LETTER REPORT ON

PHASE I
PRE-OPTIMIZATION EXPERIMENTS FOR SUBSTRATE CHARACTERIZATION FOR HUMAN RECOMBINANT AND HUMAN PLACENTAL MICROSOMES

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PREPARED FOR

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Letter Report 05 05 03

Work Assignment 2-24

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

Phase I
Pre-Optimization Experiments for Substrate Characterization for Human Recombinant and Human Placental Microsomes

1 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 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 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 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. 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. 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 mM 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 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 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 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 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 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.

Figure 1
Data Sheet for ASDN

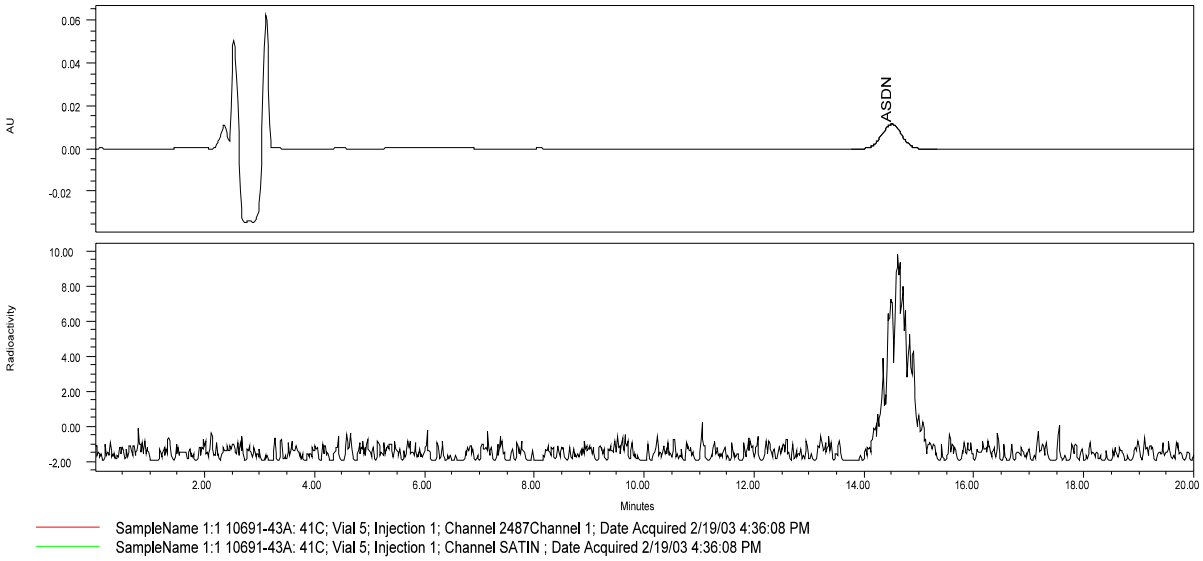
SIGMA

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 2
HPLC Radiochromatogram of ASDN and [^{3}H]ASDN



Peak Results

	Name	RT	Area	Height
1	ASDN	14.506	293714	11441
2	[3H]ASDN	14.625	337219	11235

Column: Zorbax SB-C18, USCL011903, 250 x 4.6 mm
Mobile Phase: 55:15:30 ddH2O: THF:MeOH 10691-95A
Flow Rate: 1 mL/min
Detectors: Waters 2487 at 240 nm
B-RAM with 250 ul LiGL solid cell, #11590

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

10691-5-SD 1/30/02

PerkinElmer life sciences

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

Products You Trust
NEN
Wallac

^{3}H Technical Data Certificate of Analysis

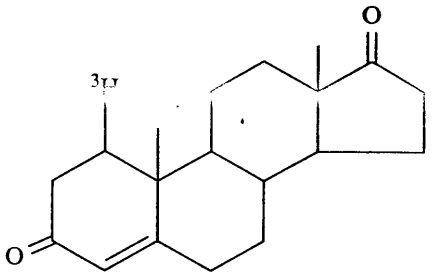
Caution: For Laboratory Use. A research chemical for research purposes only.

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:

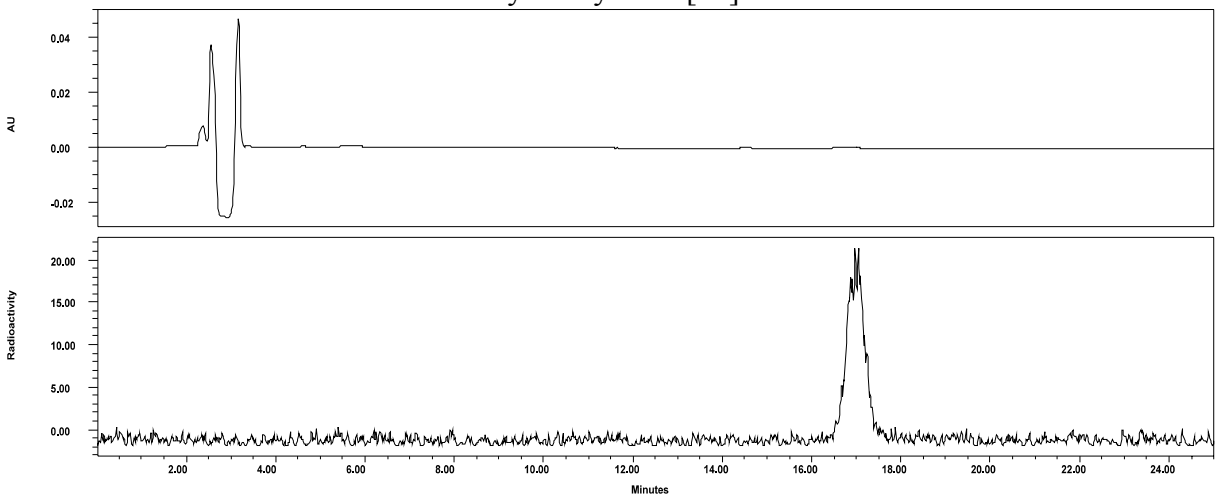
   toluene : ethyl acetate, (2:1).

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 4
HPLC Purity Analysis of [^{3}H]ASDN

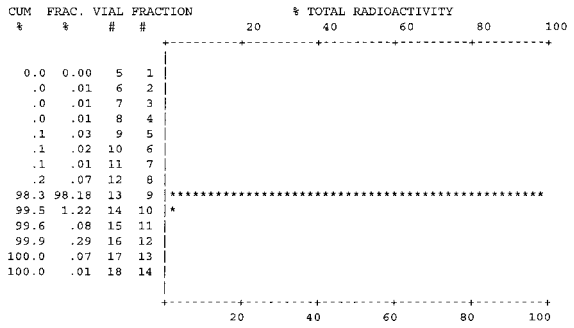


SampleName 10691-43A; Vial 2; Injection 1; Channel 2487Channel 1; Date Acquired 1/15/03 12:21:46 PM
SampleName 10691-43A; Vial 2; Injection 1; Channel SATIN ; Date Acquired 1/15/03 12:21:46 PM

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



Fraction	Time (min)
FC	2
1	0-2
2	2-4
3	4-6
4	6-8
5	8-10
6	10-12
7	12-14
8	14-16
9	16-18

Figure 5
Data Sheet for Recombinant Human CYP19

10691-19 SUP 1/13/03

A BD Biosciences Company
GENTEST
6 Henshaw St., Woburn, MA 01801 USA
Voice: (781) 935-5115, FAX: (781) 938-8644
info@gentest.com
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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

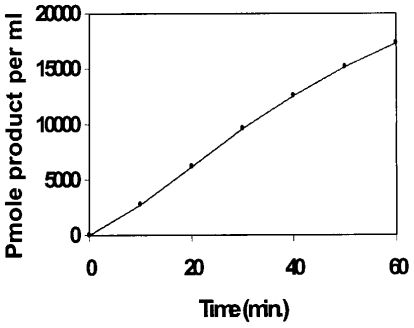
Storage Conditions..**STORE AT -80°C**
Date Released .....2000 August
Best Used by.....2003 August

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.

Time Course of Product Formation



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.

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	