included characterizing the radiolabeled substrate and preparation of placental microsomes. In addition, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and the human recombinant microsomes) was analyzed for protein concentration, cytochrome P450 (P450) content, and aromatase activity. The P450 content measurement confirms that the enzyme is present (and in what concentration/preparation type) prior to beginning the more elaborate aromatase activity assay. Finally, a single aromatase activity assay determination 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 definitive optimization experiments.

This report includes the results of the pre-optimization experiments related to bovine placental microsomes.

2.0 Materials and Methods

2.1 Chemicals

Non-radiolabeled 4-androstene-3,17-dione (ASDN) was received through Battelle from Sigma (St. Louis, MO). [1β-^{3}H(N)]Androst-4-ene-3,17-dione ([^{3}H]ASDN) was obtained from Perkin Elmer Life Science, Boston, MA. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, glycerol, niacinamide, dithiothreitol and bovine serum albumin (BSA) were purchased from Sigma. Sodium phosphate monobasic, sodium phosphate dibasic, sucrose and propylene glycol were from JT Baker (Phillipsburg, NJ). Ultima Gold scintillation cocktail was purchased from Packard Instruments (Meriden, CT). DC Protein assay kit was purchased from Biorad (Hercules, CA) .

2.2 Placental Microsome Preparation

A bovine placenta was received from NCSU's Reedy Creek Field Laboratories on February 27, 2003. Upon delivery, the placenta was placed in a plastic bag and left at ca. 0 °C. The placenta was transported on ice to RTI about 1.5 h after delivery. The tissue was placed in a tub that was nestled in a tub of ice and the cotyledons were dissected away from the membrane. Cotyledons were rinsed of soil with chilled isotonic saline prior to their transfer into beakers containing ice-cold buffer (2:1 tissue weight:buffer; 0.25 M sucrose, 0.05 M sodium phosphate (pH 7.0), 0.04 M niacinamide). The cotyledons were minced with scissors and then were homogenized in portions using a Polytron homogenizer. The homogenate was transferred to centrifuge tubes and centrifuged at a setting of 10,000*g* for 30 min at 4 °C in an IEC B-22M centrifuge. The supernatant was transferred to ultracentrifuge tubes and was centrifuged at a setting of 35,000 rpm (which is equivalent to approximately 100,000*g*) in a refrigerated Beckman L5-50B Ultracentrifuge for 1 h to obtain the crude microsomal pellet. The supernatant was decanted and discarded and the microsomal pellet was resuspended in a chilled buffer containing 0.1 M sodium phosphate buffer, pH 7.4. The sample was centrifuged again at a setting of 35,000 rpm in the Beckman L5-50B for 1 h to wash the microsomes. This washing procedure was repeated one additional time. The twice-washed microsomal pellet was resuspended in chilled 0.1 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose, 20% glycerol and 0.05 M dithiothreitol. The microsomal suspension (total volume ca. 15 mL) was divided among 20 vials, flash frozen in liquid nitrogen, and stored at ca. -70 °C.

2.3 Protein Determination

The protein concentration of the bovine placental microsome preparation was determined. A 6-point standard curve was prepared using BSA, ranging from 0.13 to 1.5 mg protein/mL. Protein concentration was determined by using a DC Protein Assay kit. 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. The absorbances are stable for about 1 h. Each sample (unknowns and standards) was transferred to disposable polystyrene cuvettes and the visible absorbance (@ 750 nm) was measured using a spectrophotometer. The protein concentration of the microsomal sample was determined by extrapolation of the absorbance value using the curve developed from the absorbance of the protein standards.

2.4 P450 Content

P450 content was determined for the bovine placental microsome preparation. Using the carbon monoxide (CO) spectral assay of Omura and Sato (1964), a single experiment was conducted as described below.

A sample of the microsomal preparation was diluted 1:20 in 0.1 M phosphate buffer (pH 7.4). The diluted sample was gently bubbled with carbon monoxide for approximately 10 s and then was divided between a pair of matched cuvettes (1 mL/cuvette). Next, a few grains of solid sodium dithionite was added to the sample cuvette with gentle mixing. The visible spectrum was then recorded from 400 to 500 nm using an Aminco split-beam spectrophotometer.

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

2.5 Aromatase Activity

Aromatase activity was determined for the bovine placental microsome preparation. A single experiment was conducted using only the substrate ([^{3}H]ASDN/ASDN). The assay was conducted as described in the following paragraph.

The [^{3}H]ASDN/ASDN substrate solution was prepared by combining solutions of [^{3}H]ASDN and ASDN. A 1 mg/mL solution of ASDN was prepared in ethanol. Serial dilutions of this solution were prepared in assay buffer to yield a solution containing ca. 1 µg ASDN/mL. The [^{3}H]ASDN stock was diluted 1:100 in assay buffer to yield a solution containing ca. 10 µCi/mL. The substrate solution was prepared by combining 275 µL of the 1 µg ASDN/mL solution, 100 µL of the 10 µCi [^{3}H]ASDN/mL solution and 625 µL buffer.

The assay was performed in duplicate in 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol was added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 µCi, 50 nM), was added to the tubes. An NADPH-generating system comprised of NADP (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) was added to each tube. The tubes were placed at 37 ± 1°C in the water bath for 5 min prior to initiation of the assay by the addition of the diluted microsomal suspension (~0.1 mg microsomal protein/mL). The total volume was 2.0 mL, and the tubes were incubated for 30 min. The incubations were stopped by the addition of methylene chloride (2.0 mL); the tubes were vortex-mixed for about 30 s. The tubes were then centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 min at a setting of

1000 rpm (which is approximately equivalent to 230*g*). The methylene chloride layer was removed to a vial and weighed; the aqueous layers were extracted again with methylene chloride (2.0 mL). This extraction procedure was performed one additional time, each time reserving and weighing the methylene chloride layer in a separate vial. The aqueous layers were transferred to vials, weighed, and duplicate aliquots (0.5 mL) were weighed into 20-mL liquid scintillation counting vials. Duplicate aliquots of each methylene chloride fraction were weighed into scintillation 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 the substrate solution was determined by analyzing 5 weighed aliquots by LSS. The substrate solution specific activity was determined by dividing the radiochemical content of the substrate solution (dpm/g) by the total concentration of ASDN in the solution (ASDN + [^{3}H]ASDN; nmol/g solution).

Analysis of the samples was performed using LSS as described in SOP METAB-610. Radiolabel found in the aqueous fractions represents ^3H_2O formed, and that in the methylene chloride fractions represents unreacted substrate.

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 solution (expressed in dpm/nmol). The activity of the enzyme reaction was expressed in nmol (mg protein)$^{-1}$ min^{-1} and was calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times the incubation time, e.g. 30 min.

3.0 Results and Discussion

A bovine placenta was obtained from a local research farm and microsomes were prepared. A sample of the microsomes was thawed rapidly in a 37 ˚C water bath and rehomogenized prior to assay for protein and P450 content and aromatase activity. The protein content of the bovine placental microsomes was determined to be ca. 45 mg/mL. The total protein yield for the preparation was calculated to be ca. 675 mg. This exceeds the 250 mg of protein criteria set in the protocol. P450 content of the bovine placental microsomes was determined to be ca. 0.031 nmol/mg protein. The aromatase activity of the bovine placental microsomes was ca. 3 pmol estrogen formed/mg protein/min; this is in good agreement with the 3.62 ± 0.78 pmol estrogen formed/mg protein/min reported by Tsumagari et al. (1993).

4.0 Conclusion

The bovine placental microsomes have sufficient protein and P450 content for the conduct of these studies. The aromatase activity for the preparation was similar to that reported in the literature and is sufficient to proceed with the optimization phase of the study.

5.0 References

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Tsumagari, S., Kamata, J., Takagi, K., Tanemura, K., Yosai, A., Takeishi, M. Aromatase activity and oestrogen concentrations in bovine cotyledons and caruncles during gestation and parturition. *J. Reprod. Fert.* 1993, **98**, 631-36.

Appendix A-4

Endocrine Disruptor Screening Program
Work Assignment 2-24: Optimize the Human Placental Aromatase Assay
Computation of K_m and Percent of Substrate Consumed and Their Variation with Assay Conditions

by

Dr. Paul Feder, Battelle

DRAFT

Endocrine Disruptor Screening Program
Work Assignment 2-24: Optimize the Human Placental Aromatase Assay
Computation of K_m and Percent of Substrate Consumed and Their Variation with Assay Conditions

This memorandum addresses the computation of K_m and percent of substrate consumed for specified protein concentrations and incubation times, based on the human placental test data generated under the U.S. EPA Endocrine Disruptor Screening Program Work Assignment 2-24, "Optimization of the Aromatase Assay". In this document is 1) a description of the computations, 2) the resulting K_m values and associated percent of substrate consumed for several scenarios, as well as relevant graphs to illustrate the results, and 3) a possible extension of this analysis - the generation of error bounds around the K_m values for specific scenarios.

Computations:

Initially, the response surface for activity was estimated. Earlier explorations related the natural logarithm of activity to the logarithm of androstenedione, protein, and incubation time. This form of the response surface proved undesirable, as the K_m value could not be computed. As a result, an alternate response surface was estimated. This response surface treated activity as a direct function of androstenedione, protein, and incubation time (no logarithms were applied).

Once the response surface was estimated, a selection of scenarios, each fixing a specific incubation time and protein level, were chosen. The response surface equation was then simplified to a scenario-specific, quadratic function of only the androstenedione level by applying the time and protein values for each scenario. This scenario-specific function was then solved algebraically to find the location of the highest point on the curve (that is, the androstenedione level which leads to the largest theoretical level of activity). Given this location, the equation was used to compute the value of the highest point on the curve (an approximation of the maximum activity in the true process). Half of this highest value is the level of activity associated with the K_m value. This results in a quadratic equation, whose smallest root corresponds to the K_m value which generated the desired level of activity. (Strictly speaking, the quadratic equation leads to two roots. We chose in each case the lower root, which was associated with the upward hill of the function, as the K_m value.)

Results:

The results in this section were generated using the estimated response surface equation which has the following form (where ASDN denotes the androstenedione level and time refers to the incubation time):

$$\begin{aligned} \text{activity} = {} & 0.03 + 0.0004 * \text{ASDN} - 0.11 * \text{Protein} - 0.0009 * \text{time} \\ & - 0.0000004 * \text{ASDN}^2 - 0.0005 * \text{Protein} * \text{ASDN} \\ & + 0.08 * \text{Protein}^2 - 0.0000007 * \text{time} * \text{ASDN} \\ & + 0.001 * \text{time} * \text{Protein} + 0.000005 * \text{time}^2 \end{aligned}$$

Nine scenarios were considered. Each scenario fixed a different time and protein level. Tables 1 - 3 present the nine scenarios, the K_m value computed for each scenario, and the percent of substrate consumed associated with that K_m value under that scenario. The K_m values range from approximately 89 nM to 107 nM, while the percent of substrate consumed ranges from roughly 8% to 37%.

Table 1. Scenarios and Results

time = 15 min	protein = 0.01 mg/mL	protein = 0.025 mg/mL	protein = 0.05 mg/mL
K_m nM	94.162	93.484	92.366
% Consumption	8.12%	19.58%	36.77%

Table 2. Scenarios and Results (continued)

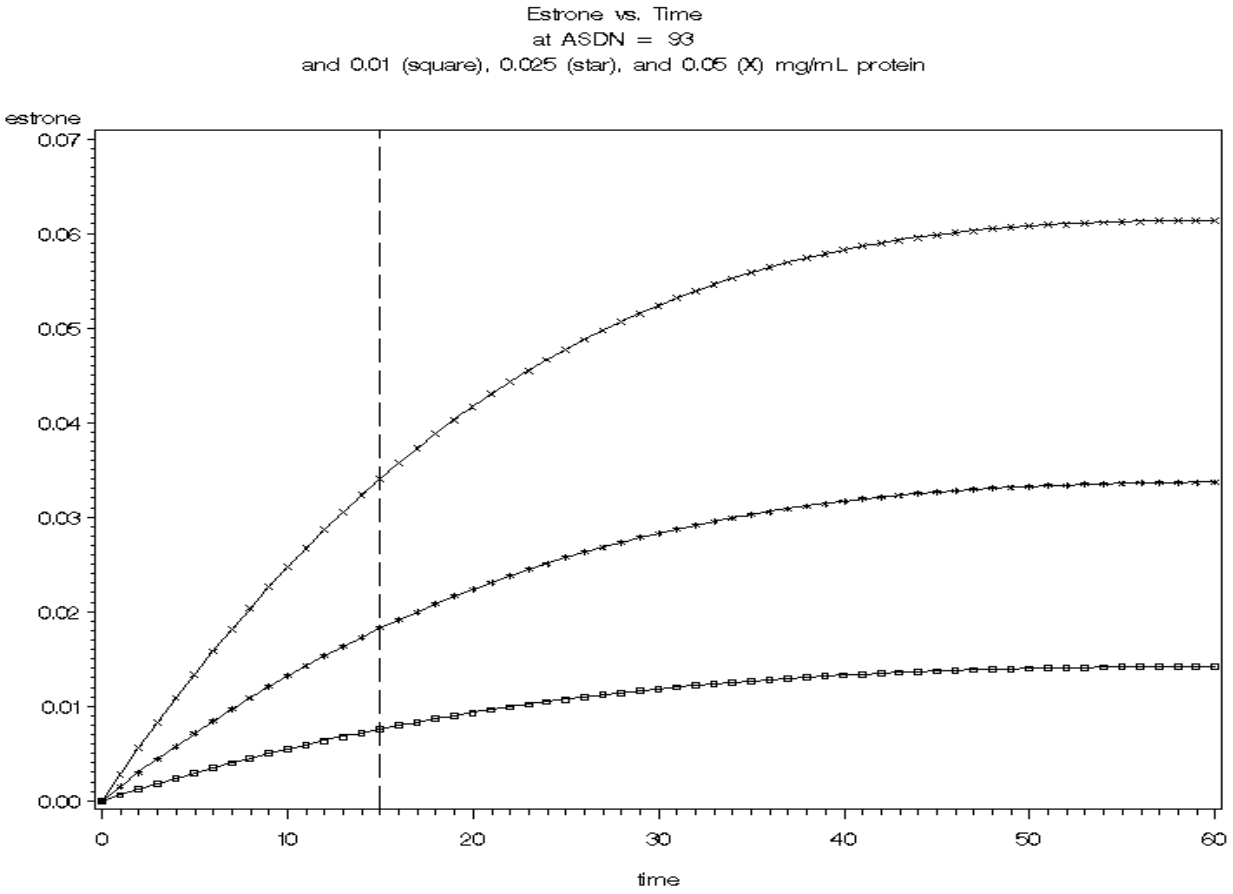
protein = 0.025 mg/mL	time = 10 min	time = 20 min	time = 30 min
K_m nM	88.687	98.080	106.602
% of Substrate Consumed	14.51%	23.58%	29.22%

Table 3. Scenarios and Results (continued)

protein = 0.025 mg/mL	time = 40 min	time = 50 min	time = 60 min
K_m nM	114.100	120.399	125.296
% of Substrate Consumed	32.73%	34.96%	36.53%

Figures 1 and 2 provide plots of the estrone levels versus time for the nine scenarios. Figure 1 shows the results from the scenarios in Table 1, while Figure 2 shows the results from Tables 2 and 3. Dashed lines are provided to illustrate the times for which the K_m was computed

for each scenario. Observe that the scenarios in Figure 1 all appear to be generating estrone in the 'linear parts' of the curves (that is, the section of the curve that is approximately linear, but before the curve flattens out towards its maximum value.) In Figure 2, however, only the scenarios associated with time equal to 10 and 20 minutes appear to be on the 'linear part' of the curve. The remaining scenarios (those associated with 30, 40, 50, and 60 minutes) appear to fall in the region of their particular curves that has started to flatten out toward the maximum value



of estrone.

Figure 1. Estrone vs. Time for the Scenarios in Table 1

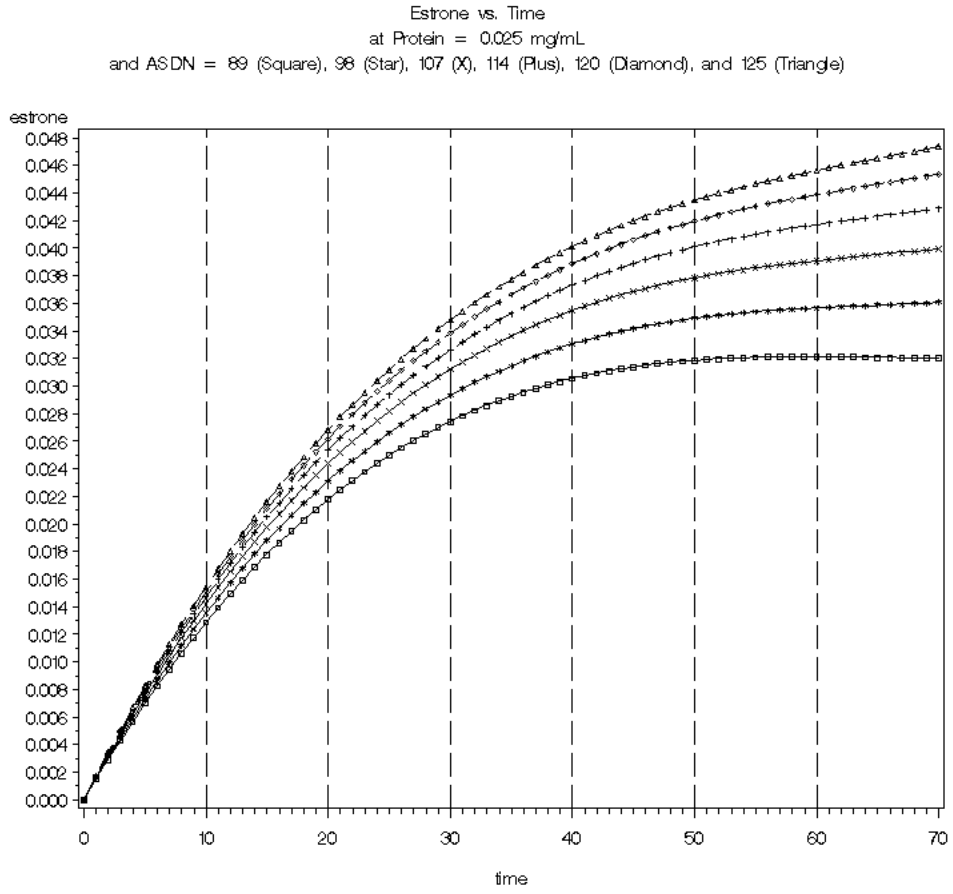


Figure 2. Estrone vs. Time for the Scenarios in Tables 2 and 3

Possible Extension:

The K_m and % of substrate consumed values presented in Tables 1 - 3 are strictly point estimates. We have provided no measure of uncertainty about these values. It is possible to put error bounds around these values using a simulation approach. In the process of estimating the response surface, we generate a variance-covariance matrix for the estimates of the regression parameters. If we treat the parameters estimates and the associated variance-covariance matrix as the center and spread of a new random distribution, we can then generate a large number (N = 100 to 1000) of realizations of the regression parameters. Then, using each set of regression parameters individually, we can compute a K_m and % of substrate consumed value for each realization for any given scenario of interest. The ordered set of K_m and % of substrate consumed values for a given scenario could then be used to generate approximate sets of error bounds of a desired confidence level (α*100%) for the true K_m and % of substrate consumed values under that scenario by selecting the appropriate center $\alpha^* N$ values of the collections. This simulation has not been carried out.

Appendix A-5

Aromatase Optimization Supplementary Studies - Experiment #1 Report

Aromatase Optimization Supplementary Studies – Experiment #1

Report

November 6, 2003

Study Objectives

To standardize the protocol for the $^3H_20$ aromatase assay using human placenta microsomes in the drug metabolism laboratories at RTI using appropriate controls plus a comparison with data from the laboratory of Dr. Robert Brueggemeier's lab. This is the first of three supplemental experiments to ensure that the placental aromatase assay is optimized and standardized before beginning the multiple chemical comparison study. The degree of inhibition of the reaction by 4-hydroxyandrostenedione (4-OH-ASDN) was investigated to demonstrate the mediation of the reaction by aromatase.

Study Results

Set 1

The aromatase assay was conducted as described in the protocol using human placental microsomes at three final protein concentrations (0.05, 0.025 and 0.0125 mg/mL). Each condition was performed in triplicate. Four conditions (for each protein concentration) were tested:

1. Complete system (microsomes, substrate, complete NADPH regenerating system)
2. Inhibited system (microsomes, substrate, complete NADPH regenerating system, 4-OH-ASDN)
3. Blank (boiled microsomes, substrate, NADPH regenerating system minus NADP)
4. No NADP (microsomes, substrate, NADPH regenerating system minus NADP)

The specific activity of the stock [^{3}H]ASDN was 25.3 Ci/mmol. [^{3}H]ASDN was combined with nonradiolabeled ASDN to prepare the substrate solution for use in the assays and the final specific activity of [^{3}H]ASDN in the substrate solution was 1.68 μCi/μg.

The spreadsheets results/data for this replicate are presented as Tables 1-3. The two higher protein concentrations yielded substrate conversion percentages (38 and 18% for 0.05 and 0.025 mg/mL, respectively) in excess of the target (10-15%). The lowest protein concentration (0.0125 mg/mL) yielded ca. 9% conversion of substrate to product in 15 min. These substrate conversion percentages are in excellent agreement with those (36.8, 19.6 and 8.1% for 0.05, 0.025 and 0.0125 mg/mL, respectively) predicted in the analysis of the previously reported

optimization data by Dr. Paul Feder. Variance within each triplicate set of tubes was low, with the coefficient of variance of aromatase activity less than 3% in both the complete and inhibited systems.

The 'No NADP' tubes show considerable activity, possibly because of residual NADP in the microsomal preparation or from cross-contamination of these tubes with NADP (during the pipetting process). Because the activity in those tubes was so high, the data from the 'Blank' tubes was used as background when calculating aromatase activity in the 'Complete' and 'Inhibited' tubes.

The results for the 'Complete' and 'Inhibited' tubes are presented in Table 4 and graphically in Figure 1.

Aromatase product formation increased linearly with increasing protein concentration. Total aromatase activity was fairly constant at 0.0501 ± 0.0016 nmol/mg/min over the range of protein concentrations tested. 4-OH-ASDN (100 nM) inhibited aromatase activity by about 76% at each protein concentration.

Set 2

An additional experiment was performed in order to address the question of whether the activity found in the 'No NADP' tubes may be due to residual NADP in the microsomes. The aromatase assay was conducted as described in the protocol using three final protein concentrations (0.05, 0.025 and 0.0125 mg/mL). Each condition was performed in triplicate. Three conditions (for each protein concentration) were tested:

1. Complete system (microsomes, substrate, complete NADPH regenerating system)
2. No NADP (microsomes, substrate, NADPH regenerating system minus NADP)
3. No NADPH regenerating system (microsomes, substrate, no NADPH regenerating system).

For this experiment, the final substrate specific activity was 1.56 µCi/µg.

The spreadsheets results/data for this experiment are presented as Tables 5-7. The two higher protein concentrations yielded substrate conversion percentages (45 and 21% for 0.05 and 0.025 mg/mL, respectively) in excess of the target 10-15%. The lowest protein concentration (0.0125 mg/mL) yielded ca. 10% conversion of substrate to product in 15 min.

The 'No NADP' tubes again showed measurable activity but that activity was much lower than was found in the first replicate. The 'No regenerating system' tubes gave essentially no turnover of substrate. The radioactive content of the aqueous portion of these tubes was very similar to that found in the 'Blank' (boiled microsomes) tubes from the first replicate and represented only about 0.1% of the radioactivity in the reaction.

Product formation was again linear with protein concentration (Table 8). Aromatase activity was slightly higher than in the first replicate (0.0573 ± 0.0026 nmol/mg/min vs. 0.0501 ± 0.0016 nmol/ mg/min, respectively).

Set 3

The aromatase assay was conducted as described in the protocol using human placental microsomes at three final protein concentrations (0.05, 0.025 and 0.0125 mg/mL). Each condition was performed in triplicate. Six conditions (for each protein concentration) were tested:

1. Complete system (microsomes, substrate, complete NADPH regenerating system)
2. Inhibited system (microsomes, substrate, complete NADPH regenerating system, 4-OH-ASDN)
3. Blank (boiled microsomes, substrate, NADPH regenerating system minus NADP)
4. No NADP (microsomes, substrate, NADPH regenerating system minus NADP)
5. No NADPH regenerating system (microsomes, substrate, no NADPH regenerating system)
6. NADPH (microsomes, substrate, 0.3 mM NADPH [instead of the regenerating system])

For this experiment, the final substrate specific activity was 2.30 µCi/µg.

The spreadsheets results/data for this experiment are presented as Tables 9-11. The two higher protein concentrations yielded substrate conversion percentages (45 and 21% for 0.05 and 0.025 mg/mL, respectively) in excess of the target (10-15%). The lowest protein concentration (0.0125 mg/mL) yielded ca. 10% conversion of substrate to product in 15 min.

The 'No NADP' tubes showed similar activity to that found in the follow-on experiment above. The aqueous portions of the 'No regenerating system' and the 'Blank' (boiled microsomes) tubes again contained very minimal radioactivity (about 0.1% of the total). The activity found in the NADPH tubes was similar to that found in the 'Complete' tubes of this set.

Product formation was again linear with protein concentration (Table 12 and Figure 2). Aromatase activity was similar to that found in the follow-on experiment (0.0567 ± 0.0030 nmol/mg/min vs. 0.0573 ± 0.0026 nmol/mg/min, respectively).

Summary and Conclusions

Product formation rates (nmol/min), substrate conversion and percent inhibition data for all three experiments are presented in Table 13. Day-to-day variance in the product formation rate was low (ca. 7.4%). Only the 0.0125 mg/mL reaction set had less than 15% substrate conversion under the reaction conditions.

Aromatase activity data for all three experiments is presented in Table 14. In each case, the radioactivity in the Blank tubes was subtracted from that in the assay tubes prior to calculation of aromatase activity. The calculated activity in the No NADP tubes of Sets 2 and 3 was much lower than that found for Set 1. A change in pipetting order was initiated between Set 1 and Set 2 that was intended to minimize the possibility of NADP carryover from tube to tube. This change may explain the widely varying activities between the Set 1 and the Set 2 'No NADP' tubes; the high apparent activity in the Set 1 'No NADP' tubes may be due to their inadvertent contamination with NADP. Only about 0.1% of the radioactivity in the reaction mixture remained in the aqueous phase of the 'Blank' and 'No system' tubes after extraction. The use of 0.3 mM NADPH instead of the regenerating system gave aromatase activities similar to those found in the tubes containing the regenerating system. At a protein concentration of 0.0125 mg/mL, the aqueous portion contained approximately 15000 DPM/mL after extraction. If the activity were inhibited 90% by a test substance, the aqueous portion would be expected to contain about 1500 DPM/mL. This is still an easily quantifiable level of radioactivity. Therefore, a protein level of 0.0125 mg/mL is suggested for use in the next studies.

Future Work

A sample of our human placenta microsomes has been shipped to Dr. Brueggemeier. Upon receipt of his data (obtained using that microsomal preparation), we will compare our data with that reported from his laboratory.

Table 1A. Set 1: Calculation of Aromatase Activity (0.05 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.05	Complete	1	2	0.5	1	20611	41222	41232	82464	0.1	218472	37.75	82241	0.0768
				0.5	2	20621	41242			0.1				
		2	2	0.5	1	20948	41896	42545	85090	0.1	218472	38.95	84867	0.0793
				0.5	2	21597	43194			0.1				
		3	2	0.5	1	20787	41574	41718	83436	0.1	218472	38.19	83213	0.0777
				0.5	2	20931	41862			0.1				
	Inhibition	1	2	0.5	1	5012	10024	10004	20008	0.1	218472	9.16	19785	0.0185
				0.5	2	4992	9984			0.1				
		2	2	0.5	1	5166	10332	10379	20758	0.1	218472	9.50	20535	0.0192
				0.5	2	5213	10426			0.1				
		3	2	0.5	1	4976	9952	9931	19862	0.1	218472	9.09	19639	0.0183
				0.5	2	4955	9910			0.1				
	Blank (boiled microsomes)	1	2	0.5	1	57	114	120	240	0.1	218472	0.11		0
				0.5	2	63	126			0.1				
		2	2	0.5	1	54	108	106	212	0.1	218472	0.10		0
				0.5	2	52	104			0.1				
		3	2	0.5	1	54	108	109	218	0.1	218472	0.10		0
				0.5	2	55	110			0.1				
	No-NADP	1	2	0.5	1	16431	32862	32966	65932	0.1	218472	30.18	65709	0.0614
				0.5	2	16535	33070			0.1				
		2	2	0.5	1	16372	32744	32881	65762	0.1	218472	30.10	65539	0.0612
				0.5	2	16509	33018			0.1				
		3	2	0.5	1	16590	33180	33802	67604	0.1	218472	30.94	67381	0.0629

Table 1B. Set 1: Calculation of Aromatase Activity (0.05 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	**Sample type**	**Replicate**	**[protein] stock microsomes (mg/mL)**	**Volume of stock microsomes used in dilution (µL)**	**Final volume of diluted microsomes (µL)**	**[protein] in dilution (mg/mL)**	**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)**	**Activity expressed independent of [protein] (nmol/min)**
0.05	Complete	1	47	64	30000	0.100	1	0.050	15	0.0511	0.0051
		2	47	64	30000	0.100	1	0.050	15	0.0527	0.0053
	Inhibition	1	47	64	30000	0.100	1	0.050	15	0.0123	0.0012
		2	47	64	30000	0.100	1	0.050	15	0.0128	0.0013
		3	47	64	30000	0.100	1	0.050	15	0.0122	0.0012
	Blank (boiled microsomes)	1	47	64	30000	0.100	1	0.050	15	0.0000	0.0000
		2	47	64	30000	0.100	1	0.050	15	0.0000	0.0000
		3	47	64	30000	0.100	1	0.050	15	0.0000	0.0000
	No-NADP	1	47	64	30000	0.100	1	0.050	15	0.0408	0.0041
		2	47	64	30000	0.100	1	0.050	15	0.0407	0.0041
		3	47	64	30000	0.100	1	0.050	15	0.0418	0.0042

Table 2A. Set I: Calculation of Aromatase Activity (0.025 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.025	Complete	1	2	0.5	1	9856	19712	19706	39412	0.1	218472	18.04	39162	0.036572
				0.5	2	9850	19700			0.1				
		2	2	0.5	1	10070	20140	19968	39936	0.1	218472	18.28	39686	0.037061
				0.5	2	9898	19796			0.1				
		3	2	0.5	1	10427	20854	20711	41422	0.1	218472	18.96	41172	0.038449
				0.5	2	10284	20568			0.1				
	Inhibition	1	2	0.5	1	2510	5020	5007	10014	0.1	218472	4.58	9764	0.009118
				0.5	2	2497	4994			0.1				
		2	2	0.5	1	2386	4772	4737	9474	0.1	218472	4.34	9224	0.008614
				0.5	2	2351	4702			0.1				
		3	2	0.5	1	2413	4826	4886	9772	0.1	218472	4.47	9522	0.008892
				0.5	2	2473	4946			0.1				
	Blank	1	2	0.5	1	61	122	126	252	0.1	218472	0.12		0
				0.5	2	65	130			0.1				
		2	2	0.5	1	63	126	132	264	0.1	218472	0.12		0
				0.5	2	69	138			0.1				
		3	2	0.5	1	55	110	117	234	0.1	218472	0.11		0
				0.5	2	62	124			0.1				
	No-NADP	1	2	0.5	1	7935	15870	15925	31850	0.1	218472	14.58	31600	0.02951
				0.5	2	7990	15980			0.1				
		2	2	0.5	1	7537	15074	15104	30208	0.1	218472	13.83	29958	0.027977
				0.5	2	7567	15134			0.1				
		3	2	0.5	1	7888	15776	15586	31172	0.1	218472	14.27	30922	0.028877
				0.5	2	7698	15396			0.1				

Table 2B. Set I: Calculation of Aromatase Activity (0.025 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.025	Complete	1	47	64	60000	0.050	1	0.025	15	0.0486	0.0024
		2	47	64	60000	0.050	1	0.025	15	0.0493	0.0025
		3	47	64	60000	0.050	1	0.025	15	0.0511	0.0026
	Inhibition	1	47	64	60000	0.050	1	0.025	15	0.0121	0.0006
		2	47	64	60000	0.050	1	0.025	15	0.0115	0.0006
		3	47	64	60000	0.050	1	0.025	15	0.0118	0.0006
	Blank	1	47	64	60000	0.050	1	0.025	15	0.0000	0.0000
		2	47	64	60000	0.050	1	0.025	15	0.0000	0.0000
		3	47	64	60000	0.050	1	0.025	15	0.0000	0.0000
	No-NADP	1	47	64	60000	0.050	1	0.025	15	0.0392	0.0020
		2	47	64	60000	0.050	1	0.025	15	0.0372	0.0019
		3	47	64	60000	0.050	1	0.025	15	0.0384	0.0019

Table 3A. Set I: Calculation of Aromatase Activity (0.0125 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.0125	Complete	1	2	0.5	1	4980	9960	10011	20022	0.1	218472	9.16	19723	0.018418
				0.5	2	5031	10062			0.1				
		2	2	0.5	1	4943	9886	9885	19770	0.1	218472	9.05	19471	0.018183
				0.5	2	4942	9884			0.1				
		3	2	0.5	1	4882	9764	10003	20006	0.1	218472	9.16	19707	0.018404
				0.5	2	5121	10242			0.1				
	Inhibition	1	2	0.5	1	1169	2338	2385	4770	0.1	218472	2.18	4471	0.004175
				0.5	2	1216	2432			0.1				
		2	2	0.5	1	1183	2366	2365	4730	0.1	218472	2.17	4431	0.004138
				0.5	2	1182	2364			0.1				
		3	2	0.5	1	1210	2420	2417	4834	0.1	218472	2.21	4535	0.004235
				0.5	2	1207	2414			0.1				
	Blank	1	2	0.5	1	74	148	148	296	0.1	218472	0.14		0
				0.5	2	74	148			0.1				
		2	2	0.5	1	82	164	151	302	0.1	218472	0.14		0
				0.5	2	69	138			0.1				
		3	2	0.5	1					0.1	218472			
				0.5	2					0.1				
	No-NADP	1	2	0.5	1	4007	8014	8047	16094	0.1	218472	7.37	15795	0.01475
				0.5	2	4040	8080			0.1				
		2	2	0.5	1	4023	8046	8003	16006	0.1	218472	7.33	15707	0.014668
				0.5	2	3980	7960			0.1				
		3	2	0.5	1	3732	7464	7468	14936	0.1	218472	6.84	14637	0.013669
				0.5	2	3736	7472			0.1				

Table 3B. Set I: Calculation of Aromatase Activity (0.0125 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.0125	Complete	1	47	64	120000	0.025	1	0.013	15	0.0490	0.0012
		2	47	64	120000	0.025	1	0.013	15	0.0484	0.0012
		3	47	64	120000	0.025	1	0.013	15	0.0489	0.0012
	Inhibition	1	47	64	120000	0.025	1	0.013	15	0.0111	0.0003
		2	47	64	120000	0.025	1	0.013	15	0.0110	0.0003
		3	47	64	120000	0.025	1	0.013	15	0.0113	0.0003
	Blank	1	47	64	120000	0.025	1	0.013	15	0.0000	0.0000
		2	47	64	120000	0.025	1	0.013	15	0.0000	0.0000
		3	47	64	120000	0.025	1	0.013	15		
	No-NADP	1	47	64	120000	0.025	1	0.013	15	0.0392	0.0010
		2	47	64	120000	0.025	1	0.013	15	0.0390	0.0010
		3	47	64	120000	0.025	1	0.013	15	0.0364	0.0009

Table 4. Summary of Aromatase Activity

[protein] mg/mL	Product formation rate (nmol/min)		Aromatase activity (nmol/mg/min)				
			Complete		Inhibited		
	Complete	Inhibited	Activity	SD	Activity	SD	% Inhibition
0.05	0.0052	0.0012	0.0518	0.0008	0.0124	0.0003	76.0
0.025	0.0025	0.0006	0.0497	0.0013	0.0118	0.0003	76.2
0.0125	0.0012	0.0003	0.0488	0.0004	0.0111	0.0001	77.2

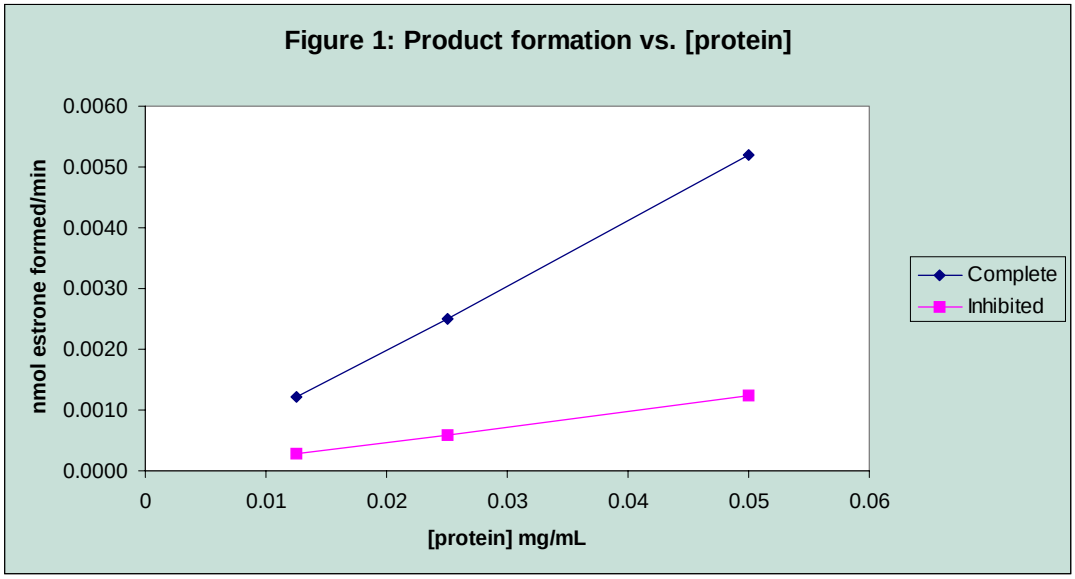


Table 5A. Set 2: Calculation of Aromatase Activity (0.05 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.05	Complete	1	2	0.5	1	22700	45400	45099	90198	0.1	199816	45.14	89921	0.090733
				0.5	2	22399	44798			0.1				
		2	2	0.5	1	22747	45494	44590	89180	0.1	199816	44.63	88903	0.089706
				0.5	2	21843	43686			0.1				
		3	2	0.5	1	22700	45400	45330	90660	0.1	199816	45.37	90383	0.0912
				0.5	2	22630	45260			0.1				
	-NADP	1	2	0.5	1	677	1354	1320	2640	0.1	199816	1.32	2363	0.002385
				0.5	2	643	1286			0.1				
		2	2	0.5	1	841	1682	1679	3358	0.1	199816	1.68	3081	0.003109
				0.5	2	838	1676			0.1				
		3	2	0.5	1	2157	4314	4230	8460	0.1	199816	4.23	8183	0.008257
				0.5	2	2073	4146			0.1				
	-Regen system	1	2	0.5	1	54	108	127	254	0.1	199816	0.13		0
				0.5	2	73	146			0.1				
		2	2	0.5	1	76	152	141	282	0.1	199816	0.14		0
				0.5	2	65	130			0.1				
		3	2	0.5	1	69	138	147	294	0.1	199816	0.15		0
				0.5	2	78	156			0.1				

Table 5B. Set 2: Calculation of Aromatase Activity (0.05 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.05	Complete	1	47	64	30000	0.100	1	0.050	15	0.0603	0.0060
		2	47	64	30000	0.100	1	0.050	15	0.0596	0.0060
		3	47	64	30000	0.100	1	0.050	15	0.0606	0.0061
	-NADP	1	47	64	30000	0.100	1	0.050	15	0.0016	0.0002
		2	47	64	30000	0.100	1	0.050	15	0.0021	0.0002
		3	47	64	30000	0.100	1	0.050	15	0.0055	0.0006
	-Regen system	1	47	64	30000	0.100	1	0.050	15	0.0000	0.0000
		2	47	64	30000	0.100	1	0.050	15	0.0000	0.0000
		3	47	64	30000	0.100	1	0.050	15	0.0000	0.0000

Table 6A. Set 2: Calculation of Aromatase Activity (0.025 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.05	Complete	1	2	0.5	1	10957	21914	21926	43852	0.1	199816	21.95	43604	0.043998
				0.5	2	10969	21938			0.1				
		2	2	0.5	1	10953	21906	21496	42992	0.1	199816	21.52	42744	0.04313
				0.5	2	10543	21086			0.1				
		3	2	0.5	1	10531	21062	21007	42014	0.1	199816	21.03	41766	0.042143
				0.5	2	10476	20952			0.1				
	-NADP	1	2	0.5	1	3021	6042	5956	11912	0.1	199816	5.96	11664	0.011769
				0.5	2	2935	5870			0.1				
		2	2	0.5	1	1822	3644	3632	7264	0.1	199816	3.64	7016	0.007079
				0.5	2	1810	3620			0.1				
		3	2	0.5	1	2458	4916	4939	9878	0.1	199816	4.94	9630	0.009717
				0.5	2	2481	4962			0.1				
	-Regen system	1	2	0.5	1	72	144	128	256	0.1	199816	0.13		0
				0.5	2	56	112			0.1				
		2	2	0.5	1	55	110	120	240	0.1	199816	0.12		0
				0.5	2	65	130			0.1				
		3	2	0.5	1	56	112	124	248	0.1	199816	0.12		0
				0.5	2	68	136			0.1				

Table 6B. Set 2: Calculation of Aromatase Activity (0.025 mg/mL protein) (continued)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.05	Complete	1	47	64	60000	0.050	1	0.025	15	0.0585	0.0029
		2	47	64	60000	0.050	1	0.025	15	0.0574	0.0029
		3	47	64	60000	0.050	1	0.025	15	0.0560	0.0028
	-NADP	1	47	64	60000	0.050	1	0.025	15	0.0157	0.0008
		2	47	64	60000	0.050	1	0.025	15	0.0094	0.0005
		3	47	64	60000	0.050	1	0.025	15	0.0129	0.0006
	-Regen system	1	47	64	60000	0.050	1	0.025	15	0.0000	0.0000
		2	47	64	60000	0.050	1	0.025	15	0.0000	0.0000
		3	47	64	60000	0.050	1	0.025	15	0.0000	0.0000

Table 7A. Set 2: Calculation of Aromatase Activity (0.0125 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.05	Complete	1	2	0.5	1	5199	10398	10512	21024	0.1	199816	10.52	20798	0.020986
				0.5	2	5313	10626			0.1				
		2	2	0.5	1	5124	10248	10291	20582	0.1	199816	10.30	20356	0.02054
				0.5	2	5167	10334			0.1				
		3	2	0.5	1	5028	10056	10030	20060	0.1	199816	10.04	19834	0.020013
				0.5	2	5002	10004			0.1				
	-NADP	1	2	0.5	1	525	1050	1056	2112	0.1	199816	1.06	1886	0.001903
				0.5	2	531	1062			0.1				
		2	2	0.5	1	1177	2354	2378	4756	0.1	199816	2.38	4530	0.004571
				0.5	2	1201	2402			0.1				
		3	2	0.5	1	1313	2626	2560	5120	0.1	199816	2.56	4894	0.004938
				0.5	2	1247	2494			0.1				
	-Regen system	1	2	0.5	1	63	126	127	254	0.1	199816	0.13		0
				0.5	2	64	128			0.1				
		2	2	0.5	1	59	118	112	224	0.1	199816	0.11		0
				0.5	2	53	106			0.1				
		3	2	0.5	1	51	102	100	200	0.1	199816	0.10		0
				0.5	2	49	98			0.1				

Table 7B. Set 2: Calculation of Aromatase Activity (0.0125 mg/mL protein) continued

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.05	Complete	1	47	64	120000	0.025	1	0.013	15	0.0558	0.0014
		2	47	64	120000	0.025	1	0.013	15	0.0546	0.0014
		3	47	64	120000	0.025	1	0.013	15	0.0532	0.0013
	-NADP	1	47	64	120000	0.025	1	0.013	15	0.0051	0.0001
		2	47	64	120000	0.025	1	0.013	15	0.0122	0.0003
		3	47	64	120000	0.025	1	0.013	15	0.0131	0.0003
	-Regen system	1	47	64	120000	0.025	1	0.013	15	0.0000	0.0000
		2	47	64	120000	0.025	1	0.013	15	0.0000	0.0000
		3	47	64	120000	0.025	1	0.013	15	0.0000	0.0000

Table 8. Summary of Aromatase Activity for Set 2

[protein] mg/mL	Product formation rate (nmol/min)	Aromatase activity (nmol/mg/min)	
		Mean	SD
0.05	0.0060	0.0602	0.0005
0.025	0.0029	0.0573	0.0012
0.0125	0.0014	0.0546	0.0013

Table 9A. Set 3: Calculation of Aromatase Activity (0.05 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.05	Complete	1	2	0.5	1	32654	65308	64723	129446	0.1	295336	43.83	129088	0.088319
				0.5	2	32069	64138			0.1				
		2	2	0.5	1	33859	67718	66896	133792	0.1	295336	45.30	133434	0.091293
				0.5	2	33037	66074			0.1				
		3	2	0.5	1	34261	68522	67070	134140	0.1	295336	45.42	133782	0.091531
				0.5	2	32809	65618			0.1				
	Inhibition	1	2	0.5	1	7436	14872	14856	29712	0.1	295336	10.06	29354	0.020083
				0.5	2	7420	14840			0.1				
		2	2	0.5	1	7581	15162	15411	30822	0.1	295336	10.44	30464	0.020843
				0.5	2	7830	15660			0.1				
		3	2	0.5	1	7740	15480	15364	30728	0.1	295336	10.40	30370	0.020779
				0.5	2	7624	15248			0.1				
	Blank	1	2	0.5	1	92	184	173	346	0.1	295336	0.12		0
	(boiled microsomes)			0.5	2	81	162			0.1				
		2	2	0.5	1	85	170	162	324	0.1	295336	0.11		0
				0.5	2	77	154			0.1				
		3	2	0.5	1	107	214	202	404	0.1	295336	0.14		0
				0.5	2	95	190			0.1				
	No-NADP	1	2	0.5	1	190	380	373	746	0.1	295336	0.25	388	0.000265
				0.5	2	183	366			0.1				
		2	2	0.5	1	193	386	411	822	0.1	295336	0.28	464	0.000317
				0.5	2	218	436			0.1				

(continued)

Table 9A. Set 3: Calculation of Aromatase Activity (0.05 mg/mL protein) (continued)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	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 (Blank Tubes)	nmol ^3H_2O formed
		3	2	0.5	1	3653	7306	7187	14374	0.1	295336	4.87	14016	0.009589
				0.5	2	3534	7068			0.1				
	- System	1	2	0.5	1	119	238	220	440	0.1	295336	0.15	82	5.61E-05
				0.5	2	101	202			0.1				
		2	2	0.5	1	101	202	198	396	0.1	295336	0.13	38	2.6E-05
				0.5	2	97	194			0.1				
		3	2	0.5	1	95	190	200	400	0.1	295336	0.14	42	2.87E-05
				0.5	2	105	210			0.1				
	+ NADPH	1	2	0.5	1	31704	63408	62923	125846	0.1	295336	42.61	125488	0.085856
				0.5	2	31219	62438			0.1				
		2	2	0.5	1	31807	63614	63269	126538	0.1	295336	42.85	126180	0.08633
				0.5	2	31462	62924			0.1				
		3	2	0.5	1	31520	63040	62261	124522	0.1	295336	42.16	124164	0.084951
				0.5	2	30741	61482			0.1				

Table 9B. Set 3: Calculation of Aromatase Activity (0.05 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	**Sample type**	**Replicate**	**[protein] stock microsomes (mg/mL)**	**Volume of stock microsomes used in dilution (µL)**	**Final volume of diluted microsomes (µL)**	**[protein] in dilution (mg/mL)**	**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)**	**Activity expressed independent of [protein] (nmol/min)**
0.05	Complete	1	47	85	40000	0.100	1	0.050	15	0.0590	0.0059
		2	47	85	40000	0.100	1	0.050	15	0.0609	0.0061
		3	47	85	40000	0.100	1	0.050	15	0.0611	0.0061
	Inhibition	1	47	85	40000	0.100	1	0.050	15	0.0134	0.0013
		2	47	85	40000	0.100	1	0.050	15	0.0139	0.0014
		3	47	85	40000	0.100	1	0.050	15	0.0139	0.0014
	Blank (boiled microsomes)	1	47	85	40000	0.100	1	0.050	15	0.0000	0.0000
		2	47	85	40000	0.100	1	0.050	15	0.0000	0.0000
		3	47	85	40000	0.100	1	0.050	15	0.0000	0.0000
	No-NADP	1	47	85	40000	0.100	1	0.050	15	0.0002	0.0000
		2	47	85	40000	0.100	1	0.050	15	0.0002	0.0000
		3	47	85	40000	0.100	1	0.050	15	0.0064	0.0006
	- System	1	47	85	40000	0.100	1	0.050	15	0.0000	0.0000
		2	47	85	40000	0.100	1	0.050	15	0.0000	0.0000
		3	47	85	40000	0.100	1	0.050	15	0.0000	0.0000
	+ NADPH	1	47	85	40000	0.100	1	0.050	15	0.0573	0.0057
		2	47	85	40000	0.100	1	0.050	15	0.0576	0.0058
		3	47	85	40000	0.100	1	0.050	15	0.0567	0.0057

Table 10A. Set 3: Calculation of Aromatase Activity (0.025 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	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 (Blank Tubes)	nmol ^3H_2O formed
0.025	Complete	1	2	0.5	1	15317	30634	30471	60942	0.1	295336	20.63	60629	0.041481
				0.5	2	15154	30308			0.1				
		2	2	0.5	1	15451	30902	30970	61940	0.1	295336	20.97	61627	0.042164
				0.5	2	15519	31038			0.1				
		3	2	0.5	1	15675	31350	30779	61558	0.1	295336	20.84	61245	0.041903
				0.5	2	15104	30208			0.1				
	Inhibition	1	2	0.5	1	3608	7216	7157	14314	0.1	295336	4.85	14001	0.009579
				0.5	2	3549	7098			0.1				
		2	2	0.5	1	3554	7108	7018	14036	0.1	295336	4.75	13723	0.009389
				0.5	2	3464	6928			0.1				
		3	2	0.5	1	3709	7418	7315	14630	0.1	295336	4.95	14317	0.009796
				0.5	2	3606	7212			0.1				
	Blank	1	2	0.5	1	81	162	163	326	0.1	295336	0.11		0
				0.5	2	82	164			0.1				
		2	2	0.5	1	66	132	147	294	0.1	295336	0.10		0
				0.5	2	81	162			0.1				
		3	2	0.5	1	72	144	159	318	0.1	295336	0.11		0
				0.5	2	87	174			0.1				
	No-NADP	1	2	0.5	1	216	432	447	894	0.1	295336	0.30	581	0.000398
				0.5	2	231	462			0.1				
		2	2	0.5	1	506	1012	1066	2132	0.1	295336	0.72	1819	0.001245
				0.5	2	560	1120			0.1				

(continued)

Table 10A. Set 3: Calculation of Aromatase Activity (0.025 mg/mL protein) (continued)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	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 (Blank Tubes)	nmol ^3H_2O formed
		3	2	0.5	1	197	394	393	786	0.1	295336	0.27	473	0.000324
				0.5	2	196	392			0.1				
	- System	1	2	0.5	1	89	178	186	372	0.1	295336	0.13	59	4.06E-05
				0.5	2	97	194			0.1				
		2	2	0.5	1	84	168	184	368	0.1	295336	0.12	55	3.79E-05
				0.5	2	100	200			0.1				
		3	2	0.5	1	121	242	229	458	0.1	295336	0.16	145	9.94E-05
				0.5	2	108	216			0.1				
	+ NADPH	1	2	0.5	1	14914	29828	29715	59430	0.1	295336	20.12	59117	0.040447
				0.5	2	14801	29602			0.1				
		2	2	0.5	1	14936	29872	29794	59588	0.1	295336	20.18	59275	0.040555
				0.5	2	14858	29716			0.1				
		3	2	0.5	1	14963	29926	29873	59746	0.1	295336	20.23	59433	0.040663
				0.5	2	14910	29820			0.1				

Table 10B. Set 3: Calculation of Aromatase Activity (0.025 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.025	Complete	1	47	85	80000	0.050	1	0.025	15	0.0554	0.0028
		2	47	85	80000	0.050	1	0.025	15	0.0563	0.0028
		3	47	85	80000	0.050	1	0.025	15	0.0559	0.0028
	Inhibition	1	47	85	80000	0.050	1	0.025	15	0.0128	0.0006
		2	47	85	80000	0.050	1	0.025	15	0.0125	0.0006
		3	47	85	80000	0.050	1	0.025	15	0.0131	0.0007
	Blank	1	47	85	80000	0.050	1	0.025	15	0.0000	0.0000
		2	47	85	80000	0.050	1	0.025	15	0.0000	0.0000
		3	47	85	80000	0.050	1	0.025	15	0.0000	0.0000
	No-NADP	1	47	85	80000	0.050	1	0.025	15	0.0005	0.0000
		2	47	85	80000	0.050	1	0.025	15	0.0017	0.0001
		3	47	85	80000	0.050	1	0.025	15	0.0004	0.0000
	- System	1	47	85	80000	0.050	1	0.025	15	0.0001	0.0000
		2	47	85	80000	0.050	1	0.025	15	0.0001	0.0000
		3	47	85	80000	0.050	1	0.025	15	0.0001	0.0000
	+ NADPH	1	47	85	80000	0.050	1	0.025	15	0.0540	0.0027
		2	47	85	80000	0.050	1	0.025	15	0.0541	0.0027
		3	47	85	80000	0.050	1	0.025	15	0.0543	0.0027

Table 11A. Set 3: Calculation of Aromatase Activity (0.0125 mg/mL protein)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	Nominal total volume (mL)	Aliq Volume (mL)	Aliq. #	DPM/aliq	DPM/mL	Ave DPM/ mL	Total DPM in 2 mL reaction	Volume of substrate solution used/assay tube (mL)	Total DPM in assay tube (initial)	% conversion to product	Total DPM corrected for background (Blank Tubes)	Total nmol ^3H_2O formed in 2 mL reaction
0.0125	Complete	1	2	0.5	1	7473	14946	14674	29348	0.1	295336	9.94	29041	0.019869
				0.5	2	7201	14402			0.1				
		2	2	0.5	1	7576	15152	14928	29856	0.1	295336	10.11	29549	0.020217
				0.5	2	7352	14704			0.1				
		3	2	0.5	1	7515	15030	15044	30088	0.1	295336	10.19	29781	0.020375
				0.5	2	7529	15058			0.1				
	Inhibition	1	2	0.5	1	1851	3702	3696	7392	0.1	295336	2.50	7085	0.004847
				0.5	2	1845	3690			0.1				
		2	2	0.5	1	1889	3778	3712	7424	0.1	295336	2.51	7117	0.004869
				0.5	2	1823	3646			0.1				
		3	2	0.5	1	1741	3482	3381	6762	0.1	295336	2.29	6455	0.004416
				0.5	2	1640	3280			0.1				
	Blank	1	2	0.5	1	84	168	156	312	0.1	295336	0.11		0
				0.5	2	72	144			0.1				
		2	2	0.5	1	72	144	153	306	0.1	295336	0.10		0
				0.5	2	81	162			0.1				
		3	2	0.5	1	69	138	152	304	0.1	295336	0.10		0
				0.5	2	83	166			0.1				
	No-NADP	1	2	0.5	1	760	1520	1521	3042	0.1	295336	1.03	2735	0.001871
				0.5	2	761	1522			0.1				
		2	2	0.5	1	640	1280	1265	2530	0.1	295336	0.86	2223	0.001521
				0.5	2	625	1250			0.1				

(continued)

Table 11A. Set 3: Calculation of Aromatase Activity (0.0125 mg/mL protein) (continued)

Sample Info			Calculate DPM in aqueous portion after extraction							Calculate % turnover				
Sample ID (mg/mL)	Sample type	Replicate	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 (Blank Tubes)	nmol ^3H_2O formed
				0.5	2	1065	2130			0.1				
	- System	1	2	0.5	1	85	170	154	308	0.1	295336	0.10	1	4.56E-07
				0.5	2	69	138			0.1				
		2	2	0.5	1	99	198	183	366	0.1	295336	0.12	59	4.01E-05
				0.5	2	84	168			0.1				
		3	2	0.5	1	100	200	202	404	0.1	295336	0.14	97	6.61E-05
				0.5	2	102	204			0.1				
	+ NADPH	1	2	0.5	1	6987	13974	14207	28414	0.1	295336	9.62	28107	0.01923
				0.5	2	7220	14440			0.1				
		2	2	0.5	1	7258	14516	14519	29038	0.1	295336	9.83	28731	0.019657
				0.5	2	7261	14522			0.1				
		3	2	0.5	1	7028	14056	14076	28152	0.1	295336	9.53	27845	0.019051
				0.5	2	7048	14096			0.1				

Table 11B. Set 3: Calculation of Aromatase Activity (0.0125 mg/mL protein)

Sample Info			Calculate final assay protein concentration								
Sample ID (mg/mL)	Sample type	Replicate	[protein] stock microsomes (mg/mL)	Volume of stock microsomes used in dilution (µL)	Final volume of diluted microsomes (µL)	[protein] in dilution (mg/mL)	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)	Activity expressed independent of [protein] (nmol/min)
0.0125	Complete	1	47	85	160000	0.025	1	0.012	15	0.0531	0.0013
		2	47	85	160000	0.025	1	0.012	15	0.0540	0.0013
		3	47	85	160000	0.025	1	0.012	15	0.0544	0.0014
	Inhibition	1	47	85	160000	0.025	1	0.012	15	0.0129	0.0003
		2	47	85	160000	0.025	1	0.012	15	0.0130	0.0003
		3	47	85	160000	0.025	1	0.012	15	0.0118	0.0003
	Blank	1	47	85	160000	0.025	1	0.012	15	0.0000	0.0000
		2	47	85	160000	0.025	1	0.012	15	0.0000	0.0000
		3	47	85	160000	0.025	1	0.012	15	0.0000	0.0000
	No-NADP	1	47	85	160000	0.025	1	0.012	15	0.0050	0.0001
		2	47	85	160000	0.025	1	0.012	15	0.0041	0.0001
		3	47	85	160000	0.025	1	0.012	15	0.0075	0.0002
	- System	1	47	85	160000	0.025	1	0.012	15	0.0000	0.0000
		2	47	85	160000	0.025	1	0.012	15	0.0001	0.0000
		3	47	85	160000	0.025	1	0.012	15	0.0002	0.0000
	+ NADPH	1	47	85	160000	0.025	1	0.012	15	0.0513	0.0013
		2	47	85	160000	0.025	1	0.012	15	0.0525	0.0013
		3	47	85	160000	0.025	1	0.012	15	0.0509	0.0013

Table 12. Summary of Aromatase Activity

[protein] mg/mL	Product formation rate (nmol/min)		Aromatase activity (nmol/mg/min)				
			Complete		Inhibited		
	Complete	Inhibited	Activity	SD	Activity	SD	% Inhibition
0.05	0.0060	0.0014	0.0603	0.0012	0.0137	0.0003	77.2
0.025	0.0028	0.0006	0.0559	0.0005	0.0128	0.0003	77.1
0.0125	0.0013	0.0003	0.0538	0.0007	0.0126	0.0007	76.6

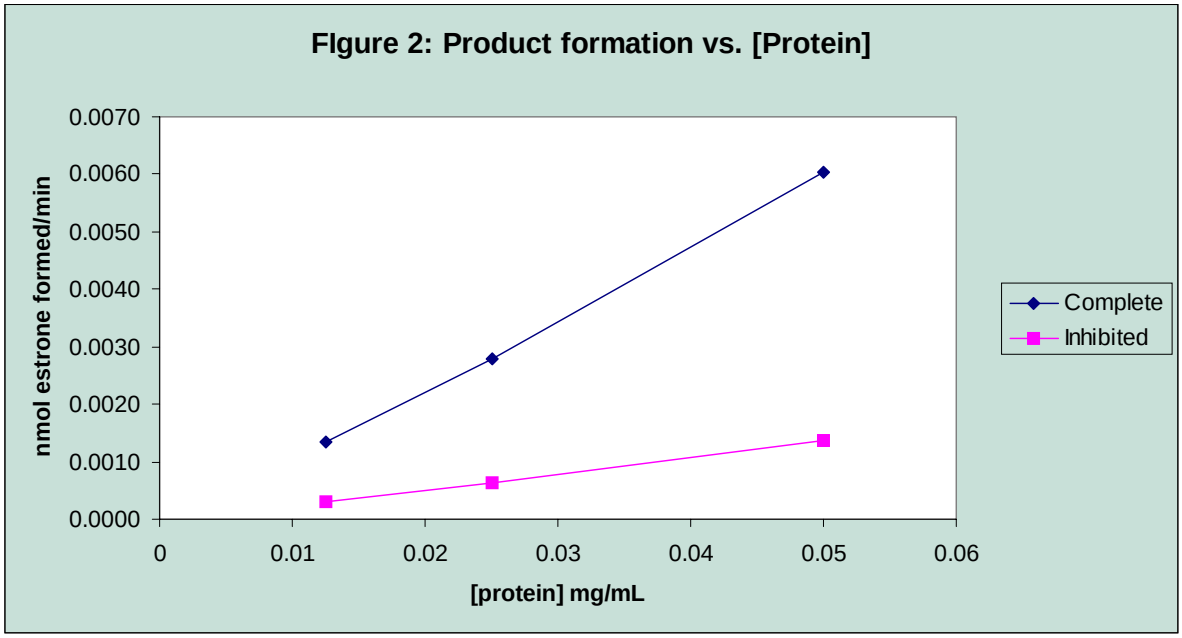


Table 13. Product Formation Rates, Substrate and Inhibition Percentages

Protein Concentration (mg/mL)	Experiment ID	Product formation rate (nmol/min)	% conversion of substrate to product	% inhibition
Complete system				
0.05	Set 1	0.0052	38.29	NA
	Set 2	0.0060	45.05	
	Set 3	0.0060	44.85	
	Mean (SD)	0.0058 (0.0004)	42.73 (3.38)	
0.025	Set 1	0.0025	18.43	
	Set 2	0.0029	21.50	
	Set 3	0.0028	20.82	
	Mean (SD)	0.0027 (0.0002)	20.25 (1.44)	
0.0125	Set 1	0.0012	9.12	
	Set 2	0.0014	10.29	
	Set 3	0.0013	10.08	
	Mean (SD)	0.0013 (0.0001)	9.83 (0.56)	
Inhibited system				
0.05	Set 1	0.0012	9.25	76.0
	Set 3	0.0014	10.30	77.2
	Mean (SD)	0.0013 (0.0001)	9.78 (0.61)	
0.025	Set 1	0.0006	4.46	76.2
	Set 3	0.0006	4.85	77.1
	Mean (SD)	0.0006 (0.0000)	4.66 (0.23)	
0.0125	Set 1	0.0003	2.19	77.2
	Set 3	0.0003	2.43	76.6
	Mean (SD)	0.0003 (0.0000)	2.33 (0.16)	

Table 14. Average Aromatase Activity (nmol/mg/min)

Conditions	Set 1	Set 2	Set 3	Mean (SD)
0.05 mg/mL protein				
Complete	0.0518	0.0602	0.0603	0.0574 (0.0043)
Inhibited	0.0124		0.0137	0.0131 (0.0008)
- NADP	0.0411	0.0030	0.0023	0.0155 (0.0193)
- System			0.0000	0.0000 (0.0000)
+ NADPH			0.0572	0.0572 (0.0005)
0.025 mg/mL protein				
Complete	0.0497	0.0573	0.0559	0.0543 (0.0036)
Inhibited	0.0118		0.0128	0.0123 (0.0006)
- NADP	0.0383	0.0127	0.0009	0.0173 (0.0166)
- System			0.0001	0.0001 (0.0000)
+ NADPH			0.0541	0.0541 (0.0001)
0.0125 mg/mL protein				
Complete	0.0488	0.0546	0.0538	0.0524 (0.0028)
Inhibited	0.0111		0.0126	0.0119 (0.0009)
- NADP	0.0382	0.0101	0.0055	0.0179 (0.0155)
- System			0.0001	0.0001 (0.0001)
+ NADPH			0.0516	0.0516 (0.0008)

Appendix A-6

Protocol and Amendments 1 through 9

TITLE: Pre-Validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

SPONSOR: Battelle Memorial Institute
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PROPOSED STUDY DATES:
SPONSOR CONTRACT NUMBER:
RTI PROJECT NUMBER: 08055.001.018
EPA Contract No.: 68-W-01-023 (Battelle Prime Contractor)

AMENDMENTS:

No.	Date	Section	Pages
1			
2			
3			
4			
5			

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1.0 OBJECTIVES

The objective of the pre-validation protocol for the aromatase assay is to design experiments that will identify the optimal factors and conditions for the assay. The assay will be optimized in four different tissue preparations, i.e., human, bovine, and porcine placental microsomes and human recombinant microsomes.

This protocol is divided into three sets of experiments:

- Pre-Optimization Experiments
- Assay Optimization Experiments using a Factorial Design
- Optimized Assay Experiments using Selected Test Substances.

The basis for each of these experiments is explained and described in the following paragraphs.

1.1 PRE-OPTIMIZATION EXPERIMENTS

The pre-optimization experiments were 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, each of the four microsomal preparations (human, bovine, and porcine placental microsomes and 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. Finally, a single aromatase activity assay run 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. These experiments are detailed in Section 2.0.

1.2 ASSAY OPTIMIZATION EXPERIMENTS USING A FACTORIAL DESIGN

The assay optimization experiments were designed to (1) identify the combination(s) of experimental factors and conditions that will maximize the rate of the aromatase reaction in each of the microsomal preparations, and (2) after the optimal factor levels and conditions are determined, then the optimized assays will be used to assess variability of the results. These experiments are detailed in Section 3.0.

The experimental factors and conditions to be optimized in the assay include the incubation time and concentrations of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, androstenedione (substrate) and protein (microsomal preparation). Each of these

factors will be tested at five different levels using a factorial experimental design. The factorial experimental design consists of four parts. Part 1, 26-1 factional runs, will permit an 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.

Finally, after optimal factors and conditions have been determined, the optimized assay using each type of microsomal preparation will be used to assess variability. Each assay will be conducted by different technicians and on different days. These results will permit an estimate to be made for the technician-to-technician and the within technician variability.

1.3 OPTIMIZED ASSAY EXPERIMENTS USING SELECTED TEST SUBSTANCES

This set of experiments was designed to use the optimized assays to evaluate the response(s) of the human, bovine, or porcine placental microsomes, as well as the human recombinant microsomes, to detect an effect of different test substances. Test substances were selected to represent a wide range of inhibitory potency and mechanisms and sites of action. In addition, some test substances were selected that do not inhibit aromatase but do have endocrine disruptor activity. These test substances were selected in order to assess the specificity of the aromatase assay.

The test substances and their basis for being tested are:

- aminoglutethimide (non-steroidal aromatase inhibitor),
- letrozole or anastrozole (potent non-steroidal aromatase inhibitor),
- 4-hydroxyandrostenedione (potent steroidal aromatase inhibitor),
- chrysin (potent flavonoid)
- genistein (weak isoflavonoid)
- ketoconazole (weak imidazole anti-fungal),
- econazole (potent imidazole anti-fungal),
- atrazine (not an inhibitor, site of action is on aromatase gene expression),
- bis-(2-ethylhexyl)phthlate (not an inhibitor, site of action is on aromatase gene expression),
- nonylphenol (not an inhibitor, site of action is on ER/AR [Estrogen Receptor/Androgen Receptor]),

- lindane (not an inhibitor, site of action is on StAR [Steroidogenic acute regulatory protein] and cholesterol metabolism),
- dibenz(a,h)anthracene (not a known inhibitor).

The optimized assays will be used to determine IC_{50} (concentration at which there is 50% inhibition of the enzymatic reaction) values for those test substances for which an IC_{50} value exists in each of the microsomal preparations. For those test substances for which an IC_{50} value may not exist (i.e., non-inhibitors) the inhibition response curve will be characterized across the range of test substance concentrations on a case-by-case basis. In addition, since each test substance will be tested over a wide concentration range (10^{-9} to 10^{-3} M), the results will be evaluated to determine what the IC_{50} might have been if only three concentrations of the test substance were tested. From this information, the results can be used to assess whether the assay can be conducted with more efficiency and less effort. These experiments are detailed in Section 4.0.

2.0 PRE-OPTIMIZATION EXPERIMENTS

2.1 SUBSTRATE CHARACTERIZATION

2.1.1 Substrate Name/Supplier

The substrate for the aromatase assay is androstenedione. Non-radiolabeled and radiolabeled androstenedione will be used. The non-radiolabeled androstenedione will be obtained from Sigma, St. Louis, MO and have a purity of greater than 98 percent. The purity will be based on information received from the supplier. The radiolabeled androstenedione will be [1β-^{3}H]-androstenedione, which will be obtained from Perkin Elmer Life Science, Boston. Information about the radiochemical purity and specific activity will be provided by the supplier. In addition, confirmation of the specific activity and radiochemical purity will be determined at RTI as described below.

2.1.2 Specific Activity Determination

The specific activity of the substrate, [1β-^{3}H]-androstenedione, will have a high enough activity so dilutions can be made to achieve a specific activity of at least 2 mCi/mmole (0.1 μCi/nM). The testing laboratory will verify the specific activity of the [1β-^{3}H]-androstenedione prior to use of the substrate for any experiments. Samples of known concentration of nonradiolabeled androstenedione will be analyzed by HPLC and a standard curve containing at least 5 points will be prepared relating peak area (or height) to mass. The radiolabeled substrate will be analyzed by the same HPLC method and the fraction containing the peak will be analyzed by using liquid scintillation counting methods. The relationship between the area (or

height) of the uv peak and the radioactivity contained in that peak will be used to calculate the specific activity of the material. The specific activity value provided by the supplier will be used for calculation purposes, if in close agreement with the value determined by the testing laboratory, i.e. ± 5 percent.

2.1.3 Radiochemical Purity

The radiochemical purity of the [1β-3H]-androstenedione will be greater than approximately 95 percent. The testing laboratory will determine the radiochemical purity of the [1β-3H]-androstenedione prior to use of the radiolabeled substrate in any experiments. Radiochemical purity will be determined at the testing laboratory using an appropriate chromatography method and liquid scintillation counting (or other appropriate analytical method) as provided by the supplier of the radiolabeled substrate. The method to be used will be added to the protocol by amendment when it is known. An androstenedione standard with known chemical purity will be obtained and used as the reference standard. If the radiochemical purity is less than 95 percent, then the Sponsor will be notified.

2.2 MICROSOME PREPARATION

2.2.1 Human, Bovine, and Porcine Placentas

2.2.1.1 Source of the Placentas. Human placenta will be obtained from a local hospital. The bovine and porcine placentas will be obtained from either an agricultural school associated with a veterinary program at a university, farmers or ranchers who call upon veterinarians, or slaughterhouses. 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 species of origin, date and time of delivery. RTI 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.

2.2.1.2 Placental Microsome Preparation. If visible soil is evident on the exterior of the placenta, the tissue may be rinsed with chilled isotonic saline. 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 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 10,000g 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 100,000g) 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 vials (labeled with the species and notebook page reference for the preparation of the microsomes) that are convenient for conducting the aromatase assay [The Cytochrome P450 spectrum assay uses 2 mg/mL and the aromatase assay uses approximately 0.1 mg/mL of microsomal protein.] The vials will be flash frozen in liquid nitrogen and then stored at approximately -70°C until removed for use. Under these storage conditions, the microsomal suspension retains aromatase activity for more than one year.

2.2.2 Human Recombinant Microsomes

2.2.2.1 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 SupersomesÔ will be stored at approximately -70°C.

2.2.2.2 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 0.1 mg/mL 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.

2.3 PROTEIN DETERMINATION

The protein concentration of the human, bovine, and porcine placental microsome preparations, as well as the human recombinant microsomal preparations, will be determined for each batch of microsomes prepared, and as necessary to determine the protein concentration of the microsomal preparations. 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 mL aliquot of unknown or standard, 125 mL 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.

2.4 CYTOCHROME P450 CONTENT

Cytochrome P450 content will be determined during the pre-optimization phase in order to demonstrate that the human, bovine, and porcine placental microsome preparations, as well as the human recombinant microsomal preparation 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.

2.5 CYTOCHROME P450 (CYP19) AROMATASE ACTIVITY

Aromatase activity will be determined during the pre-optimization phase in order to demonstrate that the human, bovine, and porcine placental microsome preparations, as well as the human recombinant microsomal preparation have sufficient activity to conduct the optimization experiments. Using the aromatase assay, a single experiment will be conducted using only the substrate (1β-^{3}H]-androstenedione/androstenedione) with each of the microsomal preparations. The assay will be conducted as described in the following paragraph.

The assays will be performed in 16x100 mm test tubes (eight - two for each microsomal preparation) maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol will be added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 µCi, 50 nM), will be added to the tubes. An NADPH-generating system comprised of NADP+ (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) will be added to each tube. 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 the diluted placental microsomal suspension (~0.1 mg microsomal protein/mL). The total volume will be 2.0 mL, and the tubes will be incubated for 30 minutes. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for about 30 seconds. The tubes will then be centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 minutes at a setting of 1000 rpm (which is approximately equivalent to 230g). The methylene chloride layer will be removed to a vial and the aqueous layers will be extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time, each time reserving the methylene chloride layer in a separate vial. The aqueous layers will be transferred to vials, and duplicate aliquots (0.5 mL) will be removed and transferred to a 20 mL liquid scintillation counting vial. Duplicate aliquots of each methylene chloride fraction will be transferred to scintillation vials. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution.

Analysis of the samples will be performed using Liquid Scintillation Counting (LSC) as described in SOP METAB-610. Radiolabel found in the aqueous fractions represents ^3H_2O formed and that in the methylene chloride fractions represents unreacted substrate. If less than 1% of the total amount of radioactivity in the incubation tube is found in any methylene chloride extract, the number of extractions per sample may be reduced for subsequent experiments.

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 [1β-^{3}H]-androstenedione substrate (expressed in nmol/dpm). 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. 30 minutes.

2.6 ACCEPTANCE CRITERIA FOR TOTAL PROTEIN YIELD, P450 CONTENT AND PRE-OPTIMIZATION AROMATASE ACTIVITY

It is essential that, for each microsomal preparation, enough protein be on hand at the start of the optimization phase for all of the planned studies. The microsomal preparations must also have sufficient P450 content/mg protein and demonstrate acceptable aromatase activity.

It is anticipated that 250 mg of protein would be necessary to run all of the proposed studies (for each preparation). Therefore, if less than that amount is obtained from a single placenta, microsomes will be prepared from additional placentas until sufficient protein is obtained. Where multiple placentas are required, the microsomes will be pooled to create a microsome stock that is homogenous for protein and P450 content.

P450 concentration in human placenta microsomes is typically 0.01 to 0.03 nmol P450/mg protein. The minimum acceptable P450 concentration for human placenta microsomes for this work is set at 0.005 nmol P450/mg protein. Aromatase activity in human placenta microsomes typically ranges from 0.15 to 0.2 nmol product/mg protein/min. The minimum acceptable aromatase activity in human placenta microsomes is set at 0.1 nmol product/mg protein/min. If the P450 content or aromatase activity for any human placenta microsomal preparation is below the standard, this preparation will not be used in further studies. In this case, new microsomal preparations would be made from additional placenta(s).

3.0 ASSAY OPTIMIZATION EXPERIMENTS USING A FACTORIAL DESIGN

The objectives of the optimization experiments are to: 1) identify the combination(s) of experimental conditions and factors that will maximize the rate of the aromatase assay in each of the different microsomal preparations and 2) assess technician-to-technician and within technician variability of the optimized assay. These two objectives will be conducted in two distinct sets of experiments and in the order listed.

3.1 CHEMICAL REAGENTS, SUBSTRATE, AND MICROSOMAL PREPARATIONS

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione, and microsomal preparations from the human, bovine, and porcine placentas, as well as human recombinant microsomes, will be obtained prior to initiation of the first set of optimization experiments to ensure that sufficient quantities are available to conduct the studies. All reagents will be prepared fresh daily, with the exception of assay buffer, which may be stored for up to one month in the refrigerator. A similar requirement will exist for the second set of experiments that are designed to estimate variability, which will be conducted following successful completion of the first set of optimization experiments.

3.2 EXPERIMENTAL DESIGN

3.2.1 Optimization of Experimental Design Factors and Conditions

The experimental design will test six different factors and five different levels of each factor. The experimental factors that will be tested and the levels for each factor are summarized below in Table 1.

The aromatase assay will be performed as follows. The assay will be performed in the required number of 16x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol will be added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (see Table 2 for concentration), will be added to the tubes. An NADPH-generating system comprised of NADP+ (see Table 2), glucose-6-phosphate (see Table 2) and glucose-6-phosphate dehydrogenase (see Table 2) will be added to each tube.

Table 1. Summary of Experimental Factors and Levels to be Optimized[a]

		Experimental Factor Levels				
Experimental Factors	Units	1	2	3	4	5
NADP+ (conc)	mM	0.1	0.5	1	2	4
Glucose-6-Phosphate (conc)	mM	0.1	1	2	3	4
Glucose-6-Phosphate Dehydrogenase (conc)	units	0.1	0.5	1	2	4
Androstenedione (substrate) (conc)	nM	10	25	50	100	500
Protein (conc)	mg/mL	0.01	0.02	0.1	0.5	1
Incubation Time	min	10	15	30	60	120

[a] Will be performed for each of the four different types of microsomal preparations.

The tubes will be placed at 37 ± 1°C in the water bath for five minutes prior to the start of the assay by the addition of the diluted placental microsomal suspension (see Table 2). The total volume will be 2.0 mL, and the tubes will be incubated for the time described in Table 2. The incubations will be stopped by the addition of methylene chloride (2.0 mL); the tubes will be vortex-mixed for ca. 30 seconds. 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 aqueous layers will be transferred to new test tubes and extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time. [The number of extractions may be modified based on data collected in Section 2.5 and any such modification would be added to the protocol by amendment.] The aqueous layers will be transferred to vials, and duplicate aliquots (0.5 mL) will be removed and transferred to a 20 mL liquid scintillation counting vial. 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 above (see Section 2.5).

The following factorial design describes the experiments that will be conducted to determine the optimum factor levels and conditions. These experiments will first be tested using only the human placental microsomal preparation. The factorial design experiments will be divided into four parts:

- Part 1. 26-1 Factorial Runs. These experiments will permit estimation of all linear main effects and linear by linear interactions.
- Part 2. Center Point Run. This experiment will estimate the response in the center of the design space and provide an overall indication of goodness-of-fit to the linear trend assumptions.
- Part 3. Axial Point Runs. These experiments will provide estimates of the quadratic main effects for each of the factors.
- Part 4. Replicate Runs. These experiments will provide estimates of the reproducibility of the response at the center of the design space and at various extremes of the design space.

The 62 test runs will be conducted in completely random order over the course of two days. The experiments to be conducted are described in Table 2.

The aromatase activity for each run will be calculated from the radioactivity in the aliquots, the protein concentration and the time of incubation using a validated Excel 97 spreadsheet. Statistical analysis of the aromatase activities under the various conditions will be performed as described in Section 4.4.1.

Before proceeding to conduct the optimization experiments listed above using the bovine and porcine placental microsomes and the human recombinant microsomes, the response outcome of the human placental microsomal preparation will be evaluated to determine whether additional experiments are needed to optimize this preparation, as well as whether all or a limited set of experiments are needed to optimize the other microsomal preparations.

Table 2. Factorial Design Experiments for Assay Optimization

Run No.	NADP Conc.	Glu-6-P Conc.	Glu-6-P D Conc.	Substrate Conc.	Protein Conc.	Incub. Time
Part 1- Factorial Runs (32 Total Runs)						
1	0.5 mM	1 mM	0.5 units	25 nM	0.02 mg/mL	15 min
2				100 nM	0.5 mg/mL	
3			2 units	25 nM		
4				100 nM	0.02 mg/mL	
5		3 mM	0.5 units	25 nM	0.5 mg/mL	
6				100 nM	0.02 mg/mL	
7			2 units	25 nM		
8				100 nM	0.5 mg/mL	
9	2 mM	1 mM	0.5 units	25 nM		
10				100 nM	0.02 mg/mL	
11			2 units	25 nM		
12				100 nM	0.5 mg/mL	
13		3 mM	0.5 units	25 nM	0.02 mg/mL	
14				100 nM	0.5 mg/mL	
15			2 units	25 nM		
16				100 nM	0.02 mg/mL	
17	0.5 mM	1 mM	0.5 units	25 nM	0.5 mg/mL	60 min
18				100 nM	0.02 mg/mL	
19			2 units	25 nM	0.02 mg/mL	
20				100 nM	0.5 mg/mL	
21		3 mM	0.5 units	25 nM	0.02 mg/mL	
22				100 nM	0.5 mg/mL	
23			2 units	25 nM	0.5 mg/mL	
24				100 nM	0.02 mg/mL	
25	2 mM	1 mM	0.5 units	25 nM	0.02 mg/mL	
26				100 nM	0.5 mg/mL	
27			2 units	25 nM	0.5 mg/mL	
28				100 nM	0.02 mg/mL	
29		3 mM	0.5 units	25 nM	0.5 mg/mL	
30				100 nM	0.02 mg/mL	
31			2 units	25 nM	0.02 mg/mL	
32				100 nM	0.5 mg/mL	

Run No.	NADP Conc.	Glu-6-P Conc.	Glu-6-P D Conc.	Substrate Conc.	Protein Conc.	Incub. Time
Part 2 – Center Point Run (1 Total Run)						
1	1 mM	2 mM	1 units	50 nM	0.1 mg/mL	30 min
Part 3 – Axial Point Runs (12 Total Runs)						
1	0.1 mM	2 mM	1 units	50 nM	0.1 mg/mL	30 min
2	4 mM					
3	1 mM	0.1 mM				
4		4 mM				
5		2 mM	0.1 units			
6			4 units			
7			1 units	10 nM		
8				500 nM		
9				50 nM	0.01 mg/mL	
10					1 mg/mL	
11					0.1 mg/mL	10 min
12						120 min
Part 4 - Replicate Runs (17 Total Runs)						
1	1 mM	2 mM	1 units	50 nM	0.1 mg/mL	30 min
2						
3						
4						
5						
6	0.1 mM					
7	4 mM					
8	1 mM	0.1 mM				
9		4 mM				
10		2 mM	0.1 units			
11			4 units			
12			1 units	10 nM		
13				500 nM		
14				50 nM	0.01 mg/mL	
15					1 mg/mL	
16					0.1 mg/mL	10 min
17						120 min

3.2.2 Variability Determination Using the Optimized Assay

After optimum conditions have been determined for the human, bovine, and porcine placental microsomes and the human recombinant microsomes, each assay will be conducted using the optimized factors to assess the variability of the results. Each assay will be conducted independently by three technicians and at three separated times per technician. Single runs will be conducted by each technician on each day for a given microsomal preparation (a total of 36 runs across assays and days). The assays must be run independently of one another. The assays will be conducted and samples will be analyzed as described above in Section 3.2.1, except that optimized factor levels and conditions will be used. The aromatase activity will be determined for each run and statistical analysis will be carried out as described in Section 4.4.2.

4.0 DETERMINATION OF THE RESPONSE OF THE OPTIMIZED ASSAY TO SELECTED TEST SUBSTANCES

This set of experiments was designed to use the optimized assays to evaluate the response(s) of the human, bovine, or porcine placental microsomes, as well as the human recombinant microsomes, to detect an effect of different test substances. Test substances were selected to represent a wide range of inhibitory potency and mechanisms and sites of action. In addition, some test substances were selected that do not inhibit aromatase but do have endocrine disruptor activity.

4.1 CHEMICAL REAGENTS, SUBSTRATE, AND MICROSOMAL PREPARATIONS

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione (substrate), and microsomal preparations from the human, bovine, and porcine placentas, as well as human recombinant microsomes, will be obtained prior to initiation of these experiments to ensure that sufficient quantities are available to conduct the study using the same lot or batch. All reagents will be prepared fresh daily, except for the assay buffer, which may be stored for up to one month in the refrigerator.

4.2 CHEMISTRY

4.2.1 Vehicle Controls

The potential vehicles for the test substances, which would also be used as controls in the assay, are ethanol (95 percent), dimethyl sulfoxide (DMSO), or distilled water. Organic solvents will be of reagent grade or better and will be stored at room temperature. Supplier and lot numbers for all solvents will be documented in the study records.

4.2.2 Test Substances

There are 12 test substances for this study. Relevant physical and chemical properties, as well as formulation and handling information are summarized below for each test substance. Information listed below as TBD (to be determined) will be documented in the study records.

4.2.2.1 Aminoglutethimide (Non-Steroidal Aromatase Inhibitor).

CAS No.:125-84-8
Molecular Formula/Weight: $C_{13}H_{16}N_2O_2$; 232.3 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.2 Letrozole (Potent Non-Steroidal Aromatase Inhibitor).

CAS No.: TBD
Molecular Formula/Weight: TBD
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: TBD

4.2.2.3 4-Hydroxyandrostenedione (Potent Steroidal Aromatase Inhibitor).

CAS No.: 566-48-3
Molecular Formula/Weight: $C_{19}H_{26}O_3$; 302.4 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Refrigerate

4.2.2.4 Chrysin (Potent Flavonoid).

CAS No.: 480-40-0
Molecular Formula/Weight: $C_{15}H_{10}O_4$; 254.2 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.5. Genistein (Weak Isoflavonoid).
CAS No.:446-72-0
Molecular Formula/Weight:$C_{15}H_{10}O_5$; 270.2 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.6. Ketoconazole (Weak Imidazole Anti-Fungal).
CAS No.: 65277-42-1
Molecular Formula/Weight: $C_{26}H_{28}Cl_2N_2O_4$; 531.48 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temerature

4.2.2.7. Econazole (Potent Imidazole Anti-Fungal).
CAS No.: 24169-02-6 (econazole nitrate)
Molecular Formula/Weight: $C_{18}H_{15}Cl_3N_2O$-HNO_3; 444.7
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.8. Atrazine (Affects Aromatase Gene Expression; no Aromatase Inhibition).
CAS No.: 1912-24-9
Molecular Formula/Weight: $C_6H_{14}ClN_5$; 215.69 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.9. Bis-(2-Ethylhexyl)Phthlate (Affects Aromatase Gene Expression; no Aromatase Inhibition).
CAS No.: 117-81-7
Molecular Formula/Weight: $C_{24}H_{36}O_4$; 390.56 g/mol
Solubility: TBD
Supplier: TBD

Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.10. Nonylphenol (Affects AR/ER; no Aromatase Inhibition).
CAS No.: 104-40-5 (4-nonylphenol)
Molecular Formula/Weight: $C_{15}H_{24}O$; 220.4 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.11. Lindane (Affects StAR and Cholesterol Metabolism; no Aromatase Inhibition).
CAS No.: 58-89-9
Molecular Formula/Weight: $C_6H_6Cl_6$; 290.8 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.2.12. Dibenz(a,h)anthracene
CAS No.: 53-70-3
Molecular Formula/Weight: $C_{22}H_{14}$, 278.35 g/mol
Solubility: TBD
Supplier: TBD
Lot No.: TBD
Purity: TBD
Storage Conditions: Room temperature

4.2.3 Preparation of Test Substance Formulations

Each test substance will be prepared in a formulation using one of the three selected vehicles, i.e. ethanol (95 percent), DMSO, or distilled water. Prior to initiation of this phase of the study, solubility tests will be conducted at Batelle to determine which of the three vehicles to use to prepare a given test substance formulation. The solubility tests will be conducted using a concentration of the test substance of 0.1 M (this concentration will allow a 10^{-3} M final concentration in a 2 mL incubation volume while holding the volume of solvent added to 1% of

the total volume). If the test substance is not soluble in any of the three vehicles at this concentration, then the concentration will be decreased by a factor of 5 and the solubility test repeated until the highest possible concentration that can be prepared is determined.

For the assay experiments, the target concentrations for each test substance will not exceed 10^{-9} M for the low concentration and 10^{-3} M for the high concentration. Up to 8 different concentrations will be tested for each test substance per microsomal preparation. The target concentrations will be specific for each test substance and will bracket the reported IC_{50} for those substances for which an IC_{50} is defined. For the other substances (i.e. the non-inhibitors) they range from 10^{-3} to 10^{-9}. The target concentrations for each test substance are summarized in Table 3. All test substance formulations will be prepared fresh daily.

Table 3. Test Substance Target Concentrations

Test Substance	Levels	Reported IC_{50}	Target Concentrations (M)
aminoglutethimide	8	1 - 6 μM	10^{-3}; 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}; 10^{-7}; 10^{-8}
letrozole	8	1 - 15 nM	10^{-6}; 10^{-7}; 2.5 and 5 x 10^{-7}; 10^{-8}; 2.5 and 5 x 10^{-8}; 10^{-9}
4-hydroxyandrostenedione	8	30 - 50 nM	10^{-6}; 10^{-7}; 2.5 and 5 x 10^{-7}; 10^{-8}; 2.5 and 5 x 10^{-8}; 10^{-9}
chrysin	8	0.5 - 10 μM	10^{-3}; 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}; 10^{-7}; 10^{-8}
genistein	8	30 - 100 μM	10^{-3}; 10^{-4}; 2.5 and 5 x 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}
econazole	8	30 - 50 nM	10^{-6}; 10^{-7}; 2.5 and 5 x 10^{-7}; 10^{-8}; 2.5 and 5 x 10^{-8}; 10^{-9}
ketoconazole	8	> 65 μM	10^{-3}; 10^{-4}; 2.5 and 5 x 10^{-4}; 10^{-5}; 2.5 and 5 x 10^{-5}; 10^{-6}
atrazine	8	–	10^{-3} to 10^{-9}
bis-(2-ethylhexyl)phthlate	7	--	10^{-3} to 10^{-9}
nonylphenol	7	–	10^{-3} to 10^{-9}
lindane	7	–	10^{-3} to 10^{-9}
Dibenz (a,h)anthracene	7	–	10^{-3} to 10^{-9}

4.2.4 Test Formulation Analysis

The formulations for each test substance will not be analyzed.

4.3 EXPERIMENTAL DESIGN

The factor levels and conditions determined as optimal (based on the optimization experiments) will be used to test the test substances using the aromatase assay. Each of 12 different test substances, at up to 8 different concentrations (ranging from no less than 10^{-9} M and no higher than 10^{-3} M), will be tested using each of the four different microsomal preparations, i.e. human, bovine, and porcine placental microsomes and human recombinant microsomes. The vehicle controls will be ethanol (95 percent), DMSO and/or distilled water, depending on the vehicle(s) used to formulate the test substances. The aromatase activity in the absence of any test substance, will be the benchmark (100 percent) activity.

4.3.1 Assay Method

The aromatase assay will be performed as described below and as shown in the flow diagram (Figure 1).

The assay will be performed in the required number of 16x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol will be added to the tubes to serve as a co-solvent. The final concentrations of substrate, NADPH-generating system components, and microsomal protein, as well as incubation time, will be those chosen based on the results of the optimization experiments and will be added to the protocol by amendment. The substrate, [1β-^{3}H]-androstenedione, will be added to the tubes. An NADPH-generating system comprised of NADP+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase will be added to each tube. The test substance will be added to the tube. The tubes will be placed at 37 ± 1°C in the water bath for five minutes prior to the start of the assay by the addition of the diluted placental microsomal suspension. The total volume will be 2.0 mL, and the tubes will be incubated for the time period determined based on the results of the optimization experiments (to be added to the protocol by amendment). The incubations will be stopped by the addition of methylene chloride (2.0 mL) and the tubes vortexed for 30 seconds. The tubes will then be centrifuged using a Beckman G2-6R centrifuge with a GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The aqueous layers will be transferred to new test tubes and extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time. [The number of extractions may be modified based on the results of the experiments in Section 2.5 and any such modification would be added to the protocol by amendment.] The aqueous layers will be transferred to vials, and duplicate aliquots (0.5 mL) will be removed and transferred to a 20 mL liquid scintillation counting vial. Liquid scintillation

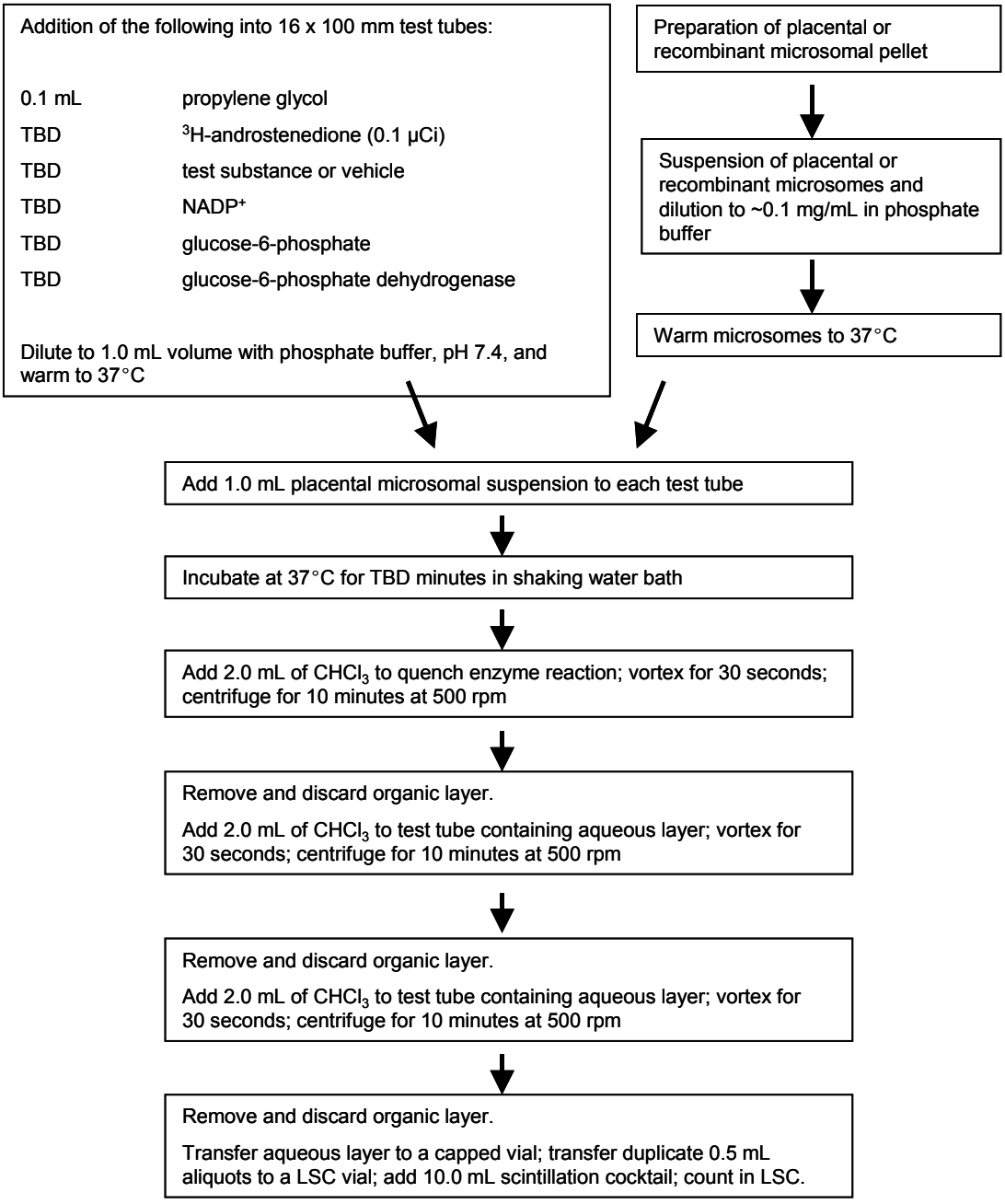

Figure 1. Flow Diagram for Aromatase Assay of Test Substances

cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The samples will be assayed for radiochemical content as described in Section 2.5.

4.3.2 Assay Sample Analysis

The radioactivity will be measured and the aromatase activity calculated as previously described (see Section 3.2.1).

4.3.3 Assay Schedule and Conduct Procedure

It is estimated that up to 48 runs/day can be reasonably performed by a trained technician. A day's runs will constitute a batch of up to 48 runs. In a given day, the following samples will be run and the number of runs of the assay for each sample will be:

Type of Assay	Number of Runs
One negative control (in quadruplicate)[1]	4
The optimized androstenedione concentration positive control (in quadruplicate)	4
Up to four test substances, each at seven (5 non-inhibitors) or eight (7 inhibitors) concentrations (in singlet)	Up to 32
Total	Up to 40/day

The assay will be conducted in quadruplicate for each test substance at each of the 7 or 8 concentrations, which will result in 364 runs of the assay. Based on conducting 32 test substance runs each day, 12 days are needed to test all of the test substances. Additional negative control runs and androstenedione positive control runs included on each of these 12 days will add 96 runs. (The actual schedule will depend on how the test substances break out with vehicle type.) Thus, the grand total number of runs for one microsomal preparation type is 460 runs, which will be completed in 12 days as designed above. This same number of runs is repeated using each of the other three microsomal preparations. Table 4 summarizes the experimental design.

[1]The negative control will include the optimal androstenedione concentration (labeled and non-labeled) but will omit the enzyme and/or the co-factor. It will estimate the background activity.

Table 4. Summary of Experimental Design for Assaying Test Substances

Day	Run Nos.	Sample Type	Replicate
1	1-4	NegativeControl(s)a	--
	5-8	Androstenedione (optimal conc)a	--
	9-40	Up to 4 Test Substancesb(7 or 8 conc/test substance)	1st for each test substance
2	1-8	As above for Day 1	--
	9-40	Up to 4 Test Substancesb(7 or 8 conc/test substance)	1st for each test substance
3	1-8	As above for Day 1	--
	9-40	Up to 4 Test Substancesb(7 or 8 conc/test substance)	1st for each test substance
4, 5 and 6	1-8	As above for Day 1	--
	9-40	Up to 4 Test Substancesb(7 or 8 conc/test substance)	2nd for each test substance
7, 8, and 9	1--8	As above for Day 1	--
	9-40	Up to 4 Test Substancesb(7 or 8 conc/test substance)	3rd for each test substance
10, 11, and 12	1-15	As above for Day 1	--
	9-40	Up to 4 Test Substancesb(7 or 8 conc/test substance)	4th for each test substance

[a] One vehicle per day

[b] The 12 test substances and their respective quadruplicate runs will be randomized across the 12 assay days. The test substances that are run on a single day will all use the same vehicle.

Data from the daily androstenedione positive control samples will yield the uninhibited aromatase activity. Data from the test substance negative control samples estimate background effects. Also, the daily androstenedione (optimized concentration) positive control samples and the negative control background samples will provide quality control data of the assay's day-to-day consistency. The quadruplicate negative control and the androstenedione (optimized conc) positive control samples will be divided such that half will be carried out at the beginning of the batch and half at the end of the batch on a given day.

4.4 STATISTICAL ANALYSES

There are three phases of the study requiring statistical analysis:

1) Assay optimization using a factorial design
2) Determination of variability of the assay using the optimized conditions
3) Determination of IC_{50} values for test substances.

The identity of software vendors and version numbers, references for statistical tests used and quantitative criteria for accepting or rejecting results will be added to the protocol by amendment.

4.4.1 Assay Optimization Using a Factorial Design

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, i.e., NADP, glucose-6-phosphate, etc. Full quadratic response surface models 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. The final response surface model will be optimized to determine the experimental conditions associated with the optimum response. The optimum 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, consideration will be given to extending the experimental region in the direction of the increasing response gradient to determine whether aromatase activity may be further improved. 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.

Data will be entered into a validated Excel97 spreadsheet and imported into a SAS Version 8.2 database. Basic data QC, data restructuring, recoding and/or transformations will be done using SAS DATA STEP programming and SAS summary statistical procedures (e.g., PROC UNIVARIATE). Response surface models will be fit using standard least square methods provided by the SAS RSREG and/or SAS REG procedures. Graphical exploratory data analyses, residual analyses, and graphical presentations of results (e.g., contour plots) will be done by employing a combination of SASGRAPH procedures and SPLUS 6.0 graphical functions.

4.4.2 Determination of Variability of the Assay Using the Optimized Conditions

After optimum conditions have been determined for the human, bovine, and porcine placental microsomes and the human recombinant microsomes, each assay will be conducted

using the optimized factors to assess the variability of the results. Differences among technicians and day-to-day variation within technicians will be estimated. Time-to-time variation within technicians will be compared with the variation determined from the regression fit to determine total uncertainty in the reaction rate at the estimated optimum conditions. 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 will be treated as a fixed effect; day-to-day variation within technician will be treated as a random effect.

4.4.3 Determination of IC_{50} Values for Test Substances

4.4.3.1. Quality Control Comparisons. The quadruplicate replicate runs on a given day for the negative control(s) and the optimal androstenedione concentration positive controls will be used for quality control comparisons. On a given day, half of the quadruplicate replicate runs conducted for the negative controls and for the androstenedione positive controls will be carried out at the beginning of the batch and half at the end. For purposes of comparison, the control responses will be combined across batches and a two-way analysis of variance will be carried out on the data. The factors in the analysis of variance will be batch, portion of batch (beginning or end) and their interaction. 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. In addition, the data will be analyzed by multiple comparisons and graphical analyses. For each of the responses, the standard deviation of the control runs within a batch will be compared between the beginning control runs and the end control runs and across days by analysis of variance and graphical techniques in a manner similar to the average values.

4.4.3.2 Concentration Response Curve Fits for the Test Substances. At each test substance concentration aromatase activity within a daily batch will be converted to percent full activity by subtracting the calculated background "activity" within the batch (from the average radiochemical content of the quadruplicate negative control runs) and normalizing by the difference of the optimal aromatase activity within the batch (average of the quadruplicate positive control runs) and the background activity. Concentration response curves will be fitted to the percent control activity values at each test substance concentration, for each test substance within each batch. 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 test substance concentration. The model will be fitted by weighted least squares with weights inversely proportional to the average activity at that test concentration, pooled across test batches for the same test substance. This process will be repeated for each batch.

Based on the results of the fit within each batch, the extent of aromatase inhibition will be expressed as the IC_{50} (concentration corresponding to 50 percent inhibition) and associated slope for those test substances for which an IC_{50} concentration exists. For the remaining test substances (i.e. the non-inhibitors) the concentration response relation will be characterized for each test substance and each assay on a case-by-case basis. The concentration response relation for each test compound will be fitted within four independent test batches. The estimated IC_{50} will be the geometric average across the four batches.

Univariate one-way random effects analyses of variance will be carried out on log10(IC_{50}), slope, and associated within batch standard errors for those test substances for which the IC_{50} exists. 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 test substance and the enzyme source. If the batch-to-batch variation is significant it will be incorporated into the standard error of the corresponding parameter and associated confidence intervals.

The principal analysis will treat the average of the four replicate negative control runs within a batch and the average of the four replicate positive control runs within a batch as constant values. If these replicates exhibit substantial variability consideration would be given to augmenting the principal analysis with an additional analysis that accounts for the variability in the normalizing averages. The normalizing values would be modeled as for example normal (lognormal) distributions with the observed sample mean (log mean) and sample standard deviation (log standard deviation). Values for the normalizing averages would be sampled from their respective distributions and the aromatase activity values will be converted to percent control values as before, but using the sampled values. The concentration response relations would be fitted within each batch and the IC_{50} s and their standard errors calculated. This process would be repeated multiple times and the total variability of the IC_{50} determined from the variation across repetitions as well as the variation within repetitions.

4.4.4 Comparison of Optimized Assays

For each test compound log10(IC_{50}), slope, and their associated standard errors, degrees of freedom, and confidence intervals will be determined with each assay for those test substances for which an IC_{50} exists. The estimates and their standard errors will be compared among assays by two-sample heterogeneous variances t-tests, adjusting for simultaneous inferences (6 pairwise comparisons among the 4 assays). Consistent patterns across test substances of inequalities among assays will be examined. Differences among assays will be judged based on the relative strength of the test substance activity inhibition, as indicated by the IC_{50}, slope, and their standard errors.

4.4.5 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 seven to 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 from 10^{-7}M to 10^{-5}M (0.1, 1, and 10 μM). Estimates of $\log10(IC_{50})$ 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.

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.

4.4.6 Statistical Software

Concentration response models will be fitted to the data using the non-linear regression analysis features in the PRISM and SAS Version 8.2 statistical packages. Summary values, graphical displays, analysis of variance fits, and multiple comparisons will be carried out using the summarization, graphics, and linear model capabilities in SAS Version 8.2.

5.0 RETENTION OF SAMPLES AND RECORDS

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

6.0 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.

7.0 REPORTING

A letter report will be submitted to the EPA after completion of each of the pre-optimization experiments (Stage 1) and assay optimization experiments (Stage 2). These two letter reports will each include an introduction, methods and materials, results, discussion, and conclusion. The purposes of the letter reports are 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 so that the EPA has the information necessary to evaluate each stage of the pre-validation studies and give its approval to continue onto the next stage.

Upon completion of the third and final stage of the pre-validation studies as described in this protocol, a final report will be written and submitted to the EPA. A draft report will be generated by the WAL/Study Director within three months of the last data collection period. Following completion of the ADQ process (see QAPP Section 18.3), the report can be finalized. Format requirements include:

a. "Final Report" and "Page 1 of _", the total number of report pages, on the title page.
b. Contract number in the header, right justified
c. Text, which includes the following sections:
   - Abstract
   - Objectives
   - Materials and Methods
   - Results
   - Discussion
   - Conclusions
   - References
   - Protocol Deviation, if any
   - Summary data with statistical analyses.

The letter reports will be included in the appendix of the final report.

8.0 PERSONNEL

Study Director:	James M. Mathews
Laboratory Supervisor:	Sherry L. Black
Statistical Advisor:	Paul Feder, Andrew Clayton
Quality Assurance:	Marcia Phillips, Doris Smith

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).

EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 1 **Date** March 18, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
Chemistry and Life Sciences
Post Office Box 12194
Research Triangle Park, NC 27709

Item 1

Section 2.1.3, Radiochemical Purity, page 8, which reads:

The radiochemical purity of the [1β-3H]-androstenedione will be greater than approximately 95 percent. The testing laboratory will determine the radiochemical purity of the [1β-3H]-androstenedione prior to use of the radiolabeled substrate in any experiments. Radiochemical purity will be determined at the testing laboratory using an appropriate chromatography method and liquid scintillation counting (or other appropriate analytical method) as provided by the supplier of the radiolabeled substrate. The method to be used will be added to the protocol by amendment when it is known. An androstenedione standard with known chemical purity will be obtained and used as the reference standard. If the radiochemical purity is less than 95 percent, then the Sponsor will be notified.

Is hereby amended as follows:

The radiochemical purity of the [1β-^{3}H]-androstenedione will be greater than approximately 95 percent. The testing laboratory will determine the radiochemical purity of the [1β-^{3}H]-androstenedione prior to use of the radiolabeled substrate in any experiments. Radiochemical purity of the [1β-^{3}H]androstenedione will be determined by HPLC using a Zorbax SB-C_{18} column (4.6 x 250 mm) with a mobile phase consisting of 55:15:30 ddH_2O: tetrahydrofuran: methanol (v:v:v) with a flow rate of 1 mL/min. The eluant will be monitored by uv absorbance and by flow-through radiochemical detection. Fractions will be collected into Ultima Gold scintillation cocktail and will be assayed for radiochemical content by liquid scintillation spectrometry. An androstenedione standard with known chemical purity will be obtained and used as the reference standard. If the radiochemical purity is less than 95 percent, then the Sponsor will be notified.

Justification:

HPLC method for purity analysis is now known. The effective date for this change is 1/8/2003 and is supported by email, a printout of which is in the study record..

Item 2

Section 2.2.1.2 Placental Microsome Preparation., page 8, which reads:

If visible soil is evident on the exterior of the placenta, the tissue may be rinsed with chilled isotonic saline. 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 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 10,000g 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 100,000g) 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 vials (labeled with the species and notebook page reference for the preparation of the microsomes) that are convenient for conducting the aromatase assay [The Cytochrome P450 spectrum assay uses 2 mg/mL and the aromatase assay uses approximately 0.1 mg/mL of microsomal protein.] The vials will be flash frozen in liquid nitrogen and then stored at approximately -70°C until removed for use. Under these storage conditions, the microsomal suspension retains aromatase activity for more than one year.

Is hereby amended as follows:

Third line, after the word Elvejhem, insert ‘or Polytron’

Fourth line from the end, replace ‘The Cytochrome P450 spectrum assay uses 2 mg/mL’ with ‘The assay for cytochrome P450 content uses 1 mg/mL’.

Justification: Dr. Brueggemeier indicates that Polytron homogenization is acceptable. Dr Mathews’ record of his conversation with Dr. Brueggemeier appears in an email dated 1/16/2003. A copy of this email is included in the study record. Second change is to correct an incorrect concentration.

Item 3

Section 2.2.2.1 Source of the Human Recombinant Microsomes, page 9, which reads:

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 SupersomesÔ will be stored at approximately -70°C.

Is hereby amended as follows:

In the last line, replace the symbol following Supersomes with a ™ symbol

Justification: Correct typographical error

Item 4

Section 2.3 PROTEIN DETERMINATION, page 9, which reads:

The protein concentration of the human, bovine, and porcine placental microsome preparations, as well as the human recombinant microsomal preparations, will be determined for each batch of microsomes prepared, and as necessary to determine the protein concentration of the microsomal preparations. 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 mL aliquot of unknown or standard, 125 mL 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.

Is hereby amended as follows:

Replace: To a 25 mL aliquot of unknown or standard, 125 mL of BioRad DC Protein Kit Reagent A will be added and mixed.

With: To a 25-µL aliquot of unknown or standard, 125-µL of BioRad DC Protein Kit Reagent A will be added and mixed.

Justification: Correct typographical error.

Item 5

Section 2.5 CYTOCHROME P450 (CYP19) AROMATASE ACTIVITY, paragraph 4, page 11, which reads:

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 $^{3}H_{2}O$ formed by the specific activity of the [1β-^{3}H]-androstenedione substrate (expressed in nmol/dpm). 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. 30 minutes.

Is hereby amended as follows:

Replace '(expressed in nmol/dpm)' with '(expressed in dpm/nmol)'
Replace 'nmol (mg protein)-1 min-1' with nmol (mg protein)$^{-1}$ (min)$^{-1}$

Justification: The first change is to correct the text, the second change is to correct a typographical error.

Item 6

Sections 2.5, 3.2.1. 4.3.1 and Figure 1, pages 11, 13, 22 and 23

are hereby amended as follows:

Replace '16 x 100 mm test tubes' with '13 x 100 mm test tubes'

Justification: The 13 x 100 mm tubes work as well as the 16 x 100 tubes and a supply of 13 x 100 tubes and racks already exists in the laboratory.

Item 7

Title page

is hereby amended as follows:

Add: November 2002-June 2003 as proposed study dates.
Delete: SPONSOR CONTRACT NUMBER

Justification: Addition of previously missing information. Deletion of extraneous heading.

Item 8

Section 2.6 ACCEPTANCE CRITERIA FOR TOTAL PROTEIN YIELD, P450 CONTENT AND PRE-OPTIMIZATION AROMATASE ACTIVITY, paragraph 3, page 12, which reads:

P450 concentration in human placenta microsomes is typically 0.01 to 0.03 nmol P450/mg protein. The minimum acceptable P450 concentration for human placenta microsomes for this work is set at 0.005 nmol P450/mg protein. Aromatase activity in human placenta microsomes typically ranges from 0.15 to 0.2 nmol product/mg protein/min. The minimum acceptable aromatase activity in human placenta microsomes is set at 0.1 nmol product/mg protein/min. If the P450 content or aromatase activity for any human placenta microsomal preparation is below the standard, this preparation will not be used in further studies. In this case, new microsomal preparations would be made from additional placenta(s).

Is hereby amended as follows:

P450 concentration in human placenta microsomes is typically 0.01 to 0.03 nmol P450/mg protein. The minimum acceptable P450 concentration for human placenta microsomes for this work is set at 0.005 nmol P450/mg protein. The minimum acceptable aromatase activity in human placenta microsomes is set at 5 pmol product/mg protein/min. If the P450 content or

aromatase activity for any human placenta microsomal preparation is below the standard, this preparation will not be used in further studies. In this case, new microsomal preparations would be made from additional placenta(s).

Justification: See attached email from Dr. Brueggemeier, dated 2/14/03. A copy of this email is included in the study record. Effective date of this change is 2/14/2003.

APPROVED BY:

Rochelle W. Tyl 3/18/03
Rochelle W. Tyl, Ph.D., DABT Date
Principal Investigator
Center for Life Sciences and Toxicology
RTI International

J. Matthews 3-18-03
James Mathews, Ph.D., DABT, Date
Study Director
RTI International

Gary E. Timm 3/25/2003
Gary E. Timm Date
Work Assignment Manager
Endocrine Disruptor Screening Program
U.S. EPA

David P. Houchens 3/19/03
David P. Houchens, Ph.D. Date
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

L. Greg Schweer
L. Greg Schweer Date
Project Officer
Endocrine Disruptor Screening Program
U.S. EPA

REVIEWED BY:

Marcia D. Phillips 3-18-2003
Marcia D. Phillips Date
Quality Assurance Officer
RTI International

Terri L. Pollock 3-24-03
Terri L. Pollock, B.A. Date
Quality Assurance Manager
Battelle Memorial Institute

EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 2 **Date** April 7, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
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Post Office Box 12194
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Item 1

Section 7.0, REPORTING, page 29, which reads:

A letter report will be submitted to the EPA after completion of each of the pre-optimization experiments (Stage 1) and assay optimization experiments (Stage 2). These two letter reports will each include an introduction, methods and materials, results, discussion, and conclusion. The purposes of the letter reports are 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 so that the EPA has the information necessary to evaluate each stage of the pre-validation studies and give its approval to continue onto the next stage.

Is hereby amended as follows:

A draft preliminary data summary will be submitted to the EPA after completion of each of the pre-optimization experiments (Stage 1) and assay optimization experiments (Stage 2). These two draft preliminary data summaries will each include an introduction, methods and materials, results, discussion, and conclusion. The purposes of the draft preliminary data summaries are 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 so that the EPA has the information necessary to evaluate each stage of the pre-validation studies and give its approval to continue onto the next stage.

Once EPA has made recommendations to continue to the next Phase of the study, the draft preliminary data summaries will be revised and submitted to RTI's QAU as a letter report for auditing purposes. The audited letter reports will be submitted to EPA.

Justification:

This change is made so that the data required for making decisions about continuing with the next phase of the study can be submitted to EPA/Battelle in a timely manner. This change is also documented in a memo to the record dated April 4, 2003.

Amendment #2

APPROVED BY:

Rochelle W. Tyl 08/01/03
Rochelle W. Tyl, Ph.D., DABT Date
Principal Investigator
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J Matthews 7-31-03
James Mathews, Ph.D., DABT, Date
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Gary E. Timm 8/05/03
Gary E. Timm Date
Work Assignment Manager
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David P. Houchens 8/4/03
David P. Houchens, Ph.D. Date
Program Manager
Endocrine Disruptor Screening Program
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L. Greg Schweer for 8/00/03
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Terri L. Pollock, B.A. Date
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EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 3 **Date** May 7, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

SPONSOR: Battelle Memorial Institute
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Item 1

Section **3.2.1 Optimization of Experimental Design Factors and Conditions**, paragraph 5, page 29, which reads:

The 62 test runs will be conducted in completely random order over the course of two days.

Is hereby amended as follows:

The 62 test runs will be conducted over the course of two days.

Justification:

As all reactions in a given day will be initiated within 10 minutes, there will be no effect of time in degrading the reaction components. However, the technician must be able to correctly add protein to the appropriate tube, and be clear also in terminating the reaction at the right time. Interspersing tubes with different protein and time requirements would add undue confusion and compromise the experiment Sponsor was notified of this change by email on 4/14/2003 and registered no objection.

Amendment #3

APPROVED BY:

Rochelle W. Tyl 08/01/03
Rochelle W. Tyl, Ph.D., DABT Date
Principal Investigator
Center for Life Sciences and Toxicology
RTI International

J Matt 7-31-03
James Mathews, Ph.D., DABT, Date
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Gary E Timm 8/05/03
Gary E Timm Date
Work Assignment Manager
Endocrine Disruptor Screening Program
U.S. EPA

Dick P. Houchens 8/4/03
David P. Houchens, Ph.D. Date
Program Manager
Endocrine Disruptor Screening Program
Battelle Memorial Institute

for 8/05/03
L. Greg Schweer Date
Project Officer
Endocrine Disruptor Screening Program
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REVIEWED BY:

Marcia D. Phillips 8-19-2003
Marcia D. Phillips Date
Quality Assurance Officer
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Dick P. Houchens for 8/4/03
Terri L. Pollock, B.A. Date
Quality Assurance Manager
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EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 4 **Date** May 7, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
Chemistry and Life Sciences
Post Office Box 12194
Research Triangle Park, NC 27709

Item 1

Section **3.2.1 Optimization of Experimental Design Factors and Conditions**, paragraph 14, page 29, which reads:

Before proceeding to conduct the optimization experiments listed above using the bovine and porcine placental microsomes and the human recombinant microsomes, the response outcome of the human placental microsomal preparation will be evaluated to determine whether additional experiments are needed to optimize this preparation, as well as whether all or a limited set of experiments are needed to optimize the other microsomal preparations.

Is hereby amended as follows:

The response outcome of each microsomal preparation will be evaluated to determine whether additional experiments are needed to optimize each preparation.

Justification:

This change is made to facilitate conduct of the optimization experiments for both human placental and human recombinant microsomes in a timely manner. Sponsor was notified of this change by email on 4/24/2003.

Amendment # 4

APPROVED BY:

Rochelle W. Tyl 08/01/03
Rochelle W. Tyl, Ph.D., DABT Date
Principal Investigator
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RTI International

J Matt 7-31-03
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REVIEWED BY:

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EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 5 **Date** July 30, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
Chemistry and Life Sciences
Post Office Box 12194
Research Triangle Park, NC 27709

The following is hereby added to the protocol. This work is conducted under WA3-5 Task 21 which is entitled "Aromatase Optimization Supplementary Studies - Experiment #1".

Study Objectives:

To standardize the protocol for the $^{3}H_{2}0$ aromatase assay using human placenta microsomes in the RTI lab using appropriate controls plus a comparison with data from Dr. Robert Brueggemeier's lab. This is the first of three supplemental experiments to ensure that the placental aromatase assay is optimized and standardized before beginning the multiple chemical comparison study.

Experiment 1: Protocol Standardization in RTI Lab

Using the general assay method described in Section 3.2.1 of the protocol, conduct the assay using final protein (enzyme) concentrations of 0.05, 0.025 and 0.0125 mg/mL as follows (all concentrations are listed as final concentrations in the 2 mL reaction mixture):

0.1 mL propylene glycol
100 nM [^{3}H]androstenedione (0.1 uCi)
1.7 mM NADP
2.8 mM glucose-6-phosphate
1.0 U glucose-6-phosphate dehydrogenase

15 minutes incubation , 37 ± 1 C

For each protein concentration, each of the following reaction conditions should be conducted in triplicate:

Blank: (boiled microsomes, no NADP)
No NADP: (microsomes, no NADP)
Total Activity: (microsomes, substrate, cofactors - complete system)
Inhibition: (complete system + 0.1 μM 4-OH-androstenedione) The 4-OH androstenedione will be added to the reaction mixture before the protein (enzyme) is added.

The term "boiled microsomes" is defined as follows: microsomes that have been heated in a boiling water bath for 10 min. Boiled microsomes will be chilled on ice prior to being reheated to 37 ± 1 °C before their inclusion in the reaction mixtures.

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the

'No NADP' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet. Activity will be reported as nmol/mg/min.

The results of the first replicate of the experiment will be reported to EPA. If EPA judges the data from that experiment to be acceptable, RTI shall repeat the experiment on a separate day using the same conditions and protein concentrations. It should be noted which technicians are conducting the assays. If the first replicate does not achieve satisfactory results, EPA and RTI/Battelle will confer to determine the reasons for discrepancies from expected results.

Report the data as follows:

(a) Spreadsheet with all raw data showing DPMs for all assay tubes, include all pertinent information such as protein concentration, with/without NADP or inhibitor, the initial specific activity of [^{3}H]androstenedione (NEN), final specific activity of [^{3}H]androstenedione (after addition of nonradiolabeled androstenedione), amount of reaction mixture that DPMs represents (volume of reaction mixture assayed by LSC), percent substrate conversion to product, and activity reported as nmol/min (to see differences due to protein concentration) and nmol/mg/min.

(b) Summary table or graph showing activity and inhibition of activity (nmol/mg/min) for each protein concentration

Evaluate the data as follows:
(a) Percentage substrate conversion to product (aiming for 10-15%) for each protein concentration

(b) Non-NADPH-dependant background activity–as determined from the 'No NADP' tubes and non-enzyme-dependant background activity-as determined from the 'boiled microsome' tubes.

(c) Variance within each triplicate set of tubes.

(d) Activity (compare protein concentrations by looking at nmol/min; and final activity nmol/mg/min)

(e) Was activity inhibited by 4-OH-androstenedione?

(f) Compare RTI data with that reported by Dr. Brueggemeier's lab using identical reaction conditions

(g) Compare results from the replicate experiments.

Select protein concentration for next set of experiments in consultation with EPA based upon the

following criteria:

10-15% substrate conversion

Sufficient DPMs (per aliquot) to provide adequate range for inhibition curve (aiming for at least 15,000 DPMs)

Reproducibility of results

Deliverables:

RTI shall forward a spreadsheet and tabular/graphical summary of data to EPA for preliminary review immediately after initial evaluation of data for each of two replicates of Exp. 1. The final report should be submitted after final QA of the data.

Amendment #5

APPROVED BY:

Rochelle W. Tyl 08/01/03
Rochelle W. Tyl, Ph.D., DABT Date
Principal Investigator
Center for Life Sciences and Toxicology
RTI International

J. Matt 7-31-03
James Mathews, Ph.D., DABT, Date
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8/05/03
Gary E Timm Date
Work Assignment Manager
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David P. Houchens 8/4/03
David P. Houchens, Ph.D. Date
Program Manager
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for 8/05/03
L. Greg Schweer Date
Project Officer
Endocrine Disruptor Screening Program
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REVIEWED BY:

Marcia D Phillips 8-15-2003
Marcia D. Phillips Date
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Terri L. Pollock, B.A. Date
Quality Assurance Manager
Battelle Memorial Institute

EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 6 **Date** September 5, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
Chemistry and Life Sciences
Post Office Box 12194
Research Triangle Park, NC 27709

The following is hereby added to the protocol. This work is conducted under WA4-5 Task 1 which is entitled “Aromatase Optimization Supplementary Studies".

Study Objectives:

This experiment follows supplementary experiment 1 (WA 3-5, Task 21) to complete work necessary to ensure that the placental aromatase assay is optimized and standardized before beginning the multiple chemical comparison study.

The purpose of these studies are to:
- Demonstrate that the substrate and enzyme (protein) concentrations used provide a linear production of product within the time constraints of the assay
- Demonstrate that NADPH is not limiting during assay reaction time
- Demonstrate that measured $^3H_20$ correlates with estradiol/estrone formed in the reaction.

Experiment 2: Linear production of product

Using the general assay method described in Section 3.2.1 of the protocol, conduct the assay with a final protein concentration of 0.0125 mg/mL per tube, with 5 different incubation times. (suggested times 5, 10, 15, 20, 30 min). Each tube will contain (final concentrations in a 2 mL reaction mixture): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN (ca. 0.1 μCi), 0.3 mM NADPH, 0.0125 mg/mL microsomal protein (from human placenta) in a 0.1 M sodium phosphate buffer, pH 7.4, unless otherwise specified in the conditions below.

For each time point, use the following conditions with all tubes in triplicate. NOTE: 1) Double the number of assay tubes (Total activity and Inhibition sets only) for the 30 min time point to use for NADPH experiment as outlined below. 2) Double the number of assay tubes for the 15 min time point (No NADPH, Total Activity and Inhibition sets, only - 9 tubes) for Estrone/Estradiol Concentration analysis. Thus, the experiment will require 75 tubes, i.e., 60 tubes for the regular runs (4x5x3), 6 for the NADPH experiment and 9 for the Estrone/Estradiol Concentration experiment.

Blank: (boiled microsomes, substrate but no NADPH)
No NADPH: (microsomes, substrate but no NADPH)
Total Activity: (microsomes, substrate, 0.3 mM NADPH)
Inhibition: (microsomes, substrate, 0.3 mM NADPH, 0.1 uM 4-OH-androstenedione) The 4-OH androstenedione will be added to the reaction mixture before the protein (enzyme) is added.

The term “boiled microsomes” is defined as follows: microsomes that have been heated in a boiling water bath for 10 min. Boiled microsomes will be chilled on ice prior to being reheated to 37 ± 1 ◦C before their inclusion in the reaction mixtures. Substrate is defined as [^{3}H]ASDN in a final concentration of 100 nM (ca. 0.1 μCi).

Estrone/Estradiol Concentration: Use one complete set of tubes for the 15 min time point and complete assay in regular manner. For the extra set of No NADPH, Total Activity and Incubation tubes freeze the reaction mixture immediately after the 15 min incubation time for measurement of estrone and estradiol. Forward samples to Dr. Susan Laws, 2525 Hwy 54, U.S. EPA, RTP, NC (919 847-8557) for assay using RIA kits from Diagnostic Products, Inc to measure estradiol and Diagnostic Systems, Inc. to measure estrone.

NADPH experiment: Use one complete set of tubes for the 30 min time point and complete assay in regular manner. In the second set of 30 min assay tubes (Set consists of Total activity and Inhibition tubes, only), supplement the NADPH concentration by adding another aliquot of NADPH (0.3 mM final concentration) at the 15 min time point. Let the reaction in the 2nd set of assay tubes progress another 15 min (e.g., a total reaction time of 30 min but spiked at 15 min with additional NADPH). Compare both sets of 30 min assay tubes to determine whether or not the spike of additional NADPH increased activity.

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'No NADPH' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet. Activity will be reported as nmol/mg/min.

Report the data as follows:

(a) Prepare a spreadsheet with all raw data showing DPMs for all assay tubes, include all pertinent information such as incubation time, with/without NADPH or inhibitor, the initial specific activity of [^{3}H]ASDN (NEN), final specific activity of [^{3}H]ASDN (after addition of inert androstenedione), amount of reaction mixture that DPMs represents (volume of reaction mixture counted), % substrate conversion to product, and activity reported as nmol/mg (to see differences due to time) and nmol/mg/min. These data should be provided to EPA in a spreadsheet. If data do not show a linear production of product or one or more time points are questionable, the Contractor shall notify EPA and the experiment will need to be replicated.

(b) Provide a linear graph showing activity and inhibition of activity (nmol/mg) versus incubation time.

(c) Provide a bar graph showing activity and inhibition of activity (nmol/mg/min) for each incubation time.

(d) After receipt of data (15 min time point) from Dr. Laws' laboratory, provide a table comparing the measured estrone/estradiol concentration versus predicted product formation using ^3H_2O.

(E) For the 30 min incubation time point, provide a table comparing the values of the NADPH spiked and unspiked samples.

Evaluate the data as follows:

(a) Determine % substrate conversion to product (aiming for 10-15%) for each protein concentration.

(b) Compare % DPMs for the 'no NADPH' tubes with the blank tubes.

(c) Calculate variability within each set of triplicate control tubes.

(d) Show in a graph how well the 15 min time point from these data compare to those for the same protein concentration from Exp. 1

Discuss in the final report:

(a) Activity as a function of time (compare time by looking at nmol/mg; and final activity nmol/mg/min)

(b) Was activity inhibited by 4-OH-androstenedione?

(d) Is the product formation linear over time? What time points?

(e) How close were predictions for product to actual estrone/estradiol concentrations?

(f) Select and justify your selection of a measurement time point for the assay based upon following:

10-15% substrate conversion
10 min. range on either side of time point shows linear product formation
Adequate DPMs for inhibition curve
Good replication for all sets of data at time point

Deliverables:

RTI shall forward a spreadsheet and tabular/graphical summary of data to Battelle/EPA for preliminary review immediately after initial evaluation of data from the experiment. The final report should be submitted after final QA of the data.

APPROVED BY:

09/05/03
Rochelle W. Tyl, Ph.D., DABT Date
Principal Investigator
Center for Life Sciences and Toxicology
RTI International

9-5-03
James Mathews, Ph.D., DABT, Date
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9/08/03
Gary E. Timm Date
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9/8/03
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9/08/03
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9-16-2003
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9-8-03
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EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 7 **Date** September 12, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
Chemistry and Life Sciences
Post Office Box 12194
Research Triangle Park, NC 27709

The following is hereby added to the protocol. This work is conducted under WA4-5 Task 2 which is entitled "Aromatase Optimization Supplementary Studies".

Study Objectives:

This experiment follows supplementary experiment 2 (WA 4-5, Task 1) to complete work necessary to ensure that the placental aromatase assay is optimized and standardized before beginning the multiple chemical comparison study.

The purposes of this study are to:
- Demonstrate the specificity of the assay through generation of a competitive inhibition curve using 4-OH-androstenedione (4-OH-ASDN)
- Develop intralaboratory variability data to measure the repeatability of the assay.

Experiment 3: Inhibition curve using 4-OH-ASDN

Using the general assay method described in Section 3.2.1 of the protocol, conduct the assay with a final protein concentration of 0.0125 mg/mL with a 15 min incubation time (Conditions selected in experiments 1 and 2, respectively). Inhibition curve samples will contain 4-OH-ASDN at 6 concentrations spanning the range 1×10^{-6} to 1×10^{-9} M. Each tube will contain (final concentrations in a 2 mL reaction mixture): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN (ca. 0.1 μCi), 0.3 mM NADPH, 0.0125 mg/mL microsomal protein (from human placenta) in a 0.1 M sodium phosphate buffer, pH 7.4, unless otherwise specified in the conditions below.

The assay will be run in triplicate for all conditions. Thus, the experiment will require 27 tubes.

Blank: (boiled microsomes, substrate but no NADPH)
No NADPH: (microsomes, substrate but no NADPH)
Total Activity: (microsomes, substrate, 0.3 mM NADPH)
Inhibition: (microsomes, substrate, 0.3 mM NADPH, 1×10^{-6} to 1×10^{-9} M 4-OH-ASDN) The 4-OH ASDN will be added to the reaction mixture before the protein (enzyme) is added.

The term "boiled microsomes" is defined as follows: microsomes that have been heated in a boiling water bath for 10 min. Boiled microsomes will be chilled on ice prior to being reheated in a 37 ± 1 °C water bath before their inclusion in the reaction mixtures. Substrate is defined as [^{3}H]ASDN in a final concentration of 100 nM (ca. 0.1 μCi).

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'No NADPH' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet. Activity will be reported as nmol/mg/min.

Report the data as follows and provide to EPA (Susan Laws and Gary Timm) as the deliverable for this QR task:

(a) Prepare a spreadsheet with all raw data showing DPMs for all assay tubes, include all pertinent information such as incubation time, with/without NADPH or inhibitor, the initial specific activity of [^{3}H]ASDN (NEN), final specific activity of [^{3}H]ASDN (after addition of inert androstenedione), amount of reaction mixture that DPMs represents (volume of reaction mixture counted), % substrate conversion to product, and activity reported as nmol/mg/min.

(b) Provide a graph showing activity vs. inhibitor concentration.

(c) Using PRISM or other appropriate software, calculate an IC50 for each trial.

(d) Determine % substrate conversion to product (aiming for 10-15%) for 'total activity' tubes.

(e) Compare % DPMs for the 'no NADPH' tubes with that of the 'Blank' tubes

(f) Calculate variability within each set of triplicate control tubes and inhibitor concentration.

Contact Susan Laws (EPA) if there is any difficulty experienced in performing any part of this analysis.

Guidance for inclusion in WA 2-24 Final Report

Discuss in the final report and show data graphically where indicated:

(a) Activity (nmol/mg/min) versus inhibitor concentration

(b) How well does the activity in the 'Total activity' tubes compare with data from Exp. 1 and 2? (Plot all three.)

(c) How well does the IC50 compare with IC50 data from Dr. Brueggemeier's lab? (Bar graph)

APPROVED BY:

09/12/03
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Principal Investigator
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9-12-03
James Mathews, Ph.D., DABT, — Date
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David P. Houchens, Ph.D. — Date
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9-12-03
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9-30-2003
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9-12-03
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Battelle Memorial Institute

EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 8 **Date** September 12, 2003

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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

TESTING FACILITY: RTI International
Chemistry and Life Sciences
Post Office Box 12194
Research Triangle Park, NC 27709

The following is hereby added to the protocol. This work is conducted under WA4-5 Task 3 which is entitled "Aromatase Optimization Supplementary Studies".

Study Objectives:

Experiments 4 and 5 (using recombinant microsomes) are analogous to experiments 2 and 3, respectively, conducted with the placental aromatase assay. In lieu of conducting an experiment analogous to experiment 1 conducted with the placental assay, the protein concentration to achieve 10-15% conversion in the study below will be estimated by Battelle. Experiment 4 will follow the protocol for experiment 2. Experiment 5 will follow the protocol from experiment 3.

The purpose of these studies are to:
- Demonstrate that the substrate and enzyme (protein) concentrations used provide a linear production of product within the time constraints of the assay
- Demonstrate that NADPH is not limited during assay reaction time
- Demonstrate that measured $^{3}H20$ correlates with estradiol/estrone formed in the reaction.
-Demonstrate the specificity of the assay (estrone/estradiol production and competitive inhibition curve with 4-OH-androstenedione)
- Develop intralaboratory variability data to measure the repeatability of the assay.

Experiment 4: Linear production of product

Using the general assay method described in Section 3.2.1 of the protocol, conduct the assay using the final protein concentration as determined from Battelle's modeling data per tube, with 5 different incubation times. (5, 10, 15, 20, 30 min). Each tube will contain (final concentrations in a 2 mL reaction mixture): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN (ca. 0.1 μCi), 0.3 mM NADPH, microsomal protein (recombinant microsomes, protein concentration from Battelle) in a 0.1 M sodium phosphate buffer, pH 7.4, unless otherwise specified in the conditions below.

For each time point, use the following conditions with all tubes in triplicate. NOTE: 1) Double the number of assay tubes (Total activity and Inhibition sets only) for the 30 min time point to use for NADPH experiment as outlined below. 2) Double the number of assay tubes for the 15 min time point (No NADPH, Total Activity and Inhibition sets, only - 9 tubes) for Estrone/Estradiol Concentration analysis. Thus, the experiment will require 75 tubes, i.e., 60 tubes for the regular runs (4x5x3), 6 for the NADPH experiment and 9 for the Estrone/Estradiol Concentration experiment.

Blank: (boiled microsomes, substrate but no NADPH)
No NADPH: (microsomes, substrate but no NADPH)
Total Activity: (microsomes, substrate, 0.3 mM NADPH)
Inhibition: (microsomes, substrate, 0.3 mM NADPH, 0.1 uM 4-OH-androstenedione) The 4-OH androstenedione will be added to the reaction mixture before the protein (enzyme) is added.

The term "boiled microsomes" is defined as follows: microsomes that have been heated in a boiling water bath for 10 min. Boiled microsomes will be chilled on ice prior to being reheated in a 37 ± 1 ◦C water bath before their inclusion in the reaction mixtures. Substrate is defined as [^{3}H]ASDN in a final concentration of 100 nM (ca. 0.1 μCi).

Estrone/Estradiol Concentration: Use one complete set of tubes for the 15 min time point and complete assay in regular manner. For the extra set of No NADPH, Total Activity and Incubation tubes freeze the reaction mixture immediately after the 15 min incubation time for measurement of estrone and estradiol. Forward samples to Dr. Susan Laws, 2525 Hwy 54, U.S. EPA, RTP, NC (919 847-8557) for assay using

RIA kits from Diagnostic Products, Inc to measure estradiol and Diagnostic Systems, Inc. to measure estrone.

NADPH experiment: Use one complete set of tubes for the 30 min time point and complete assay in regular manner. In the second set of 30 min assay tubes (Set consists of Total activity and Inhibition tubes, only), supplement the NADPH concentration by adding another aliquot of NADPH (0.3 mM final concentration) at the 15 min time point. Let the reaction in the 2nd set of assay tubes progress another 15 min (e.g., a total reaction time of 30 min but spiked at 15 min with additional NADPH). Compare both sets of 30 min assay tubes to determine whether or not the spike of additional NADPH increased activity.

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'Blank' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet. Activity will be reported as nmol/mg/min.

Report the data as follows and provide to EPA as the deliverable for this QR task:

(a) Prepare a spreadsheet with all raw data showing DPMs for all assay tubes, include all pertinent information such as incubation time, with/without NADPH or inhibitor, the initial specific activity of trace (NEN), final specific activity of trace (after addition of inert androstenedione), amount of reaction mixture that DPMs represents (volume of reaction mixture counted), % substrate conversion to product, and activity reported as nmol/mg (to see differences due to time) and nmol/mg/min. These data should be provided to EPA in a spreadsheet. If data do not show a linear production of product or one or more time points are questionable, the Contractor shall notify EPA and the experiment will need to be replicated.

(b) Provide a linear graph showing activity and inhibition of activity (nmol/mg) versus incubation time.

(c) Provide a bar graph showing activity and inhibition of activity (nmol/mg/min) for each incubation time.

(d) After receipt of data (15 min time point) from Dr. Laws' laboratory, provide a table comparing the measured estrone/estradiol concentration versus predicted product formation using ^3H_2O.

(e) For the 30 minute incubation time point, provide a table comparing the values of the NADPH spiked and unspiked samples.

(f) Determine % substrate conversion to product (aiming for 10-15%) for each time point.

(g) Compare % DPMs for the 'no NADPH' tubes with the blank tubes.

(h) Calculate variability within each set of triplicate control tubes.

Experiment 5: Inhibition curve using 4-OH-androstenedione

Using the general assay method described in Section 3.2.1 of the protocol, conduct the assay using the estimated protein concentration and the assay time point selected from Exp. 4 above. Inhibition curve samples will contain 4-OH-ASDN at 6 concentrations spanning the range 1×10^{-6} to 1×10^{-9} M. Each tube will contain (final concentrations in a 2 mL reaction mixture): 0.1 mL propylene glycol, 100 nM [^{3}H]ASDN (ca. 0.1 μCi), 0.3 mM NADPH, microsomal protein (recombinant) in a 0.1 M sodium phosphate buffer, pH 7.4, unless otherwise specified in the conditions below.

The assay will be run in triplicate for all conditions. Thus, the experiment will require 27 tubes.

Blank: (boiled microsomes, substrate but no NADPH)
No NADPH: (microsomes, substrate but no NADPH)
Total Activity: (microsomes, substrate, 0.3 mM NADPH)
Inhibition: (microsomes, substrate, 0.3 mM NADPH, 1×10^{-6} to 1×10^{-9} M 4-OH-ASDN) The 4-OH ASDN will be added to the reaction mixture before the protein (enzyme) is added.

The term "boiled microsomes" is defined as follows: microsomes that have been heated in a boiling water bath for 10 min. Boiled microsomes will be chilled on ice prior to being reheated in a 37 ± 1 °C water bath before their inclusion in the reaction mixtures. Substrate is defined as [^{3}H]ASDN in a final concentration of 100 nM (ca. 0.1 μCi).

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'No NADPH' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet. Activity will be reported as nmol/mg/min.

Report the data as follows and provide to EPA (Susan Laws and Gary Timm) as the deliverable for this QR task:

(a) Prepare a spreadsheet with all raw data showing DPMs for all assay tubes, include all pertinent information such as incubation time, with/without NADPH or inhibitor, the initial specific activity of [^{3}H]ASDN (NEN), final specific activity of [^{3}H]ASDN (after addition of inert androstenedione), amount of reaction mixture that DPMs represents (volume of reaction mixture counted), % substrate conversion to product, and activity reported as nmol/mg/min.

(b) Provide a graph showing activity vs. inhibitor concentration.

(c) Using PRISM or other appropriate software, calculate an IC50 for each trial.

(d) Determine % substrate conversion to product (aiming for 10-15%) for 'total activity' tubes.

(e) Compare % DPMs for the 'no NADPH' tubes with that of the 'Blank' tubes

(f) Calculate variability within each set of triplicate control tubes and inhibitor concentration.

Contact Susan Laws (EPA) if there is any difficulty experienced in performing any part of this analysis.

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EPA Contract No. 68-W-01-023 (Battelle Prime Contractor)
RTI Contract No.: 65U-08055.001.018
RTI Master Protocol No.: RTI-869-AN

Amendment 9 **Date** February 19, 2004

TITLE: Pre-validation of the Aromatase Assay using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes

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Many of the following amended items arose from the outcome of the optimization and quick response task experiments.

Item 1

Section 3.2.2 Variability Determination Using the Optimized Assay, paragraph 1, page 17, which reads:

After optimum conditions have been determined for the human, bovine, and porcine placental microsomes and the human recombinant microsomes, each assay will be conducted using the optimized factors to assess the variability of the results. Each assay will be conducted independently by three technicians and at three separated times per technician. Single runs will be conducted by each technician on each day for a given microsomal preparation (a total of 36 runs across assays and days). The assays must be run independently of one another. The assays will be conducted and samples will be analyzed as described above in Section 3.2.1, except that optimized factor levels and conditions will be used. The aromatase activity will be determined for each run and statistical analysis will be carried out as described in Section 4.4.2.

Is hereby amended as follows:

After optimum conditions have been determined for the human placental microsomes and the human recombinant microsomes, each assay will be conducted using the optimized factors to assess the variability of the results. Each assay will be conducted independently by three technicians and at three separated times per technician. Triplicate runs will be conducted by each technician on each day for a given microsomal preparation (a total of 54 runs across assays and days). The assays must be run independently of one another. The assays will be conducted and samples will be analyzed as described above in Section 3.2.1, except that optimized factor levels and conditions will be used. The aromatase activity will be determined for each run and statistical analysis will be carried out as described in Section 4.4.2.

Justification:

Bovine and porcine placental assays are no longer being pursued, as decided at EDMVS meeting. Conduct of each assay in triplicate tubes will allow for better statistics and more closely models the expected final assay design. Also, since the bulk of the labor involved in the assay is in the solution preparation, little additional time will be required to produce the additional data. Documentation related to this change is in NB 10786-51.

Item 2

Section 4.2.2.2 Letrozole is hereby amended as follows:

It is deleted in it's entirety.

Justification Letrozole was not available so it is deleted from the list of test substances. See email dated 2/28/2003 in the record

Item 3

Table 3, page 21 is hereby amended as follows:

The number in the levels column for atrazine is changed from 8 to 7 since there are only 7 factors of 10 in the range 10^{-3} to 10^{-9}. Delete the row for Letrozole.

Justification: Correct typographical error. Letrozole was not available and is deleted from the list of test substances. See email dated 2/28/2003 in the record

Item 4

Section 4.3 Experimental Design, that reads:

The factor levels and conditions determined as optimal (based on the optimization experiments) will be used to test the test substances using the aromatase assay. Each of 12 different test substances, at up to 8 different concentrations (ranging from no less than 10^{-9} M and no higher than 10^{-3} M), will be tested using each of the four different microsomal preparations, i.e. human, bovine, and porcine placental microsomes and human recombinant microsomes. The vehicle controls will be ethanol (95 percent), DMSO and/or distilled water, depending on the vehicle(s) used to formulate the test substances. The aromatase activity in the absence of any test substance, will be the benchmark (100 percent) activity.

Is hereby amended as follows:

The factor levels and conditions determined as optimal (based on the optimization experiments) will be used to test the test substances using the aromatase assay. Each of 11 different test substances, at up to 8 different concentrations (ranging from no less than 10^{-9} M and no higher than 10^{-3} M), will be tested using each of the two different microsomal preparations, i.e. human placental microsomes and human recombinant microsomes. The vehicle controls will be ethanol, DMSO and/or distilled water, depending on the vehicle(s) used to formulate the test substances. The aromatase activity in the absence of any test substance, will be the benchmark (100 percent) activity.

Justification: Bovine and porcine placental assays are no longer being pursued. Letrozole was unavailable and has been removed from the list of test chemicals, so the number of test substances to be tested dropped from 12 to 11. See email dated 2/28/2003 in the record.

Item 5

Section 4.3.1 Assay Method, which reads:

The aromatase assay will be performed as described below and as shown in the flow diagram (Figure 1).

The assay will be performed in the required number of 16x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol will be added to the tubes to serve as a co-solvent. The final concentrations of substrate, NADPH-generating system components, and microsomal protein, as well as incubation time, will be those chosen based on the results of the

optimization experiments and will be added to the protocol by amendment. The substrate, [1β-^{3}H]-androstenedione, will be added to the tubes. An NADPH-generating system comprised of NADP+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase will be added to each tube. The test substance will be added to the tube. The tubes will be placed at 37 ± 1°C in the water bath for five minutes prior to the start of the assay by the addition of the diluted placental microsomal suspension. The total volume will be 2.0 mL, and the tubes will be incubated for the time period determined based on the results of the optimization experiments (to be added to the protocol by amendment). The incubations will be stopped by the addition of methylene chloride (2.0 mL) and the tubes vortexed for 30 seconds. The tubes will then be centrifuged using a Beckman G2-6R centrifuge with a GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The aqueous layers will be transferred to new test tubes and extracted again with methylene chloride (2.0 mL). This extraction procedure will be performed one additional time. [The number of extractions may be modified based on the results of the experiments in Section 2.5 and any such modification would be added to the protocol by amendment.] The aqueous layers will be transferred to vials, and duplicate aliquots (0.5 mL) will be removed and transferred to a 20 mL liquid scintillation counting vial. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The samples will be assayed for radiochemical content as described in Section 2.5.

Is hereby amended as follows:

The aromatase assay will be performed as described below and as shown in the flow diagram (Figure 1).

The assay will be performed in the required number of 13x100 mm test tubes maintained at 37 ± 1°C in a shaking water bath. An aliquot (100 µL) of propylene glycol will be added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione, will be added to the tubes to give a final concentration of 100 nM with about 0.1 µCi/tube. The test substance or test substance vehicle and assay buffer will be added to the tube. NADPH will be added to each tube (except negative controls) in a final concentration of 0.3 mM. The tubes will be placed at 37 ± 1°C in the water bath for five minutes prior to the start of the assay by the addition of the diluted placental microsomal suspension (for human placental microsomes the target final protein concentration is about 0.0125 mg/mL and for the recombinant microsomes, the target final protein concentration is about 0.004 mg/mL). The total 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) and the tubes vortexed for ca 5 s and placed on ice. After all incubations in the set have been stopped, each tube will be vortexed an additional 20-25 s. The tubes will then be centrifuged using a Beckman GS-6R centrifuge with a GH-3.8 rotor for 10 minutes at a setting of 1000 rpm. The methylene chloride layers will be removed and discarded and the aqueous layer will be extracted again with methylene chloride (2.0 mL, 20-25 s vortex followed by centrifugation as before). This extraction procedure will be performed one additional time. The aqueous layers will be transferred to vials, and duplicate aliquots (0.5 mL) will be removed and transferred to a 20 mL liquid scintillation counting vial. Liquid scintillation cocktail (Ultima Gold, Packard, 10 mL) will be added to each counting vial and shaken to mix the solution. The samples will be assayed for radiochemical content as described in Section 2.5.

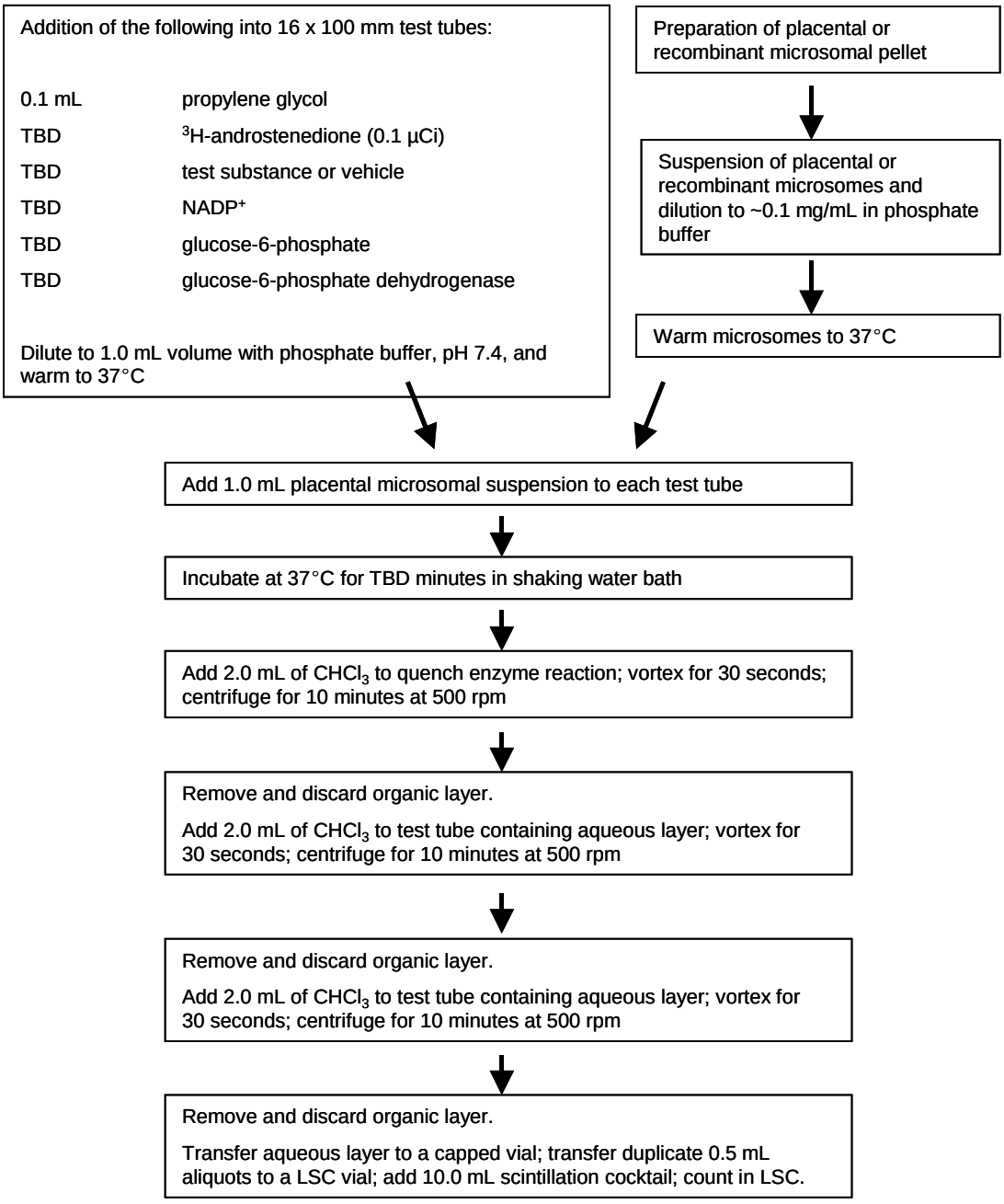

Figure 1. Flow Diagram for Aromatase Assay of Test Substances

Figure 1 in that section is revised as follows:

1. Change tube size to 13 x 100
2. Add 100 nM as [^{3}H]androstenedione concentration
3. Add 20 µL as volume for test substance or vehicle. Add "Concentration varies with level".
4. Delete all references to NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase.
5. Add 0.3 mM NADPH
6. Delete reference to "dilution to ~0.1 mg/mL in phosphate buffer" (as refers to protein concentration). Add "dilution to appropriate concentration - see text".
7. Add 15 min for incubation time
8. Change all references to $CHCl_3$ to CH_2Cl_2.
9. Change all references to 500 rpm to "at a setting of 1000 rpm".
10. Change 'vortex for 30 seconds' to 'vortex as described in text'.

Justification: Addition of experimental details. Documentation of these changes (email) is in the record

Item 6

The footnote to the Table in Section 4.3.3 Assay Schedule and Conduct Procedure, which reads:

The negative control will include the optimal androstenedione concentration (labeled and non-labeled) but will omit the enzyme and/or co-factor. It will estimate the background activity.

is hereby amended as follows:

The negative control will include the optimal androstenedione concentration (labeled and non-labeled) but will omit the co-factor. It will estimate the background activity.

Justification: Addition of chosen method of control preparation based on optimization results. Documentation is in the record.

Item 7

Section 4.4.4 Comparison of Optimized Assays, which reads:

For each test compound log10(IC_{50}), slope, and their associated standard errors, degrees of freedom, and confidence intervals will be determined with each assay for those test substances for which an IC_{50} exists. The estimates and their standard errors will be compared among assays by two-sample heterogeneous variances t-tests, adjusting for simultaneous inferences (6 pairwise comparisons among the 4 assays). Consistent patterns across test substances of inequalities among assays will be examined. Differences among assays will be judged based on the relative strength of the test substance activity inhibition, as indicated by the IC_{50}, slope, and their standard errors.

Is hereby amended as follows:

For each test compound $\log_{10}(IC_{50})$, slope, and their associated standard errors, degrees of freedom, and confidence intervals will be determined with each assay for those test substances for which an IC_{50} exists. The estimates and their standard errors will be compared among the two assays by a two-sample heterogeneous variances t-test. Differences among assays will be judged based on the relative strength of the test substance activity inhibition, as indicated by the IC_{50}, slope, and their standard errors.

Justification: Reduced number of comparisons since bovine and porcine plancentas are no longer included as decided at June 2003 EDMVS meeting.

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Appendix A-7

Protocol Deviations

Protocol Deviation 1

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 8, Section 2.2.1.2 Placental Microsome Preparation, line 13

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.

DEVIATION:

All placental microsomal preparations made prior to 3/24/03 were resuspended in a buffer containing 0.05 M dithiothreitol (not 0.05 mM as specified). This affects human, porcine and bovine preparations that are intended for use in the study.

REASON/IMPACT OF CHANGE:

No adverse effects are expected.

Protocol Deviation 2

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 10, Section 2.4 CYTOCHROME P450 CONTENT, paragraph 2, line 3:

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 (1mL/cuvette).

DEVIATION:

Human placental and human recombinant microsomes were bubbled with CO for approximately 15-20 s.

REASON/IMPACT OF CHANGE:

No adverse effects are expected.

Protocol Deviation 3

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 14, 3.2.1 Optimization of Experimental Design Factors and Conditions, paragraph 5,:

The 62 test runs will be conducted in completely random order over the course of two days**.**

DEVIATION:

Because of errors in assay conduct on the second testing day for both human placental and human recombinant microsomes, some tubes in each set were repeated on another day. In both cases, additional tubes (at the centerpoint) of the study design were included on the additional day to allow for day-to-day variance assessment.

REASON/IMPACT OF CHANGE:

No adverse effects are expected. The activity values for the centerpoint tubes show very little day-to day variance. Sponsor was notified by email on 4/24/2003.

Protocol Deviation 4

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol Amendment 5, page 2

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'No NADP' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet.

DEVIATION:

Aromatase activity for each run was calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'Blank' tubes after extraction)

REASON/IMPACT OF CHANGE:

No adverse effects are expected. Email from Dr. Brueggemeier (8/7/03) expresses his reservations about the use of 'No NADP' tubes as blanks since any NADP still present in the microsomes can utilize the other regenerating system components to provide some aromatase activity. For this reason, and because the 'No NADP' tubes did show aromatase activity, the 'Blank' tubes were substituted as a more representative background level.

Protocol Deviation 5

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol Amendment 5

The amendment calls for two replicates of the experiment.

DEVIATION:

An unexpectedly high aromatase activity was found in the 'No NADP' tubes. An additional experiment consisting of repeating the assay using the following conditions in triplicate for each of the three protein concentrations (0.05, 0.025 and 0.0125 mg/mL) was undertaken to investigate that high activity:

1) Complete incubation (microsomes, all co-factors, substrate)
2) No NADP (microsomes, all co-factors except NADP, substrate)
3) No NADPH regenerating system (microsomes, no co-factors, substrate)

This set of assays is collectively referred to as the follow-on experiment in the data records and reports.

REASON/IMPACT OF CHANGE:

No adverse effects are expected. Additional data on the activity in assays containing no NADP and no regenerating system were obtained. Email from Dr. Brueggemeier (8/7/03) expresses his reservations about the use of 'No NADP' tubes as blanks since any NADP still present in the microsomes can utilize the other regenerating system components to provide some aromatase activity.

Protocol Deviation 6

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol Amendment 5, page 2

For each protein concentration, each of the following reaction conditions should be conducted in triplicate:

Blank: (boiled microsomes, no NADP)
No NADP: (microsomes, no NADP)
Total Activity: (microsomes, substrate, cofactors - complete system)
Inhibition: (complete system + 0.1 μM 4-OH-androstenedione) The 4-OH androstenedione will be added to the reaction mixture before the protein (enzyme) is added.

DEVIATION:

For the second replicate of the experiment, the following additional conditions were tested:
No regenerating system (microsomes, no co-factors, substrate)
NADPH (microsomes, no co-factors, 0.3 mM NADPH, substrate)

REASON/IMPACT OF CHANGE:

No adverse effects are expected. The NADPH set was added at the suggestion of Dr. Laws. The no regenerating system set was added as an alternate 'blank' reaction and to provide additional data for these conditions for comparison with that obtained from the follow-on experiment (see protocol deviation 5).

Protocol Deviation 7

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol page 8, Section 2.1.2

The specific activity value provided by the supplier will be used for calculation purposes, if in close agreement with the value determined by the testing laboratory, i.e. ± 5%.

DEVIATION:

The supplier provided specific activity of 25.3 Ci/mmol was used in calculations even though it was > 5% different from that determined by the testing laboratory for the second lot of [^{3}H]ASDN.

REASON/IMPACT OF CHANGE:

Experimentally determined specific activity was about 86-88% of the supplier provided specific activity. Because the mass of [^{3}H]ASDN in the substrate solutions is only ca 2% of the total mass, the minor discrepancy in specific activity of the stock has no significant effect (< 0.5%) on the final specific activity of the substrate solutions and subsequently no significant effect on the aromatase activity calculations.

Protocol Deviation 8

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol page 17, Section 4.1

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione (substrate), and microsomal preparations from the human, bovine, and porcine placentas, as well as human recombinant microsomes, will be obtained prior to initiation of these experiments to ensure that sufficient quantities are available to conduct the study using the same lot or batch.

DEVIATION:

Two different batches of DMSO were used in the test substance phase of the study.

REASON/IMPACT OF CHANGE:

Additional DMSO was required to complete the experiments. No impact is expected.

Protocol Deviation 9

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol page 30, Table 3 Test Substance Target Concentrations

Ketoconazole:10^{-3}

Dibenz(*a*,*h*)anthracene: 7 levels, 10^{-3}

DEVIATION:

Highest concentration of ketoconazole tested was 0.8×10^{-3} M and the highest concentration of dibenz(*a*,*h*)anthracene was 10^{-4}. Only 6 levels of dibenz(*a*,*h*)anthracene were tested.

REASON/IMPACT OF CHANGE:

Concentrations were modified from the target concentrations due to solubility problems at the initial target concentrations. Documented in NB 10914-4. No expected impact..

Protocol Deviation 10

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol page 30, Table 3 Test Substance Target Concentrations

Ketoconazole:10^{-3}

Dibenz(*a*,*h*)anthracene: 7 levels, 10^{-3}

DEVIATION:

Highest concentration of ketoconazole tested was 0.8×10^{-3} M and the highest concentration of dibenz(*a*,*h*)anthracene was 10^{-4}. Only 6 levels of dibenz(*a*,*h*)anthracene were tested.

REASON/IMPACT OF CHANGE:

Concentrations were modified from the target concentrations due to solubility problems at the initial target concentrations. Documented in NB 10914-4. No expected impact..

Appendix A-8

QAPP Deviations

Deviation 1

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 20, Section 10.1.2.1.2 Placental Microsome Preparation, line 13

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.

DEVIATION:

All placental microsomal preparations made prior to 3/24/03 were resuspended in a buffer containing 0.05 M dithiothreitol (not 0.05 mM as specified). This affects human, porcine and bovine preparations that are intended for use in the study.

REASON/IMPACT OF CHANGE:

No adverse effects are expected.

Deviation 2

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 22, Section 10.1.4 CYTOCHROME P450 CONTENT, paragraph 2, line 3:

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 (1mL/cuvette).

DEVIATION:

Human placental and human recombinant microsomes were bubbled with CO for approximately 15-20 s.

REASON/IMPACT OF CHANGE:

No adverse effects are expected.

Deviation 3

ORIGINAL DOCUMENT SPECIFICATIONS:

Page 25, Section 10.2.2.1 Optimization of Experimental Design Factors and Conditions, paragraph 3, reads::

The 62 test runs will be conducted in completely random order over the course of two days.

DEVIATION:

Because of errors in assay conduct on the second testing day for both human placental and human recombinant microsomes, some tubes in each set were repeated on another day. In both cases, additional tubes (at the centerpoint) of the study design were included on the additional day to allow for day-to-day variance assessment.

REASON/IMPACT OF CHANGE:

No adverse effects are expected. The activity values for the centerpoint tubes show very little day-to day variance. Sponsor was notified by email on 4/24/2003.

Deviation 4

ORIGINAL DOCUMENT SPECIFICATIONS:

QAPP Amendment 12, page 2

The aromatase activity for each run will be calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'No NADP' tubes after extraction), the protein concentration and the time of incubation using a validated Excel 97 spreadsheet.

DEVIATION:

Aromatase activity for each run was calculated from the radioactivity remaining in the aqueous layer after extraction (less the mean radiochemical content of the aqueous layer of the 'Blank' tubes after extraction)

REASON/IMPACT OF CHANGE:

No adverse effects are expected. Email from Dr. Brueggemeier (8/7/03) expresses his reservations about the use of 'No NADP' tubes as blanks since any NADP still present in the microsomes can utilize the other regenerating system components to provide some aromatase activity. For this reason, and because the 'No NADP' tubes did show aromatase activity, the 'Blank' tubes were substituted as a more representative background level.

Deviation 5

ORIGINAL DOCUMENT SPECIFICATIONS:

QAPP Amendment 12

The amendment calls for two replicates of the experiment.

DEVIATION:

An unexpectedly high aromatase activity was found in the 'No NADP' tubes. An additional experiment consisting of repeating the assay using the following conditions in triplicate for each of the three protein concentrations (0.05, 0.025 and 0.0125 mg/mL) was undertaken to investigate that high activity:

1) Complete incubation (microsomes, all co-factors, substrate)
2) No NADP (microsomes, all co-factors except NADP, substrate)
3) No NADPH regenerating system (microsomes, no co-factors,substrate)

This set of assays is collectively referred to as the follow-on experiment in the data records and reports.

REASON/IMPACT OF CHANGE:

No adverse effects are expected. Additional data on the activity in assays containing no NADP and no regenerating system were obtained. Email from Dr. Brueggemeier (8/7/03) expresses his reservations about the use of 'No NADP' tubes as blanks since any NADP still present in the microsomes can utilize the other regenerating system components to provide some aromatase activity.

Deviation 6

ORIGINAL DOCUMENT SPECIFICATIONS:

QAPP Amendment 12, page 1

For each protein concentration, each of the following reaction conditions should be conducted in triplicate:

Blank: (boiled microsomes, no NADP)
No NADP: (microsomes, no NADP)
Total Activity: (microsomes, substrate, cofactors - complete system)
Inhibition: (complete system + 0.1 μM 4-OH-androstenedione) The 4-OH androstenedione will be added to the reaction mixture before the protein (enzyme) is added.

DEVIATION:

For the second replicate of the experiment, the following additional conditions were tested:
No regenerating system (microsomes, no co-factors, substrate)
NADPH (microsomes, no co-factors, 0.3 mM NADPH, substrate)

REASON/IMPACT OF CHANGE:

No adverse effects are expected. The NADPH set was added at the suggestion of Dr. Laws. The no regenerating system set was added as an alternate 'blank' reaction and to provide additional data for these conditions for comparison with that obtained from the follow-on experiment (see protocol deviation 5).

Deviation 7

ORIGINAL DOCUMENT SPECIFICATIONS:

QAPP, page 19, Section 10.1.1.2

The specific activity value provided by the supplier will be used for calculation purposes, if in close agreement with the value determined by the testing laboratory, i.e. ± 5%.

DEVIATION:

The supplier provided specific activity of 25.3 Ci/mmol was used in calculations even though it was > 5% different from that determined by the testing laboratory for the second lot of [^{3}H]ASDN.

REASON/IMPACT OF CHANGE:

Experimentally determined specific activity was about 86-88% of the supplier provided specific activity. Because the mass of [^{3}H]ASDN in the substrate solutions is only ca 2% of the total mass, the minor discrepancy in specific activity of the stock has no significant effect (< 0.5%) on the final specific activity of the substrate solutions and subsequently no significant effect on the aromatase activity calculations.

Deviation 8

ORIGINAL DOCUMENT SPECIFICATIONS:

QAPP page 28, Section 10.3.1

A sufficient supply of chemical reagents, radiolabeled and non-radiolabeled androstenedione (substrate), and microsomal preparations from the human, bovine, and porcine placentas, as well as human recombinant microsomes, will be obtained prior to initiation of these experiments to ensure that sufficient quantities are available to conduct the study using the same lot or batch.

DEVIATION:

Two different batches of DMSO were used in the test substance phase of the study.

REASON/IMPACT OF CHANGE:

Additional DMSO was required to complete the experiments. No impact is expected.

Deviation 9

ORIGINAL DOCUMENT SPECIFICATIONS:

QAPP Amendment 14, page 1 and **QAPP Amendment 15**, page 2:

Inhibition: (microsomes, substrate, 0.2 mM NADPH, 1×10^{-6} to 1×10^{-9} M 4-OH-ASDN)

DEVIATION:

Actual range of concentrations of 4-OH ASDN used was 5×10^{-6} to 1×10^{-9} M.

REASON/IMPACT OF CHANGE:

Error in preparation of solutions discovered after experiments were run. No adverse impact is expected.

Deviation 10

ORIGINAL DOCUMENT SPECIFICATIONS:

Protocol page 29, Table 10-3 Test Substance Target Concentrations

Ketoconazole 10^{-3}

Dibenz(*a*,*h*)anthracene: 7 levels, 10^{-3}

DEVIATION:

Highest concentration of ketoconazole tested was 0.8×10^{-3} M and the highest concentration of dibenz(*a*,*h*)anthracene was 10^{-4}. Only 6 levels of dibenz(*a*,*h*)anthracene were tested..

REASON/IMPACT OF CHANGE:

Concentrations were modified from the target concentrations due to solubility problems at the initial target concentrations. Documented in NB 10914-4. No expected impact.

Appendix A-9

QMP Deviations

Deviation 1

ORIGINAL DOCUMENT SPECIFICATIONS:

Section 4.2.2 Study/Work Assignment Records

All data generated during the conduct of a study or work assignment, except those generated by automated data collection systems, must be recorded directly and promptly.

DEVIATION:

Original data for a Pig microsome P450 scan was lost, but a reduced-size photocopy of the data is in the record.

REASON/IMPACT OF CHANGE:

No expected impact since pig microsome analysis was deleted from the study scope.

RTI INTERNATIONAL

Quality Assurance Statement

Study Title: **Pre-Validation of the Aromatase Assay Using Human, Bovine, and Porcine Placental Microsomes, and Human Recombinant Microsomes**

Sponsor: **Battelle Memorial Institute**

Study Code: **Rt02-ED05**

Protocol Number: **RTI-869-AN**

This study was audited by the Sciences and Engineering – Health Sciences Quality Assurance Unit and the results of the inspections and audits were reported to the task leader 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 Task Leader and Management
Protocol Review	October 11, 15–17, 2002	October 18, 2002
Specific Activity Determination Inspection	January 21, 2003	January 24, 2003
Protocol Amendment Review	February 4, 2003	February 6, 2003
Placental Microsome Sample Preparation Inspection and Record Review	January 30, and February 3, 2003	March 7, 2003
Data and Report Audit	March 10–11, 13-14, and 17–21, 2003	March 24, 2003
Data Audit	April 10, 16-18, 21, and 22, 2003	April 28, 2003
Data and Report Audit	May 1, 6, and 7, 2003	May 7, 2003
Data and Report Audit	May 14, 2003	May 14, 2003

Data and Report Audit	August 28, and 29, 2003	September 2, 2003
Data and Report Audit Follow-up	October 28, 2003	October 28, 2003
Data and Report Audit	January 12-14, 16, 19, 23, 28, 29, and February 2-4, 2004	February 6, 2004

K. Collier
Quality Assurance Specialist

6/30/2005
Date

Approval:

Debra A. Drissel
Debra Drissel
Quality Assurance Manager

06/30/05
Date