LETTER REPORT ON

PHASE I
PRE-OPTIMIZATION EXPERIMENTS FOR SUBSTRATE CHARACTERIZATION FOR BOVINE MICROSOMES

EPA Contract Number 68-W-01-023
WA 2-24

May 8, 2003

PREPARED FOR

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Letter Report

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 Bovine 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 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 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

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 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 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 References

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