



8EHQ-1293-12809

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December 16, 1993



Document Processing Center (Room L-100)  
Office of Pesticides and Toxic Substances  
US Environmental Protection Agency  
401 M Street, S.W.  
Washington, DC 20460



ATTN.: TSCA 8(d) Coordinator

Dear Sir/Madam:

On behalf of the Program For Alternative Fluorocarbon Toxicity Testing, enclosed is a copy of the following report:

Testosterone Metabolism by Liver Microsomes From Rats Exposed to HFA-134A in Protocol 90612

In this study, the potential for HFC 134a to alter the metabolism of testosterone by the liver enzymes cytochrome P450 and UDP-glucuronosyl transference was studies. Had an effect been seen, this effect could have resulted in a stress on the Leydig cells in the testes, and could therefore have had an indirect role in the development of the benign tumors seen in that organ. No effects were seen. Therefore, either HFA 134a does not affect this system, or the effect is too small to be measured.

**This report is not considered confidential.**

The U.S. members of PAFT welcome this opportunity to continue to share the results of our program with the US EPA. If there are any questions, please call me (201/455-3672) at your convenience.

Sincerely,

George M. Rusch, Ph.D., DABT  
Chairman, PAFT Toxicology Committee

Enc.  
GMR:rl

- cc: R.E. Boberg
- M.R. Harris
- R. Jones
- R. Rubenstein
- H.J. Trochimowicz
- A.F. Vogelsberg
- T.W. Werkema



REC'D  
OFFICE OF POLLUTION  
PREVENTION AND TOXICS  
93 DEC 22 PM 2:15

PROTOCOL KUMC 111692:

TESTOSTERONE METABOLISM BY LIVER MICROSOMES  
FROM RATS EXPOSED TO HFA-134a IN PROTOCOL 90612

*Prepared by*

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*Sponsored by*

International Pharmaceutical Aerosol Consortium for Toxicology Testing of HFA-134a  
c/o Gardner, Carton & Douglas  
1301 K Street, N.W.,  
Suite 900, East Tower  
Washington, DC 2000  
USA

April 5, 1993

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COPY No. 16

TOX Computer Entry No.  
MA-250B-82-208 11/22/93

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Boehringer Ingelheim Pharmaceuticals, Inc.  
900 Ridgebury Road  
Ridgefield, CT 06877
- Samples:** Rat liver microsomes (n = 50) from rats dosed according to BioResearch protocol 90612 will be received from BioResearch Laboratories and kept frozen at -70°C until analysis .
- Regulatory Compliance:** The conduct of this study complied with the FDA Good Laboratory Practice Regulations, 21 CFR Part 58.
- Quality Assurance:** The protocol, critical phases of the study conduct and the final report were reviewed and audited by a Quality Assurance Unit comprising Mr. Peter Bullock, B.S. and Robin Pearce, B.S., M.S.

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**Timetable of Study Conduct and Quality Assurance**

<b>Procedure</b>	<b>Date</b>	<b>Quality Assurance</b>
Protocol Review	November 15-16, 1992	Robin Pearce & Peter Bullock
Cytochrome b <sub>5</sub> determination	November 23, 1992	Robin Pearce
Cytochrome P450 determination	November 23, 1992	Robin Pearce
Protein determination	November 24, 1992	Peter Bullock
Testosterone oxidation	December 3-6, 1993	Robin Pearce & Peter Bullock
Testosterone glucuronidation	January 15, 1993	Peter Bullock
Analysis of raw & processed data	February-April 1993	Robin Pearce & Peter Bullock
Final report review	May 1993	Robin Pearce & Peter Bullock

## **SUMMARY**

The purpose of this study was to determine if repeated exposure of rats to HFA-134a induces liver microsomal cytochrome P450, and if that exposure changes the metabolism of testosterone by rat liver microsomes. Mature male and female rats were exposed to a low (2,500 ppm), mid (10,000 ppm) or high dose (50,000 ppm) of HFA-134a by inhalation six hours/day, five days/week for four weeks at BioResearch Laboratories according to protocol 90612. Untreated rats and rats that received 10% ethanol in drinking water for 26 days served as negative and positive controls. Liver microsomes were prepared at BioResearch Laboratories and were shipped on dry ice by overnight courier to the University of Kansas Medical Center (KUMC). Each microsomal sample was analyzed to determine the specific content of cytochrome P450 and cytochrome b<sub>5</sub>, the rate of testosterone oxidation by cytochrome P450, and the rate of testosterone glucuronidation by 17-hydroxysteroid UDP-glucuronosyltransferase. Liver microsomes from rats treated with various cytochrome P450 inducers at KUMC were included as additional controls.

Treatment of male and female rats with HFA-134a caused no changes in the specific content of liver microsomal cytochrome P450 or cytochrome b<sub>5</sub>, with the exception of a 17% increase in cytochrome P450 in female rats treated with the low dose of HFA-134a. Treatment of female rats with HFA-134a caused no statistically significant changes either in the overall rate of testosterone oxidation or in the specific pathways of testosterone oxidation. In male rats, the overall rate of testosterone oxidation was decreased with increasing dose of HFA-134a. Only the highest dose of HFA-134a caused a statistically significant decrease in testosterone oxidation (19%), which was comparable to that caused by treating male rats with 10% ethanol. Treatment of male rats with the lowest dose of HFA-134a caused approximately a 28% increase in those pathways of testosterone oxidation catalyzed by the male-specific P450 enzyme CYP2C11 (i.e., 2 $\alpha$ -hydroxylation, 16 $\alpha$ -hydroxylation and 17-oxidation to androstenedione). CYP2C11 activity was unaffected by the mid dose HFA-134a, and was slightly decreased (~17%) by the highest dose of HFA-134a. Treatment of male rats with the lowest dose of HFA-134a caused approximately a 40% decrease in those pathways of testosterone oxidation catalyzed by the male-predominant P450 enzyme CYP3A2 (i.e., 1 $\beta$ -, 2 $\beta$ -, 6 $\beta$ -, 15 $\beta$ - and 18-hydroxylation and oxidation to 6-dehydrotestosterone). In terms of its effect on CYP3A2 activity, the low dose of HFA-134a mimicked the effect of 10% ethanol in drinking water. In rats treated with the mid and high dose of HFA-134a, CYP3A2 activity did not differ statistically from untreated controls, but showed a trend to decrease with dose. Those pathways of testosterone oxidation catalyzed by the female-predominant P450 enzyme CYP2A1 (i.e., 6 $\alpha$ - and 7 $\alpha$ -hydroxylation) were unaffected by treatment of male or female rats with HFA-134a. Similarly, treatment of male or female rats with HFA-134a had no effect on the glucuronidation of testosterone by liver microsomes, with the exception of a 14% increase in testosterone glucuronidation in male rats treated with the mid dose of HFA-134a.

These results suggest that exposure of rats to 2,500-50,000 ppm HFA-134a has little or no effect on the metabolism of testosterone by liver microsomal cytochrome P450 and UDP-glucuronosyltransferase.

## INTRODUCTION

The aim of this study was to determine whether treatment of mature male and female rats with HFA-134a alters the metabolism of testosterone (17 $\beta$ -hydroxy-4-androstene-3-one) by liver microsomal cytochrome P450 and UDP-glucuronosyltransferase. Mature male and female rats were exposed to a low, mid or high dose of HFA-134a (2,500, 10,000 and 50,000 ppm, respectively) by inhalation six hours/day, five days/week for four weeks at BioResearch Laboratories according to protocol 90612. Untreated rats and rats that received 10% ethanol in drinking water for 26 days served as negative and positive controls. Liver microsomes were prepared at BioResearch Laboratories and were shipped on dry ice by overnight courier to the University of Kansas Medical Center (KUMC). These samples (5 per group) were analyzed to determine whether HFA-134a is an inducer and/or suppressor of cytochrome P450 and UDP-glucuronosyltransferase in rats. Liver microsomes from rats treated with known inducers of cytochrome P450 served as additional controls. These microsomal samples were prepared previously at the University of Kansas Medical Center (KUMC) under non-GLP conditions.

## BACKGROUND INFORMATION

$\beta$ -Naphthoflavone, phenobarbital, isoniazid, dexamethasone and clofibrate represent five different classes of P450 enzyme inducers (1-6). Treatment of rats with  $\beta$ -naphthoflavone causes a marked (> 20-fold) induction of cytochromes CYP1A1 and CYP1A2 (P450c and P450d), which are structurally related enzymes (69% identical), but which catalyze different reactions. Liver microsomes from untreated rats contain low levels of CYP1A2 and virtually undetectable levels of CYP1A1. In addition to flavones, such as  $\beta$ -naphthoflavone, inducers of the CYP1A enzymes include numerous polycyclic aromatic hydrocarbons (e.g., 3-methylcholanthrene), polyhalogenated aromatic hydrocarbons (e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin or TCDD) and derivatives of indole-3-carbinol. CYP1A1 and CYP1A2 have little or no capacity to metabolize testosterone (7,8).

Treatment of rats with phenobarbital causes a marked (> 20-fold) induction of cytochromes CYP2B1 and CYP2B2 (P450b and P450e), which are structurally related enzymes (97% identical) with very similar substrate specificities (1-6). However, CYP2B1 is generally much more catalytically active than CYP2B2. Liver microsomes from untreated rats contain very low levels of CYP2B2 and virtually undetectable levels of CYP2B1. In addition to barbiturates, such as phenobarbital, inducers of the CYP2B enzymes include drugs (e.g., phenytoin, lorazepam), pesticides (e.g., DDT, chlordane), food additives (e.g., BHT and BHA) and certain polyhalogenated aromatic hydrocarbons (e.g., polychlorinated biphenyls). Although it primarily induces the CYP3A enzymes (see below), dexamethasone is also an inducer of the CYP2B enzymes. CYP2B1 and, to a much lesser extent, CYP2B2 convert testosterone to three main metabolites:

16 $\alpha$ -hydroxytestosterone, 16 $\beta$ -hydroxytestosterone and androstenedione. Of these three metabolites, only 16 $\beta$ -hydroxytestosterone is formed specifically by the CYP2B enzymes (7-9).

Treatment of rats with isoniazid causes a 2- to 5-fold induction of CYP2E1 (P450j) (1-6). In sexually mature rats, the levels of liver microsomal CYP2E1 are slightly greater (~2 fold) in female than in male rats. In addition to isoniazid, inducers of CYP2E1 include ethanol, acetone, pyrazole and uncontrolled diabetes. A common feature of CYP2E1 inducers is their ability to inhibit alcohol dehydrogenase and/or their ability to increase serum ketone bodies. CYP2E1 has little or no capacity to metabolize testosterone (5).

Treatment of male rats with dexamethasone causes a marked induction of CYP3A1 (P450p) and CYP3A2 (P450l), which are two independently regulated CYP3A enzymes with very similar structures (87%) and substrate specificities (1-6). In contrast to CYP3A1, CYP3A2 is present in liver microsomes from untreated rats, although the levels of this enzyme decline markedly after puberty in female rats. As a result of this decline in female rats, CYP3A2 is a male-specific protein in mature rats, and is inducible in mature male but not mature female rats (whereas CYP3A1 is inducible in mature male and female rats). In addition to dexamethasone, inducers of CYP3A enzymes include steroids (*e.g.*, pregnenolone-16 $\alpha$ -carbonitrile and spironolactone) and macrolide antibiotics (*e.g.*, troleandomycin and erythromycin estolate). Although it primarily induces the CYP2B enzymes (see above), phenobarbital is also an inducer of the CYP3A enzymes. CYP3A1 and CYP3A2 convert testosterone to 4 major and 4 minor metabolites. The major metabolites are 2 $\beta$ -hydroxytestosterone, 6 $\beta$ -hydroxytestosterone, 15 $\beta$ -hydroxytestosterone and 6-dehydrotestosterone (4,6-androstadien-17 $\beta$ -ol-3-one). The minor metabolites are 1 $\beta$ -hydroxytestosterone, 16 $\beta$ -hydroxytestosterone, 18-hydroxytestosterone and androstenedione. For all practical purposes, the 4 major metabolites are formed specifically by the CYP3A enzymes (10).

Treatment of rats with clofibric acid causes a marked induction (up to 20 fold) of CYP4A1, CYP4A2 and CYP4A3, which are three independently regulated CYP4A enzymes with similar substrate specificities. Some of the CYP4A enzymes are expressed in both the liver and kidney, although CYP4A levels in these tissues are sexually differentiated. In addition to clofibric acid, inducers of CYP4A enzymes include 2,4-dichlorophenoxyacetic acid (2,4-D), perfluorodecanoic acid and ciprofibrate. A feature common to all CYP4A enzyme inducers is their ability to cause proliferation of hepatic peroxisomes. Although the expression of the CYP4A enzymes is under the control of testosterone, this androgen is not a substrate for the CYP4A enzymes.

Several inducers cause a 2- to 5-fold increase the levels of CYP2A1 (P450a), the levels of which are ~2-fold higher in mature female than in male rats (1-6). CYP2A1 catalyzes the 7 $\alpha$ -hydroxylation and, to a lesser extent, the 6 $\alpha$ -hydroxylation of testosterone. The induction of cytochrome P450 in mature male rats is often associated with a 30-80% decrease in the levels of CYP2C11 (P450h), which is present only in adult male rats and is regulated by growth hormone

(5,9). CYP2C11 converts testosterone to 3 major metabolites: 2 $\alpha$ -hydroxytestosterone, 16 $\alpha$ -hydroxytestosterone and androstenedione (7,8). Of these three metabolites, only 2 $\alpha$ -hydroxytestosterone is formed specifically by the CYP2C11.

The effects of treating rats with phenobarbital or dexamethasone on the levels of liver CYP2A1, CYP2B1/2, CYP2C11 and CYP3A1/2 can be monitored by changes in specific pathways of testosterone oxidation (5-10). For all practical purposes, the rate of testosterone 2 $\alpha$ -, 7 $\alpha$ - and 16 $\beta$ -hydroxylation accurately reflects the levels of CYP2C11, CYP2A1 and CYP2B1/2, respectively. CYP2A1 also catalyzes the 6 $\alpha$ -hydroxylation of testosterone, whereas CYP2C11 and CYP2B1/2 also catalyze the 16 $\alpha$ -hydroxylation of testosterone, as well as the 17-oxidation of testosterone to androstenedione (4-androstene-3,17-dione). In mature male rats, the overall effect of phenobarbital on the rate of formation of 16 $\alpha$ -hydroxytestosterone and androstenedione depends on the extent to which CYP2C11 is suppressed *versus* the extent to which CYP2B1/2 is induced. The 2 $\beta$ -, 6 $\beta$ - and 15 $\beta$ -hydroxylation of testosterone collectively reflect the levels of CYP3A1 and/or CYP3A2 (the relative contribution of these two isozymes to testosterone oxidation depends on the source of the liver microsomes). CYP3A1/2 also oxidize testosterone to 6-dehydrotestosterone (11), although this metabolite, which absorbs maximally at 280 nm, can be difficult to quantify when testosterone metabolites are monitored at 254 nm.

In mature rats, the levels of certain P450 enzymes are sexually differentiated, i.e., they are higher in either male or female rats (9,12). Male-specific enzymes include CYP2C11 (P450h), CYP2C13 (P450g), CYP2A2 (P450m) and CYP3A2 (P450l) and CYP4A1/2. The only known female-specific P450 enzyme is CYP2C12 (P450i), although the levels of several other P450 enzymes are greater in female than male rats, including CYP2A1 (P450a), CYP2C7 (P450f) and CYP2E1 (P450j). These gender-related differences in P450 enzyme expression are due in large part to sex differences in the pattern of secretion of growth hormone, which is pulsatile in male rats and more or less continuous in females. Sex differences in growth secretion are regulated by testosterone, due to neonatal imprinting and postpubertal modulation. Castration or hypophysectomy of mature male rats causes a "feminization" of P450 enzyme expression (9,12).

In addition to inducing cytochrome P450, treatment of rats with phenobarbital induces 17 $\beta$ -hydroxysteroid glucuronosyltransferases (13). Induction of this microsomal enzyme by phenobarbital causes a 2- to 3-fold increase in the rate of glucuronidation of testosterone.

## **MATERIALS AND METHODS**

### **Chemicals**

The sources of the reagents used in this study are described in the Standard Operating Procedures, and batch or lot numbers are detailed in the Experimental Worksheets.

### **Animal treatments and preparation of liver microsomes**

Mature male and female rats were exposed to a low, mid or high dose of HFA-134a (2,500, 10,000 and 50,000 ppm, respectively) by inhalation six hours/day, five days/week for four weeks at BioResearch Laboratories according to protocol 90612. Untreated rats and rats that received 10% ethanol in drinking water for 26 days served as negative and positive controls. Liver microsomes were prepared at BioResearch Laboratories and were shipped on dry ice by overnight courier to the University of Kansas Medical Center. In addition to the liver microsomal samples, BioResearch Laboratories provided information on the concentration of protein in each sample. However, the concentration of protein was determined at University of Kansas Medical Center (see below), and these values were used throughout the remainder of the study.

Liver microsomes from 2-month-old male and female Sprague Dawley rats (Charles River Labs, Wilmington, MA) were prepared at the University of Kansas Medical Center, and were included in each enzyme assay as additional controls. These microsomal samples were prepared under non-GLP conditions according to SOP 03:00 (KUMC Notebook 4017, page 23). Liver microsomes were prepared four days after treating both male and female rats with a single intraperitoneal injection of corn oil (5 ml/kg, vehicle control) or perfluorodecanoic acid (PFDA, 40 mg/kg). Liver microsomes were also prepared from male rats 24 hours after four consecutive daily injections (intraperitoneal) of corn oil (5 ml/kg, vehicle control), saline (5 ml/kg, vehicle control), clofibrilic acid (400 mg/kg), 2,4-dichlorophenoxyacetic acid (2,4-D, 200 mg/kg), phenobarbital (80 mg/kg),  $\beta$ -naphthoflavone (100 mg/kg), isoniazid (200 mg/kg) or dexamethasone (50 mg/kg). These treatments were designed to increase the levels of various P450 enzymes, as described in *Background Information*.

### **Protein**

The concentration of protein was determined with a commercially available kit (BCA Protein Assay, Pierce Chemical Co. (Rockford, IL) as described by the manufacturer (14), according to SOP 07.00. Bovine serum albumin served as standard (supplied with the BCA kit).

### **Cytochrome $b_5$**

The concentration of cytochrome  $b_5$  was determined from the difference spectrum between NADH-reduced and oxidized microsomes, as described by Omura and Sato (15), according to SOP 08.00. Briefly, liver microsomes were diluted to ~1 mg/ml in 100 mM potassium phosphate buffer,

pH 7.4, and divided between two 1-ml cuvettes. After a baseline of equal light absorbance was obtained between 400 and 500 nm, 5  $\mu$ l of 20 mM NADH was added to the 1-ml sample cuvette, and the difference spectrum was recorded. The concentration of cytochrome  $b_5$  was determined from the absorbance difference between 410 nm (trough) and 425 nm (peak), based on an extinction coefficient of 185  $\text{mM}^{-1} \text{cm}^{-1}$ .

### Cytochrome P450

The concentration of cytochrome P450 was determined by the method of Omura and Sato (15) according to SOP 08.00. The same samples used to determine the concentration of cytochrome  $b_5$  were used to determine the concentration of cytochrome P450. After the cytochrome  $b_5$  spectrum was recorded, 5  $\mu$ l of 20 mM NADH was added to the reference cuvette (to balance the NADH previously added to the sample cuvette), and a few grains of solid sodium dithionite (1-2 mg) were added to both cuvettes (slightly more was added to the reference cuvette to give a positive absorbance reading at 400 nm). Immediately following the addition of dithionite, the contents of the sample cuvette were saturated with carbon monoxide (30-40 bubbles over a 15- to 20-sec period), and the carbon monoxide-difference spectrum of reduced microsomes was repeatedly recorded between 400 and 500 nm until two coincidental spectra were obtained (NB: In contrast to the cytochrome  $b_5$  spectrum, which develops in seconds, the cytochrome P450 spectrum takes 30-120 sec to develop fully). The concentration of cytochrome P450 was determined from the absorbance difference between 450 nm (peak) and 490 nm (isosbestic point), based on an extinction coefficient of 91  $\text{mM}^{-1} \text{cm}^{-1}$ .

### Testosterone oxidation

The pathways of testosterone oxidation catalyzed by liver microsomes were determined by high performance liquid chromatography (HPLC), essentially as described by Sonderfan *et al.* (8,16), according to SOP 16.00. Liver microsomes (0.2 mg/ml) were incubated for 4, 8 or 16 min at 37° in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4),  $\text{MgCl}_2$  (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/ml), the steroid  $5\alpha$ -reductase inhibitor, 17 $\beta$ -*N,N*-diethylcarbamoyl-4-methyl-4-aza-5 $\alpha$ -androstan-3-one (1  $\mu$ M) and testosterone (250  $\mu$ M), at the final concentrations indicated. Reactions were started by addition of the NADPH-generating system (50  $\mu$ l of 20 mM NADP, 100 mM glucose-6-phosphate and 20 Units/ml of glucose-6-phosphate dehydrogenase). Reactions were stopped by addition of 6 ml dichloromethane. Each sample was spiked with 3 nmol of 11  $\beta$ -hydroxytestosterone (in 100  $\mu$ l dichloromethane) and vigorously mixed on a batch vortexer (3 x 1 min). After the two phases were separated by low-speed centrifugation (2000 g for 10 min), the aqueous (upper) phase was aspirated and discarded. An aliquot (4 ml) of the organic phase was transferred to a culture tube (12 x 75 mm) and evaporated in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY). The residue was redissolved in 200  $\mu$ l solvent A (see below), and a 50- $\mu$ l aliquot was analyzed by HPLC.

Testosterone, androstenedione, 6-dehydrotestosterone, and 14 monohydroxylated testosterone isomers were resolved on a Supelcosil reverse-phase C<sub>18</sub> column (Supelco, Bellefonte, PA) with a Shimadzu LC-6A binary gradient HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The method was similar to that described by Sonderfan *et al.* (8), with slight changes in the mobile phase A and the gradient system. The ratio of water-methanol-acetonitrile (v/v/v) was 64-35-1 for mobile phase A, and 18-80-2 for mobile phase B. Testosterone and its metabolites were eluted from the column with 100% A for 10 min followed by a linear gradient from 100% A to 45% B from 10 to 28 min. To wash the column, the concentration of B was increased linearly from 45% to 100% from 28 to 29 min, and maintained at 100% B for 2 min. Between 31 and 32 min the concentration of B was decreased linearly from 100% to 0%. The column was re-equilibrated with 100% A from 32 to 34 min. The total flow rate was 2.0 ml/min and the column temperature was 40°. Total analysis time was 37 min per sample. All other conditions (e.g., guard column, auto-injection etc.) were the same as described previously (8). The slight changes in mobile phase A and the gradient system enabled 6 $\beta$ - and 15 $\alpha$ -hydroxytestosterone to be resolved, whereas they co-eluted in the original system described by Sonderfan *et al.* (8). Testosterone and its potential metabolites were monitored at 254 nm with a variable wavelength uv detector (Shimadzu SPD-6A). Metabolites were determined by comparison of their peak area (integrated by a Shimadzu C-R3A recording data processor) with that of each authentic standard. The overall rate of testosterone oxidation was calculated as the sum of the rates of formation of the individual metabolites (i.e., all isomeric hydroxytestosterones + androstenedione + 6-dehydrotestosterone).

All microsomal samples were incubated with testosterone and NADPH for 8 min at a final protein concentration of 0.2 mg/ml. However, to ensure that product formation was proportional to incubation time, selected microsomal samples were also incubated with testosterone for 4 and 16 min (i.e., half and twice the usual incubation period). Similarly, to ensure that product formation was proportional to the concentration of microsomal protein, selected samples were also incubated at a final protein concentration of 0.1 and 0.4 mg/ml (i.e., half and twice the usual protein concentration). The samples selected for these studies were liver microsomes from control rats and from rats treated with phenobarbital or dexamethasone, two cytochrome P450 inducers that increase the rate of testosterone oxidation.

#### Testosterone glucuronidation

The rate of testosterone glucuronidation by liver microsomes was determined by a solvent partition method, essentially as described by Jacobson *et al.* (13) and Arlotto *et al.* (17). Microsomal protein (5 mg/ml) was solubilized for 15 min with an equal volume of 10 mM CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane) in 250 mM sucrose and 400 mM Tris.HCl (pH 8.0 at room temperature) prior to its addition (in 50  $\mu$ l aliquots) to incubation mixtures. The

final incubation volume was 500  $\mu$ l, and contained Tris.HCl buffer (200 mM, pH 8.0), MgCl<sub>2</sub> (10 mM), EDTA (1 mM), D-saccharic acid-1,4-lactone (0.1 mM), UDP-glucuronic acid (4 mM), CHAPS (0.5 mM), [<sup>14</sup>C]-testosterone (134  $\mu$ M, 0.5  $\mu$ Ci/ml) and solubilized microsomal protein (0.25 mg/ml), at the final concentrations indicated. Reactions were started with UDP-glucuronic acid and stopped after a 15-min incubation at 37°C with 6 ml of dichloromethane, followed by 1.1 ml of water (to increase the volume of the aqueous phase to 1.6 ml). After vigorous mixing on a batch vortex mixer (3 x 1 min), the aqueous and organic phase were separated by centrifugation (2,000 g for 10 min). The aqueous phase was extracted a second time with 6 ml of dichloromethane. An aliquot (400  $\mu$ l  $\approx$  25%) of the aqueous (upper) phase (containing testosterone glucuronide) was mixed with 5 ml of BioSafe Biodegradable Counting Cocktail (Research Products International Corporation, Mount Prospect, IL), and the amount of radioactivity was determined with a Packard 2000 CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL). Zero-time incubations served as blanks.

Each 500- $\mu$ l incubation consisted of 50  $\mu$ l of microsomes, 50  $\mu$ l of UDP-glucuronic acid, and 400  $\mu$ l of substrate solution (Tris.HCl, MgCl<sub>2</sub>, EDTA, D-saccharic acid-1,4-lactone, CHAPS and [<sup>14</sup>C]-testosterone). Aliquots of this substrate solution (100  $\mu$ l = 25% of the amount added to each incubation) were also analyzed by liquid scintillation counting, exactly as described above for the aqueous phase. The amount of radioactivity in 25% of the aqueous phase (400  $\mu$ l) equals the amount of radioactivity in 25% of the substrate solution (100  $\mu$ l) only if 100% of the substrate (testosterone) is converted to water-soluble metabolites (testosterone glucuronide). Therefore, the ratio of the radioactivity in the aqueous phase to the radioactivity in the substrate solution represents the fraction of substrate converted to metabolites. This ratio was used to determine the overall rate of testosterone glucuronidation, based on the following equation:

Rate of testosterone glucuronidation (nmol/mg protein/min)

$$= \frac{\text{dpm in 400 } \mu\text{l of aqueous phase}}{\text{dpm in 100 } \mu\text{l of substrate solution}} \times \frac{67 \text{ nmol testosterone}}{\text{incubation}} \times \frac{1}{\text{mg protein/incubation}} \times \frac{1}{\text{time}}$$

All microsomal samples were incubated with testosterone and UDP-glucuronic acid for 15 min at a final protein concentration of 0.25 mg/ml. However, to ensure that product formation was proportional to incubation time, liver microsomes from phenobarbital-treated rats were also incubated with testosterone for 7.5, 22.5 and 30 min (i.e., half, one-and-a-half and twice the usual incubation period). Similarly, to ensure that product formation was proportional to the concentration of microsomal protein, liver microsomes from phenobarbital-treated rats were also incubated at a final protein concentration of 0.125, 0.375 and 0.5 mg/ml (i.e., half, one-and-a-half and twice the usual protein concentration). Liver microsomes from phenobarbital-treated rats

were selected for this analysis because phenobarbital treatment increases the rate of testosterone glucuronidation.

Statistical analysis

Data were analyzed for statistically significant differences between controls and the multiple treatment groups by Dunnett's two-way analysis of variance at the 5% level of significance ( $\alpha = 0.05$ ) (18). The microsomal samples prepared at KUMC were from pooled livers, which precluded a statistical evaluation of the results obtained with these samples. These samples were included in measurements of enzyme activity as additional controls.

## **RESULTS AND DISCUSSION**

### **Samples received from BioResearch Laboratories**

The samples of rat liver microsomes prepared at BioResearch Laboratories were received in good condition. None of the samples had thawed, and none of the tubes was lost, broken or uncapped. The samples were stored at  $-80^{\circ}\text{C}$  until used, during which time the freezer temperature did not increase above the *Warm Set-Point* of  $-60^{\circ}\text{C}$ .

### **Protein concentration**

The concentration of protein in the microsomal samples was determined at BioResearch Laboratories and at the University of Kansas Medical Center. There was good agreement between the two data sets ( $r = 0.858$ ). Although enzymatic rates were calculated based on the protein concentration values determined at the University of Kansas Medical Center, the results of this study would be essentially the same if the enzymatic rates had been calculated on the protein values determined at BioResearch Laboratories.

### **Cytochrome P450 and cytochrome $b_5$**

Treatment of male and female rats with HFA-134a caused no changes in the specific content of liver microsomal cytochrome P450 or cytochrome  $b_5$ , with the exception of 17% increase in cytochrome P450 in female rats treated with the low dose of HFA-134a (Table 1a). The lack of change in the concentration of total cytochrome P450 does not preclude the possibility that treatment of rats with HFA-134a causes an increase or decrease in the concentration of specific P450 enzymes. This principle is illustrated by the results in Table 1a, which shows that 10% ethanol in drinking water, which significantly increases the levels of CYP2E1, caused no statistically significant changes in the concentration of total cytochrome P450.

In the spectrophotometric measurement of cytochrome P450, which involves chemical reduction of cytochrome P450 and complexation with carbon monoxide, none of the liver microsomal samples contained a chromophore that produced a distinct absorbance peak at 420 nm. This indicates that the liver microsomal samples were not excessively contaminated with hemoglobin. It also indicates that treatment of rats with HFA-134a did not convert cytochrome P450 to its catalytically inactive form, cytochrome P420 (15). In terms of the concentration of cytochrome P450 and cytochrome  $b_5$ , liver microsomes from the untreated control male and female rats were similar to the corresponding microsomal samples prepared at the University of Kansas Medical Center (Table 1b).

### **Testosterone oxidation**

The effects of treating male and female rats with HFA-134a on the overall rate of testosterone oxidation by liver microsomes are shown in Fig. 1. Exposure of female rats to HFA-134a or ethanol caused no statistically significant changes in the overall rate of testosterone oxidation,

which represents the sum of the oxidative metabolites identified by HPLC. In male rats, the overall rate of testosterone oxidation was decreased with increasing dose of HFA-134a. Only the highest dose of HFA-134a caused a statistically significant decrease in testosterone oxidation (19%), which was comparable to that caused by giving male rats 10% ethanol in drinking (Fig. 1). In terms of the overall rate of testosterone oxidation by liver microsomes, male rats were considerably more active than female rats, as previously reported (7-9,16).

The effects of treating male rats with HFA-134a on individual pathways of testosterone oxidation are shown in Table 2. Those pathways of testosterone oxidation catalyzed by the female-predominant P450 enzyme CYP2A1 (i.e., 6 $\alpha$ - and 7 $\alpha$ -hydroxylation) were unaffected by treatment of male rats with HFA-134a. Treatment of male rats with the lowest dose of HFA-134a caused a ~28% increase in those pathways of testosterone oxidation catalyzed by the male-specific P450 enzyme CYP2C11 (i.e., 2 $\alpha$ -hydroxylation, 16 $\alpha$ -hydroxylation and 17-oxidation to androstenedione). CYP2C11 activity was unaffected by the mid dose HFA-134a, and was slightly suppressed (~17%) by the highest dose of HFA-134a. Treatment of male rats with the lowest dose of HFA-134a caused a ~40% decrease in those pathways of testosterone oxidation catalyzed by the male-predominant P450 enzyme CYP3A2 (i.e., 1 $\beta$ -, 2 $\beta$ -, 6 $\beta$ -, 15 $\beta$ - and 18-hydroxylation and oxidation to 6-dehydrotestosterone). In terms of its effect on CYP3A2 activity, the low dose of HFA-134a mimicked the effect of treating rats with 10% ethanol. In rats treated with the mid and high dose of HFA-134a, CYP3A2 activity did not differ statistically from untreated controls, but showed a trend to decrease with increasing dose.

In Fig. 1, treatment of male rats with the high dose of HFA-134a was shown to cause a statistically significant decrease (~19%) in the overall rate of testosterone oxidation. This suppressive effect can be attributed to a ~17% decrease in CYP2C11 activity (which was statistically significant) and a ~21% decrease in CYP3A2 activity (which was not statistically significant).

Treatment of female rats with HFA-134a caused no statistically significant changes in the individual pathways of testosterone oxidation, as shown in Table 3. Compared with those from male rats, liver microsomes from female rats catalyzed low rates of testosterone 2 $\alpha$ -hydroxylation, 16 $\alpha$ -hydroxylation and 17-oxidation to androstenedione (which are catalyzed by the male-specific enzyme CYP2C11). Similarly, liver microsomes from female rats also supported low rates of testosterone 1 $\beta$ -, 2 $\beta$ -, 6 $\beta$ -, 15 $\beta$ - and 18-hydroxylation and oxidation to 6-dehydrotestosterone (which are catalyzed by the male-specific enzyme CYP3A2). In contrast, liver microsomes from female rats were more active than those from male rats at converting testosterone to 6 $\alpha$ - and 7 $\alpha$ -hydroxytestosterone (which is catalyzed by the female-predominant enzyme CYP2A1).

The effects of treating rats with HFA-134a on liver microsomal testosterone oxidation was determined under conditions where metabolite formation was directly proportional to incubation

time and the concentration of liver microsomes. The absolute and relative rates of testosterone oxidation by liver microsomes from untreated control male and female rats were similar to those determined with liver microsomes prepared from control rats at the University of Kansas Medical Center (Table 4). Table 4 also illustrates the effects of treating male rats with various P450 enzyme inducers on liver microsomal testosterone oxidation. Like HFA-134a and ethanol, several chemicals suppressed the activity of CYP2C11 and/or CYP3A2, including the CYP4A enzyme inducers and peroxisome proliferators (perfluorodecanoic acid, clofibric acid and 2,4-dichlorophenoxyacetic acid), the CYP1A enzyme inducer  $\beta$ -naphthoflavone, and the CYP2E1 inducer isoniazid. Although they suppressed CYP2C11 activity, phenobarbital and dexamethasone increased CYP3A activity (due to their ability to induce CYP3A1 and CYP3A2). Treatment of rats with phenobarbital also caused a marked (18-fold) increase in the 16 $\beta$ -hydroxylation of testosterone, due to the marked induction of CYP2B1 and CYP2B2. The results presented in Table 4 are comparable to those published previously (7-9).

#### Testosterone glucuronidation

As shown in Table 5, treatment of male or female rats with HFA-134a had no effect on the glucuronidation of testosterone by liver microsomes, with the exception of a 14% increase in testosterone glucuronidation in male rats treated with the mid dose of HFA-134a. The effects of treating rats with HFA-134a on liver microsomal testosterone glucuronidation was determined under conditions where metabolite formation was directly proportional to incubation time and the concentration of liver microsomes. The absolute and relative rates of testosterone glucuronidation by liver microsomes from control male and female rats were similar to those determined with liver microsomes prepared from control rats at the University of Kansas Medical Center (Table 6). The highest rate of testosterone glucuronidation was catalyzed by liver microsomes from rats treated with phenobarbital, which has long been recognized as an inducer of 17 $\beta$ -hydroxysteroid glucuronosyltransferase (13).

#### Summary

The results of this study suggest that exposure of rats to 2,500-50,000 ppm HFA-134a has little or no effect on the metabolism of testosterone by liver microsomal cytochrome P450 and UDP-glucuronosyltransferase. In male rats, HFA-134a caused a dose-related decrease (up to 19%) in the overall rate of testosterone oxidation. At the high dose of HFA-134a, this suppressive effect was due to a decrease in CYP2C11 activity (-17%, significant) and CYP3A2 activity (-21%, not significant). The magnitude of the effects of the highest concentration of HFA-134a on testosterone metabolism were comparable to those caused by giving male rats 10% ethanol in drinking water, and were considerably less than those caused by treating rats with known inducers of cytochrome P450 and UDP-glucuronosyltransferase. Treatment of female rats with HFA-134a caused no statistically significant changes in the overall rate of testosterone oxidation, in the specific pathways of testosterone oxidation or in the rate of testosterone glucuronidation.

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**TABLE 1A**  
**CYTOCHROMES P450 AND b<sub>5</sub> IN LIVER MICROSOMES**  
**FROM RATS TREATED WITH HFA-134a**

<b>Sample/ Treatment<sup>a</sup></b>	<b>Cytochrome P450<sup>b</sup> (nmol/mg protein)</b>	<b>Cytochrome b<sub>5</sub><sup>b</sup> (nmol/mg protein)</b>
<b>MALE</b>		
1016-1020 (Control)	1.19 ± 0.06	0.57 ± 0.04
2013-2017 (2,500 ppm) <sup>c</sup>	1.16 ± 0.100	0.60 ± 0.05
3013-3017 (10,000 ppm) <sup>c</sup>	1.14 ± 0.050	0.60 ± 0.06
4088-4092 (50,000 ppm) <sup>c</sup>	1.22 ± 0.110	0.55 ± 0.04
5001-5005 (10% Ethanol) <sup>d</sup>	1.30 ± 0.040	0.59 ± 0.04
<b>FEMALE</b>		
1516-1520 (Control)	0.884 ± 0.032	0.53 ± 0.02
2513-2517 (2,500 ppm) <sup>c</sup>	1.03 ± 0.035*	0.60 ± 0.04
3513-3517 (10,000 ppm) <sup>c</sup>	0.845 ± 0.041	0.51 ± 0.04
4588-4592 (50,000 ppm) <sup>c</sup>	0.890 ± 0.054	0.50 ± 0.04
5501-5505 (10% Ethanol) <sup>d</sup>	0.936 ± 0.031	0.55 ± 0.03

<sup>a</sup>Rats (n=5/group) were treated with HFA-134a (6 hours/day, 5 days /week for 4 weeks) or 10% ethanol (positive control) or they were allowed to breathe normally (air control).

<sup>b</sup>Values represent means ± standard deviations of 5 determinations/group.

<sup>c</sup>Airborne concentration of HFA-134a.

<sup>d</sup>10% ethanol in drinking water *ad libitum* for 26 days.

\*Significantly different from corresponding control according to Dunnett's test at the 5% level of significance ( $\alpha < 0.05$ ).

**TABLE 1B**  
**CYTOCHROMES P450 AND b<sub>5</sub> IN POOLED LIVER**  
**MICROSOMES FROM RATS TREATED AT KUMC<sup>a</sup>**

<b>Sample/Treatment</b>	<b>Cytochrome P450 (nmol/mg protein)</b>	<b>Cytochrome b<sub>5</sub> (nmol/mg protein)</b>
<b>FEMALE</b>		
Com oil (1 day)	1.02	0.65
Perfluorodecanoic acid	0.91	0.57
<b>MALE</b>		
Com oil (1 day)	1.46	0.76
Perfluorodecanoic acid	2.25	0.66
Clofibric acid	1.48	0.62
2,4-Dichlorophenoxyacetic acid	1.37	0.73
Saline (4 days)	1.37	0.66
Phenobarbital	3.60	0.78
β-Naphthoflavone	2.00	0.78
Isoniazid	1.00	0.77
Dexamethasone	1.48	0.47
Com oil (4 days)	1.48	0.68

<sup>a</sup>Rats were treated at the University of Kansas Medical Center as described in notebook reference 4017-36. Each microsomal sample is a pool of 4 individual samples.

TABLE 2. TESTOSTERONE OXIDATION BY LIVER MICROSOMES FROM MALE RATS TREATED WITH HFA-134a

TREATMENT <sup>a</sup>	RATE OF TESTOSTERONE OXIDATION (mean ± SD, n = 5) <sup>b</sup> (pmol metabolite formed/mg protein/min)												
	1αβ	2α	2β	6α	6β	7α	15α	15β	16α	16β	18/12α	ASD	DHT
1016-1020 (Air control)	175 (33)	2590 (319)	432 (56)	24 (3)	5080 (666)	360 (93)	133 (16)	166 (10)	3520 (406)	141 (19)	111 (20)	1380 (108)	1120 (165)
2013-2017 (2,500 ppm) <sup>c</sup>	96* (23)	3310* (311)	284* (56)	20 (4)	3140* (632)	322 (65)	85 (29)	108* (18)	4510* (409)	110* (12)	70* (15)	1740* (144)	691* (158)
3013-3017 (10,000 ppm) <sup>c</sup>	148 (21)	2350 (216)	383 (38)	22 (1)	4550 (477)	316 (44)	111 (43)	145 (21)	3180 (309)	104* (12)	97 (13)	1290 (108)	947 (82)
4088-4092 (50,000 ppm) <sup>c</sup>	131 (13)	1991* (160)	346 (29)	23 (4)	4120 (383)	306 (75)	50* (17)	124* (8)	2630* (202)	98* (13)	84 (9)	1100* (55)	915 (101)
5001-5005 (10% ethanol) <sup>d</sup>	93* (13)	2350 (186)	263* (30)	27 (3)	3210* (443)	403 (56)	128 (34)	113* (9)	3190* (236)	121 (10)	70 (9)*	1350 (103)	712* (86)

<sup>a</sup>Rats (n = 5/group) were treated with HFA-134a or 10% ethanol (positive control) or they were allowed to breathe normally (air control).

<sup>b</sup>With the exception of androstenedione (ASD) and 6-dehydrotestosterone (DHT), the abbreviations denote the hydroxytestosterone metabolite formed, e.g., 7α denotes 7α-hydroxytestosterone. This HPLC system does not resolve 1α-hydroxytestosterone from 1β-hydroxytestosterone nor does it separate 18-hydroxytestosterone from 12α-hydroxytestosterone.

<sup>c</sup>Airborne concentrations of HFA-134a (6 hours/day, 5 days/week for 4 weeks).

<sup>d</sup>10% Ethanol in drinking water *ad libitum* for 28 days

\*Significantly different from corresponding control according to Dunnett's test at the 5% level of significance (α < 0.05).

TABLE 3. TESTOSTERONE OXIDATION BY LIVER MICROSOMES FROM FEMALE RATS TREATED WITH HFA-134a

TREATMENT <sup>a</sup>	RATE OF TESTOSTERONE OXIDATION (mean ± SD, n = 5) <sup>b</sup> (pmol metabolite formed/mg protein/min)													DHT
	1α/β	2α	2β	6α	6β	7α	15α	15β	16α	16β	18/12α	ASD		
1516-1520 (Air control)	<20	<20	41 (7)	45 (4)	178 (28)	1290 (86)	<20	28 (6)	61 (14)	70 (8)	<20	316 (110)	<20	
2513-2517 (2,500 ppm) <sup>c</sup>	<20	<20	41 (18)	52 (6)	159 (59)	1480 (196)	<20	33 (12)	83 (28)	89 (15)	<20	356 (40)	<20	
3513-3517 (10,000 ppm) <sup>c</sup>	<20	<20	46 (9)	39 (4)	193 (32)	1140 (78)	<20	34 (3)	57 (13)	60 (8)	<20	258 (21)	<20	
4588-4592 (50,000 ppm) <sup>c</sup>	<20	<20	41 (12)	44 (4)	192 (51)	1270 (126)	<20	32 (10)	63 (19)	72 (20)	<20	298 (42)	<20	
5501-5005 (10% ethanol) <sup>d</sup>	<20	<20	59 (10)	46 (6)	270* (31)	1260 (212)	115* (14)	42 (5)	51 (2)	63 (8)	<20	277 (29)	138 (28)	

<sup>a</sup>Rats (n = 5/group) were treated with HFA-134a or 10% ethanol (positive control) or they were allowed to breathe normally (air control).

<sup>b</sup>With the exception of androstenedione (ASD) and 6-dehydrotestosterone (DHT), the abbreviations denote the hydroxytestosterone metabolite formed, e.g., 7α denotes 7α-hydroxytestosterone. This HPLC system does not resolve 1α-hydroxytestosterone from 1β-hydroxytestosterone nor does it separate 18-hydroxytestosterone from 12α-hydroxytestosterone.

<sup>c</sup>Airborne concentrations of HFA-134a (6 hours/day, 5 days/week for 4 weeks).

<sup>d</sup>10% Ethanol in drinking water *ad libitum* for 26 days.

\*Significantly different from corresponding control according to Dunnett's test at the 5% level of significance.

TABLE 4. TESTOSTERONE OXIDATION BY LIVER MICROSOMES FROM POOLED RAT LIVER MICROSOMES PREPARED AT THE UNIVERSITY OF KANSAS MEDICAL CENTER

SEX/TREATMENT	TESTOSTERONE OXIDATION <sup>a</sup>											ASD	DHT
	(pmol metabolite formed/mg protein/min)												
	1 $\alpha$ / $\beta$	2 $\alpha$	2 $\beta$	6 $\alpha$	6 $\beta$	7 $\alpha$	15 $\alpha$	15 $\beta$	16 $\alpha$	16 $\beta$	18/12 $\alpha$		
<b>FEMALE/</b>													
Com oil (1 day)	<20	<20	49	54	226	1440	92	41	236	175	<20	546	<20
Perfluorodecanoic acid	<20	18	108	49	512	1240	143	85	347	278	28	662	192
<b>MALE/</b>													
Com oil (1 day)	76	3680	232	24	2850	370	140	89	5270	216	64	1870	619
Perfluorodecanoic acid	120	2400	361	30	4490	525	185	109	3430	27	95	1610	1070
Clofibrac acid	139	1450	320	34	4060	639	187	112	2360	461	96	1240	1020
2,4-Dichlorophenoxy-acetic acid	54	1680	169	25	2030	329	118	69	2510	171	45	1100	503
Saline (4 days)	57	3380	195	26	2520	343	149	65	4780	197	59	1880	557
Phenobarbital	287	1370	571	61	6420	862	331	234	4970	3570	235	2530	1500
$\beta$ -Naphthoflavone	<20	237	75	32	1070	812	101	23	356	36	.33	284	302
Isoniazid	51	776	163	27	1820	424	154	114	1290	202	40	823	416
Dexamethasone	307	1160	919	44	7490	636	273	548	1930	270	266	1000	1810
Com oil (4 days)	66	4810	233	28	2650	370	164	101	6960	265	65	2380	552

<sup>a</sup> With the exception of androstenedione (ASD) and 6-dehydrotestosterone (DHT), the abbreviations denote the hydroxytestosterone metabolite formed, e.g., 7 $\alpha$  denotes 7 $\alpha$ -hydroxytestosterone. This HPLC system does not resolve 1 $\alpha$ -hydroxytestosterone from 1 $\beta$ -hydroxytestosterone nor does it separate 18-hydroxytestosterone from 12 $\alpha$ -hydroxytestosterone.

**TABLE 5. TESTOSTERONE GLUCURONIDATION BY LIVER MICROSOMES FROM MALE AND FEMALE RATS TREATED WITH HFA-134a**

TREATMENT	TESTOSTERONE GLUCURONIDATION (nmol/mg protein/min)	
	MALE RATS	FEMALE RATS†
Air Control	12.1 ± 1.1	8.24 ± 0.49
HFA-134a 2,500 ppm	11.1 ± 0.5	9.28 ± 0.36
HFA-134a 10,000 ppm	13.8 ± 1.6*	8.06 ± 1.01
HFA-134a 50,000 ppm	12.0 ± 0.9	9.38 ± 0.39
Ethanol (10%)	13.4 ± 0.4	8.74 ± 0.41

\*Significantly different from corresponding control according to Dunnett's test at the 5% level of significance ( $\alpha < 0.05$ ).

†For each treatment group (including controls), the rates determined with liver microsomes from female rats were significantly lower than the corresponding values for male rats according to Dunnett's test ( $\alpha < 0.05$ )

**TABLE 6. TESTOSTERONE GLUCURONIDATION BY POOLED RAT LIVER  
 MICROSOMES PREPARED AT THE UNIVERSITY OF KANSAS MEDICAL CENTER**

TREATMENT	TESTOSTERONE GLUCURONIDATION (nmol/mg protein/min)	
	MALE RATS	FEMALE RATS
Control (Corn oil, 1 day)	14.5	11.6
PFDA	11.2	9.1
Clofibric acid	12.5	ND
2,4-D	12.4	ND
Control (saline, 4 days)	15.5	ND
Phenobarbital	23.9	ND
$\beta$ -Naphthoflavone	10.4	ND
Isoniazid	11.6	ND
Dexamethasone	13.2	ND
Control (Corn oil, 4 days)	12.7	ND

PFDA: Perfluorodecanoic acid

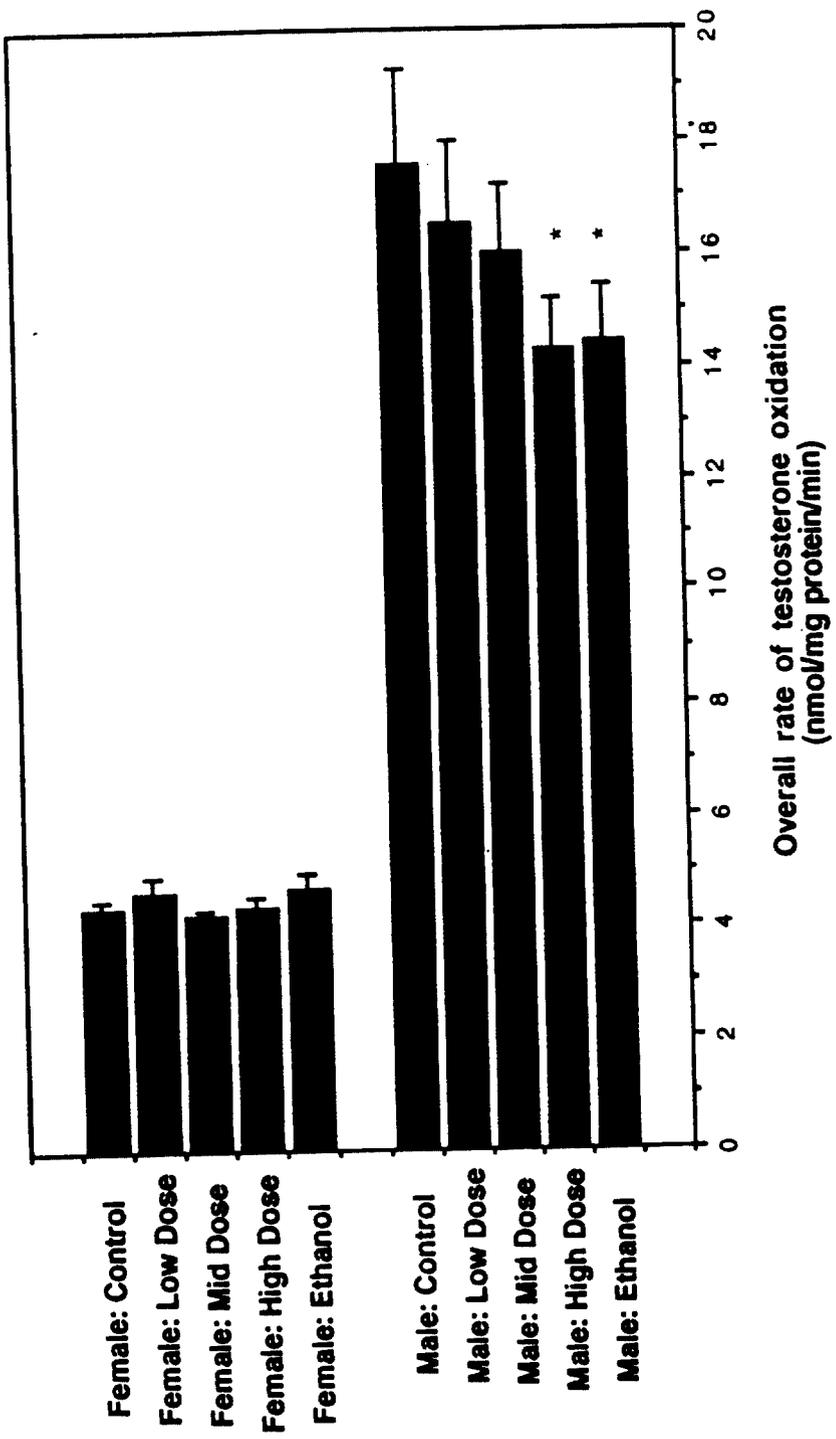
2,4-D: 2,4-Dichlorophenoxyacetic acid

**Fig. 1. Overall rate of testosterone oxidation by liver microsomes from rats treated with HFA-134a or ethanol**

Rats (n = 5/group) were treated with 2,500, 10,000 or 50,000 ppm HFA-134a (low, mid and high dose) or 10% ethanol (positive control), or they were allowed to breathe normally (control). The overall rate of testosterone oxidation was calculated as the sum of the rates of the individual pathways of testosterone oxidation, data for which are summarized in Table 2 (for male rats) and Table 3 (for female rats). Values are mean  $\pm$  standard deviation of 5 determinations/group.

\*Significantly different from corresponding control according to Dunnett's test at the 5% level of significance ( $\alpha < 0.05$ )

Overall rate of testosterone oxidation by liver microsomes from rats treated with HFA-134a or ethanol (mean + std dev)



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**PROTOCOL**

**KUMC 111692**

**TESTOSTERONE METABOLISM BY LIVER MICROSOMES  
FROM RATS EXPOSED TO HFA-134a IN PROTOCOL 90612**

**Department of Pharmacology, Toxicology & Therapeutics  
University of Kansas Medical School  
Kansas City, Kansas**

**This study conduct will comply with current Good Laboratory Practices as promulgated by the United States Food and Drug Administration.**

**Purpose:** To determine if repeated exposure of rats to HFA-134a induces liver microsomal cytochrome P450, and if that exposure changes the metabolism of testosterone by rat liver microsomes.

**Project No.:** KUMC 111692

**Protocol Date:** November 16, 1992

**Sponsor:** International Pharmaceutical Aerosol Consortium for Toxicology Testing of HFA-134a (IPACT-I)  
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Denise Greenway, B.S.

**Samples:** Rat liver microsomes (n = 50) from rats dosed according to BioResearch protocol 90612 will be received from BioResearch Laboratories and kept frozen at -70°C until analysis .

**Methods:** Rat liver microsomes (n = 50) will be analyzed to determine the concentration of protein, cytochrome b<sub>5</sub> and cytochrome P-450, and the rate of testosterone glucuronidation and testosterone oxidation. Experimental protocols for these methods are outlined below.

## Outline of experimental protocols

### Protein

The concentration of protein will be determined with a commercially available kit (BCA Protein Assay, Pierce Chemical Co., Rockford, IL) as described by the manufacturer. Bovine serum albumin will serve as standard (supplied with the BCA kit).

### Cytochrome $b_5$

The concentration of cytochrome  $b_5$  will be determined from the difference spectrum between NADH-reduced and oxidized microsomes, as described by Omura and Sato (1). Liver microsomes will be diluted to ~1 mg/ml in 100 mM potassium phosphate buffer, pH 7.4, and divided between two 1-ml cuvettes. After a baseline of equal light absorbance is obtained between 400 and 500 nm, 5  $\mu$ l of 20 mM NADH will be added to the 1-ml sample cuvette, and the difference spectrum will be recorded. The concentration of cytochrome  $b_5$  will be determined from the absorbance difference between 410 nm (trough) and 425 nm (peak), based on an extinction coefficient of 185  $\text{mM}^{-1} \text{cm}^{-1}$ .

### Cytochrome P450

The concentration of cytochrome P450 will be determined by the method of Omura and Sato (1). The same samples used to determine the concentration of cytochrome  $b_5$  will also be used to determine the concentration of cytochrome P450. After the cytochrome  $b_5$  spectrum is recorded, 5  $\mu$ l of 20 mM NADH will be added to the reference cuvette (to balance the NADH previously added to the sample cuvette), and a few grains of solid sodium dithionite (1-2 mg) will be added to both cuvettes (slightly more will be added to the reference cuvette to give a positive absorbance reading at 400 nm). Immediately following the addition of dithionite, the contents of the sample cuvette will be saturated with carbon monoxide (30-40 bubbles over a 15- to 20-sec period), and the carbon monoxide-difference spectrum of reduced microsomes will be repeatedly recorded between 400 and 500 nm until two coincidental spectra are obtained. The concentration of cytochrome P450 will be determined from the absorbance difference between 450 nm (peak) and 490 nm (isosbestic point), based on an extinction coefficient of 91  $\text{mM}^{-1} \text{cm}^{-1}$ .

### Testosterone glucuronidation

The rate of testosterone glucuronidation by liver microsomes will be determined by a solvent partition method, essentially as described by Jacobson *et al.* (2) and Ariotto *et al.* (3). Microsomal protein (5 mg/ml) will be solubilized for 15 min with an equal volume of 10 mM CHAPS (3-[[3-cholanidopropyl]-dimethyl-ammonio]-1-propane) in 250 mM sucrose and 400 mM Tris.HCl (pH 8.0 at room temperature) prior to its addition (in 50  $\mu$ l aliquots) to incubation mixtures. The final incubation volume will be 500  $\mu$ l, and will contain Tris.HCl buffer (200 mM, pH 8.0),  $\text{MgCl}_2$  (10 mM), EDTA (1 mM), D-saccharic acid-1,4-lactone (1.25 mM), UDP-glucuronic acid (4 mM), CHAPS (0.5 mM), [ $^{14}\text{C}$ ]-testosterone (130  $\mu\text{M}$ , 0.5  $\mu\text{Ci/ml}$ ) and solubilized microsomal protein (0.25 mg/ml), at the final concentrations indicated. Reactions will be started with UDP-glucuronic acid and stopped after a 15-min incubation at 37°C with 6 ml of dichloromethane, followed by 1.1 ml of water (to increase the volume of the aqueous phase to 1.6 ml). After vigorous mixing on a batch vortex mixer (3 x 1 min), the aqueous and organic phase will be separated by centrifug-

ation (2,000 g for 10 min). An aliquot (400  $\mu$ l  $\approx$  25%) of the aqueous (upper) phase (containing testosterone glucuronide) will be mixed with 5 ml of BioSafe Biodegradable Counting Cocktail (Research Products International Corporation, Mount Prospect, IL), and the amount of radioactivity will be determined with a Packard 2000 CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL). Zero-time incubations will serve as blanks.

Each 500- $\mu$ l incubation will consist of 50  $\mu$ l of microsomes, 50  $\mu$ l of UDP-glucuronic acid, and 400  $\mu$ l of substrate solution (Tris.HCl, MgCl<sub>2</sub>, EDTA, D-saccharic acid-1,4-lactone, CHAPS and [<sup>14</sup>C]-testosterone). Aliquots of this substrate solution (100  $\mu$ l  $\approx$  25% of the amount added to each incubation) will also be analyzed by scintillation spectrometry. The amount of radioactivity in 25% of the aqueous phase (400  $\mu$ l) will equal the amount of radioactivity in 25% of the substrate solution (100  $\mu$ l) only if 100% of the substrate (testosterone) is converted to water-soluble metabolites (testosterone glucuronide). Therefore, the ratio of the radioactivity in the aqueous phase to the radioactivity in the substrate solution represents the fraction of substrate converted to metabolites. This ratio can be used to determine the overall rate of testosterone glucuronidation, based on the following equation:

Rate of testosterone glucuronidation (nmol/mg protein/min)

$$= \frac{\text{dpm in 400 } \mu\text{l of aqueous phase}}{\text{dpm in 100 } \mu\text{l of substrate solution}} \times \frac{95 \text{ nmol testosterone}}{\text{incubation}} \times \frac{1}{\text{mg protein/incubation}} \times \frac{1}{\text{time}}$$

#### Testosterone oxidation

The pathways of testosterone oxidation catalyzed by liver microsomes will be determined by reverse-phase HPLC, essentially as described by Wood *et al.* (4) and Sonderfan *et al.* (5,6). Liver microsomes (0.2 mg) will be incubated at 37°C in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl<sub>2</sub> (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/ml), testosterone (250  $\mu$ M) and the steroid 5 $\alpha$ -reductase inhibitor, 4-MA (1  $\mu$ M), at the final concentrations indicated. Testosterone (12.5 mM) and 4-MA (1 mM) will be added to each 1-ml incubation in 20  $\mu$ l methanol and 1  $\mu$ l acetone, respectively. Reactions will be started by addition of the NADPH-generating system, and will be stopped after 10 min by addition of 6 ml of dichloromethane. Zero-time incubations will serve as blanks.

Each sample will be spiked with 3 nmol of 11 $\beta$ -hydroxytestosterone (in 100  $\mu$ l dichloromethane) and vigorously mixed on a batch vortexer (3 x 1 min). After the two phases are separated by low-speed centrifugation (2000 g for 10 min), the aqueous (upper) phase will be aspirated and discarded. An aliquot (4 ml) of the organic phase will be transferred to a culture tube (12 x 75 mm) and evaporated in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY). The residue will be redissolved in 200  $\mu$ l mobile phase A (see below). A 50- $\mu$ l aliquot will be analyzed by HPLC with a Shimadzu LC-6A binary gradient HPLC system equipped with a SIL-6A autosampler, SPD-6A variable wavelength UV detector and column heater (Shimadzu Scientific Instruments, Columbia, MD). Testosterone, androstenedione, 6-dehydrotestosterone, and 12

hydroxytestosterone isomers (6 $\alpha$ -, 15 $\beta$ -, 6 $\beta$ -, 15 $\alpha$ -, 7 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ -, 1 $\beta$ -, 18-, 11 $\beta$ -, 2 $\alpha$ -, 2 $\beta$ -hydroxytestosterone), will be resolved on a Supelcosil LC-18 octyldecylsilane (C<sub>18</sub>) column (5  $\mu$ m particle size, 15 cm x 4.6 mm i. d.), which will be preceded by a Supelcosil LC-18 guard column (40  $\mu$ m particle size, 2 cm x 4.6 mm, i.d.) (Supelco, Bellefonte, PA). The HPLC method will be similar to that described by Sonderfan *et al.* (5), with slight changes in mobile phase A and the solvent program. The ratio of water:methanol:acetonitrile (v/v/v) will be 64:35:1 for mobile phase A, and 18:80:2 for mobile phase B. The total flow rate will be 2.0 ml/min and the column temperature will be 40°C. The solvent program will be as follows: 100% A from zero to 10 min, a linear gradient to 45% B from 10-28 min, a linear gradient from 45% to 100% B from 28-29 min, 100% B from 29 to 31 min, a linear gradient to 100% A from 31-32 min followed by re-equilibration with 100% A from 32 to 34 min. Total analysis time will be 37 min per sample. The slight changes in mobile phase A and the solvent program enable 6 $\beta$ - and 15 $\alpha$ -hydroxytestosterone to be resolved, whereas they co-eluted in the original system described by Sonderfan *et al.* (5). Testosterone and its potential metabolites will be monitored at 254 nm. However, this HPLC system does not resolve 1 $\alpha$ - from 1 $\beta$ -hydroxytestosterone, nor does it resolve 18- from 12 $\beta$ -hydroxytestosterone. Metabolites will be quantified by comparison of their peak areas (integrated by a Shimadzu C-R3A recording data processor) with those of authentic standards. The recovery of testosterone and its metabolites from incubation mixtures is essentially quantitative (>95%). Nevertheless, sample-to-sample variations in extraction efficiency will be corrected based on recovery of the internal standard, 11 $\beta$ -hydroxytestosterone.

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## **SOP 01.00. BUFFERS & COMMONLY USED STOCK REAGENTS**

**DATE:** 15.11.91

1. Potassium phosphate buffers (1.0 M, various pH values)
2. Tris.HCl buffers (1.0 M, various pH values)
3. Sucrose (250 mM)
4. Magnesium chloride (100 mM)
5. Ethylenediaminetetraacetic acid (100 mM EDTA, pH 7.4)
6. Sodium hydroxide solutions (5.0, 1.0 and 0.1 M)
7. Hydrochloric acid solutions (5.0, 1.0 and 0.1 M)

## **1. POTASSIUM PHOSPHATE BUFFERS (1.0 M, various pH values)**

Potassium phosphate buffers with a pH value in the range of 6.5 to 7.7 are prepared by dissolving pre-determined amounts of monobasic potassium phosphate ( $\text{KH}_2\text{PO}_4$ ) and dibasic potassium phosphate ( $\text{K}_2\text{HPO}_4$ ) in de-ionized water. Over the pH range of 6.5 to 7.7, the mono- and dibasic salts of potassium phosphate serve as conjugate acid and base, respectively.

### **Potassium dihydrogen phosphate (monobasic)**

**Source:** J.T. Baker, Inc., Phillipsburg, New Jersey  
**Catalog Number :** 3246-05  
**Molecular weight:** 136.1 g/mol  
**Storage:** Room temperature  
**Expiration:** Five years

### **Dipotassium hydrogen phosphate (dibasic)**

**Source:** J.T. Baker, Inc., Phillipsburg, New Jersey  
**Catalog Number :** 3252-05  
**Molecular weight:** 174.2 g/mol  
**Storage:** Room temperature  
**Expiration:** Five years

Potassium phosphate salts are purchased from J.T. Baker because of their low iron content. If an alternate supplier must be used, choose a supplier that can provide potassium phosphate salts with an iron content as low as that guaranteed by J.T. Baker. A low iron content is desirable because too much iron can lead to lipid peroxidation when microsomal membranes are incubated with NADPH.

**Procedure to prepare 2 liters of buffer with pH values 6.5 To 7.7**

pH	KH <sub>2</sub> PO <sub>4</sub> monobasic	K <sub>2</sub> HPO <sub>4</sub> dibasic
6.50	184.2 g	110.9 g
6.80	137.5 g	172.4 g
7.00	101.2 g	202.6 g
7.25	77.1 g	247.4 g
7.40	53.1 g	280.6 g
7.60	37.4 g	300.2 g
7.70	35.8 g	304.2 g

**Procedure to prepare 2 liters of 1.0 M potassium phosphate buffer**

Weigh out the designated amounts of mono- and dibasic potassium phosphate (see Table above).

Combine and dissolve in a final volume of 2 liters of de-ionized water.

Add 10 ml of resultant buffer to 90 ml of de-ionized water. Check that the pH value is  $\pm 0.05$  of the theoretical value.

**Note:** It is critical that the stock buffer be diluted 10 fold prior to verification of pH value because the pH of potassium phosphate buffers is concentration dependent.

Filter through a 0.45  $\mu\text{m}$  nylon membrane to remove any particulate matter.

**Storage:** Store in a polypropylene or polyethylene container at 4°C.

**Expiration date:** Two years from the date of preparation.

## 2. TRIS.HCL BUFFERS (1.0 M, various pH values)

Tris.HCl buffers are prepared by dissolving a pre-determined amount of Tris base (also known as Trizma base or tris[hydroxymethyl]aminomethane) in de-ionized water and adjusting the pH to the desired value by addition of hydrochloric acid. During the addition of hydrochloric acid, the pH is monitored continuously with a pH meter. The final pH value can range from 7.0 to 9.0.

The pH of Tris-HCl buffers is influenced by temperature. The temperature coefficient is  $-0.02$  pH units/ $^{\circ}\text{C}$  (*i.e.*, the pH increases as the buffer is cooled and, conversely, decreases as the buffer is warmed). When a Tris.HCl buffer prepared at  $22^{\circ}\text{C}$  is cooled to  $4^{\circ}\text{C}$ , the pH increases  $0.36$  pH units ( $-18^{\circ}\text{C} \times -0.02$  pH units/ $^{\circ}\text{C} = 0.36$  pH units). Consequently, a Tris.HCl buffer that was pH 7.0 at room temperature would have a pH close to 7.4 when placed on ice or in a refrigerator. Conversely, when a Tris.HCl buffer prepared at  $22^{\circ}\text{C}$  is warmed to  $37^{\circ}\text{C}$ , the pH decreases  $0.30$  pH units ( $-15^{\circ}\text{C} \times -0.02$  pH units/ $^{\circ}\text{C} = 0.30$  pH units). Consequently, a Tris.HCl buffer that was pH 7.7 at room temperature would have a pH close to 7.4 when placed at body temperature or in metabolic water bath. Due to the temperature dependence of Tris.HCl buffers, it is important to consider the temperature at which the buffer will be used, and it is important to indicate on the label the temperature at which the buffer was prepared.

### Tris (Trizma base or tris[hydroxymethyl]aminomethane)

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog Number :** T1503  
**Molecular weight:** 121.1 g/mol  
**Storage:** Room temperature  
**Expiration:** Five years

### Procedure to prepare 2 liters of 1.0 M Tris.HCl buffer

Dissolve 242.2 g Trizma base in ~1900 ml of de-ionized water at room temperature. Use a 2-liter beaker and stir on a magnetic stirrer.

Calibrate the pH meter with the standard buffer (pH 7 or 10) that is closest to the desired pH of the Tris buffer.

Wash the pH electrode and monitor the pH of the Tris solution (which will be very alkaline).

Slowly add hydrochloric acid until the pH reaches the desired value. Begin by adding concentration hydrochloric acid until the pH approaches the desired value, then make fine pH adjustments with 5.0 M or 1.0 M hydrochloric acid (see Reagent 7).

During the addition of the acid (which causes an exothermic reaction), periodically check the temperature of the buffer. This can be achieved with the temperature probe attached to the pH meter.

If the temperature rises above 24°C, allow the solution to cool to room temperature (20-24°C) before making final adjustments to the pH.

Adjust the final volume to 2000 ml with de-ionized water.

Filter through a 0.45 µm nylon membrane to remove any particulate matter.

**Storage:** Store in a polypropylene or polyethylene container at 4°C.

**Expiration date:** Two years from the date of preparation.

**3. SUCROSE (250 mM)**

A 250 mM solution of sucrose is prepared by dissolving sucrose in de-ionized water to give a final concentration of 85.6 g/liter

**Sucrose**

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog Number :** S9378  
**Molecular weight:** 342.3 g/mol  
**Storage:** Room temperature  
**Expiration:** Five years

**Procedure to prepare 2 liters of 250 mM sucrose**

Dissolve 171.2 g sucrose in 2 liters of de-ionized water.  
Filter through a 0.45  $\mu$ m nylon membrane to remove any particulate matter.

**Storage:** Store in a polypropylene or polyethylene container at 4° C.

**Expiration date:** Two months from the date of preparation.

**4. MAGNESIUM CHLORIDE (100 mM)**

A 100 mM solution of magnesium chloride is prepared by dissolving magnesium chloride in de-ionized water to give a final concentration of 20.33 g/liter

**Magnesium chloride (hexahydrate)****MgCl<sub>2</sub>•6 H<sub>2</sub>O****Source:** Sigma Chemical Co., St. Louis, Missouri**Catalog Number :** M-9272**Formula weight:** 203.3 g/mol**Storage:** Room temperature**Expiration:** Five years**Procedure to prepare 500 ml of 100 mM magnesium chloride**

Dissolve 10.17 g MgCl<sub>2</sub>.6H<sub>2</sub>O in a final volume of 500 ml of de-ionized water.

Filter through a 0.45 μm nylon membrane to remove any particulate matter.

**Storage:** Store in a glass, polypropylene or polyethylene container at 4° C.**Expiration date:** Two years from the date of preparation.

**5. ETHYLENEDIAMINETETRAACETIC ACID (100 mM EDTA, PH 7.4)**

A 100 mM solution of EDTA is prepared by dissolving EDTA tetrasodium salt in de-ionized water (to give a final concentration of 38.02 g/liter) and adjusting the pH to 7.4 with 1 N HCl.

**Ethylenediaminetetraacetic acid, tetrasodium salt**

**Source:** Aldrich Chemical Company, Milwaukee, Wisconsin  
**Catalog Number :** E2,629-0  
**Molecular weight:** 380.2 g/mol  
**Storage:** Room temperature  
**Expiration:** Five years

**Procedure to prepare 2 liters of 100 mM EDTA, pH 7.4**

Dissolve 76.04 g EDTA, tetrasodium salt to ~1800 ml of de-ionized water. Use a 2-liter beaker and stir on a magnetic stirrer.

Calibrate the pH meter with the pH 7.0 or 7.4 standard buffer.

Wash the pH electrode and monitor the pH of the EDTA solution (which will be alkaline).

Slowly add hydrochloric acid until the pH reaches the desired value. Begin with 5.0 M HCl/NaOH until the pH approaches 7.4, then make fine pH adjustments with 1.0 or 0.1 M HCl/NaOH.

Adjust the final volume to 2000 ml with de-ionized water.

Filter through a 0.45  $\mu\text{m}$  nylon membrane to remove any particulate matter.

**Storage:** Store in a polypropylene or polyethylene container at 4° C.  
**Expiration date:** Two years from the date of preparation.

## 6. SODIUM HYDROXIDE SOLUTIONS

A 5.0 M solution of sodium hydroxide is prepared by dissolving sodium hydroxide in de-ionized water to give 200 g/liter . The molarity of this solution is not determined by acid titration, hence, the concentration of sodium hydroxide is only an approximation. 1.0 M and 0.1 M solutions of sodium hydroxide are prepared by diluting the 5.0 M solution with de-ionized water.

### Sodium hydroxide

**Source:** Fisher Scientific, Chicago, Illinois  
**Catalog Number:** S318-3  
**Molecular weight:** 40.0 g  
**Storage:** Room temperature  
**Expiration:** Five years

### Procedure to prepare 500 ml of 5.0 M NaOH

Dissolve 100 g of sodium hydroxide in a final volume of 500 ml de-ionized water.

**Caution:** This procedure should be carried out in a safety hood.

Add 100 ml of 5.0 M sodium hydroxide to 400 ml of water to prepare 500 ml of a 1.0 N solution of sodium hydroxide.

Add 50 ml of 1.0 N sodium hydroxide to 450 ml of water to prepare 500 ml of a 0.1 N solution of sodium hydroxide.

**Storage:** Store in a polypropylene or polyethylene container at room temperature.  
DO NOT STORE IN GLASS BECAUSE IT WILL DISSOLVE IN STRONG BASES  
**Expiration date:** Five years from the date of preparation.

## 7. HYDROCHLORIC ACID SOLUTIONS

A 5.0 M solution of hydrochloric acid is prepared by diluting concentration hydrochloric acid 2.4 fold in de-ionized water. The molarity of this solution is not determined by titration, hence, the concentration of hydrochloric acid is only an approximation. 1.0 M and 0.1 M solutions of hydrochloric acid are prepared by diluting the 5.0 M solution with de-ionized water.

### Hydrochloric acid

**Source:** Fisher Scientific, Chicago, Illinois  
**Catalog Number:** A144c-212  
**Concentration:** 36.5-38%  
**Molarity:** 12 M  
**Storage:** Room temperature in a safety cabinet  
**Expiration:** Five years

### Procedure to prepare 500 ml of 5.0 M HCl

Very slowly add 208 ml of concentrated hydrochloric acid to 292 ml of ice-cold de-ionized water.

**Note:** Add acid to water, not *vice versa*.

**Caution:** This procedure should be carried out in a safety hood.

Add 100 ml of 5.0 M hydrochloric acid to 400 ml of water to prepare 500 ml of a 1.0 N solution of hydrochloric acid.

Add 50 ml of 1.0 N hydrochloric acid to 450 ml of water to prepare 500 ml of a 0.1 N solution of hydrochloric acid

**Storage:** Store in a glass, polypropylene or polyethylene container at room temperature.

**Expiration date:** Five years from the date of preparation.

**SOP 02.00. NADPH-REGENERATING SYSTEM****DATE:** 03.07.92

NADPH is added to microsomal incubations to support cytochrome P450-dependent reactions. To reduce the cost of purchasing chemically reduced NADPH and to maintain NADPH in the reduced state during microsomal incubations, NADPH is generated from NADP by glucose-6-phosphate, according to the following scheme:

**SPECIAL REAGENTS** **$\beta$ -NADP ( $\beta$ -Nicotinamide Adenine Dinucleotide Phosphate, oxidized form)**

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog Number:** N-3886  
**Formula weight:** 765.4 g/mol (This value may change from batch-to-batch, depending on the water-ethanol content of the material)  
**Storage:** The dry solid is stored in a desiccator at -20°C.  
**Expiration:** Five years

**Glucose-6-Phosphate**

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog Number:** G-7879  
**Formula weight:** 282.1 g/mol  
**Storage:** The dry solid is stored at room temperature  
**Expiration:** Five years

**Glucose-6-Phosphate Dehydrogenase**

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog Number:** G-8878  
**Concentration:** The enzyme is suspended in 2.6 M ammonium sulfate at a concentration of up to 1,000 to 5000 Units/ml.  
**Storage:** The enzyme suspension is stored in a desiccator at 4°C.  
**NOTE: THE ENZYME SUSPENSION MUST NOT BE FROZEN**  
**Expiration:** One year

## COMMON REAGENTS

### 20 mM Potassium Phosphate Buffer, pH 7.4

Prepared from the stock 1.0 M potassium phosphate buffer (pH 7.4) (see SOP 01.00).

Add 2 ml of 1.0 M stock buffer to 98 ml of de-ionized water.

Use to prepare the solutions described below, and discard the excess.

### Sodium hydroxide solutions

See SOP 01.00

## PREPARATION OF STOCK SOLUTIONS OF COMPONENTS OF THE NADPH-GENERATING

In a typical microsomal incubation, the NADPH-generating system consists of 1 mM NADP(H), 5 mM glucose-6-phosphate and 1 Unit/ml of glucose-6-phosphate dehydrogenase. Stock solutions of these three components are prepared and stored frozen at -20°C. The stock solutions are 100x more concentrated than the final incubation concentration, and they are pre-mixed to generate NADPH prior to initiating microsomal reactions. The three stock solutions are 100 mM NADP, 500 mM glucose-6-phosphate and 100 Units/ml of glucose-6-phosphate dehydrogenase.

### Procedure to prepare 10 ml of 100 mM $\beta$ -NADP

1. Dissolve 0.765 g NADP in ~7 ml of 20 mM potassium phosphate buffer, pH 7.4. This solution will appear faintly yellow.

**Note:** NADP is strongly acidic, hence, the pH is adjusted to 7.0 - 7.4 with sodium hydroxide.

2. Calibrate the pH meter with a pH 7.0 or 7.4 standard buffer.
3. Wash the pH electrode and monitor the pH of the NADP solution.
4. While the solution is stirred with a magnetic stirrer, add 5.0 M NaOH dropwise to pH ~6.0, then add 1.0 M NaOH dropwise to pH 6.5 - 6.8, and finally add 0.1 M NaOH dropwise to pH 7.0 - 7.4. Adjust volume to 10 ml with 20 mM potassium phosphate buffer, pH 7.4.

**Storage:** Store frozen at -20°C in 0.5-ml aliquots. Traditionally, 2-ml plastic vials with blue caps have been used to store the stock solution of NADP.

After an aliquot is thawed for experimental purposes, do not re-freeze any unused reagent (i.e., discard any reagent remaining at the end of an experiment).

**Expiration date:** One year

**Procedure to prepare 10 ml of 500 mM glucose-6-phosphate**

1. Dissolve 1.41 g glucose-6-phosphate in ~7 ml of 20 mM potassium phosphate buffer, pH 7.4.  
**Note:** Glucose-6-phosphate is strongly acidic, hence, the pH is adjusted to 7.0 - 7.4 with sodium hydroxide.
2. Calibrate the pH meter with a pH 7.0 or 7.4 standard buffer.
3. Wash the pH electrode and monitor the pH of the glucose-6-phosphate solution.
4. While the solution is stirred with a magnetic stirrer, add 5.0 M NaOH dropwise to pH ~6.0, then add 1.0 M NaOH dropwise to pH 6.5 - 6.8, and finally add 0.1 M NaOH dropwise to pH 7.0 - 7.4. Adjust volume to 10 ml with 20 mM potassium phosphate buffer, pH 7.4.

**Storage:** Store frozen at -20°C in 0.5-ml aliquots. Traditionally, 2-ml plastic vials with **yellow** caps have been used to store the stock solution of glucose-6-phosphate.

After an aliquot is thawed for experimental purposes, do not re-freeze any unused reagent (i.e., discard any reagent remaining at the end of an experiment).

**Expiration date:** One year

**Procedure to prepare 10 or 20 ml of glucose-6-phosphate dehydrogenase**

1. Dilute the enzyme to 100 Units/ml in 20 mM potassium phosphate buffer, pH 7.4. The enzyme is supplied in 1000 or 2000 Unit aliquots and is suspended in ~0.5 or ~1.0 ml of 2.6 M ammonium sulfate.
2. For 1,000 Units of enzyme, adjust the volume to 10 ml.
3. For 2,000 Units of enzyme, adjust the volume to 20 ml.

**Storage:** Store frozen at -20°C in 0.5-ml aliquots. Traditionally, 2-ml plastic vials with **green** caps have been used to store the stock solution of glucose-6-phosphate dehydrogenase.

After an aliquot is thawed for experimental purposes, do not re-freeze any unused enzyme (i.e., discard any reagent remaining at the end of an experiment).

**Expiration date:** One year

## **SPECTROPHOTOMETRIC VERIFICATION of NADPH PRODUCTION**

Although NADP and NADPH both absorb at 260 nm, only NADPH absorbs strongly at 340 nm. Consequently, the reduction of NADP to NADPH is associated with an increase in absorbance at 340 nm. The rate of formation of NADPH can be determined from the rate of change in absorbance at 340 nm. This is the basis for verifying that, when added together, the three components of the NADPH-generating system (*i.e.*, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) can generate NADPH.

NADPH production is examined when new components of the NADPH-generating system are prepared, and each time the NADPH-generating system is prepared from frozen aliquots. In the first case, the rate of NADPH production is determined to ascertain whether the new system can generate NADPH at  $> 0.2 \mu\text{mol/ml/min}$ . In the second case, the concentration of NADPH is measured to ascertain whether at least 20% of the NADP is reduced to NADPH prior to its addition to microsomal incubation (which verifies that the concentration of NADPH in the microsomal incubations is at least 0.2 mM).

### **Procedure to verify NADPH production by newly prepared components of the NADPH-generating system**

The rate of change in absorbance at 340 nm is determined with a DW 2C dual beam spectrophotometer (SLM Aminco, Urbana, Illinois). The instrument is used as described by the manufacturer with the following settings:

Lamp	UV (5 minute warm-up required)
Wavelength	340 nm
Beam Chopper Speed	Low (15 times/sec)
Time Base	2 seconds/inch
Wavelength Scan	Off
Beam Selector	Sample and Reference
Filter	Open
Full Scale Absorbance	2.0

**Procedure to measure the rate of NADPH production**

1. Dispense 970  $\mu\text{l}$  of 100 mM potassium phosphate buffer (pH 7.4) into a 1-ml sample cuvette at room temperature.
2. Dispense 980  $\mu\text{l}$  of the same buffer into a 1-ml matched reference cuvette at room temperature.
3. Add 10  $\mu\text{l}$  of 500 mM glucose-6-phosphate to both cuvettes.
4. Add 10  $\mu\text{l}$  of 100 units/ml glucose-6-phosphate dehydrogenase to the sample cuvette.
5. Add 10  $\mu\text{l}$  of 100 mM NADP to reference cuvette.

**Note:** At this stage, the reference cuvette contains all compounds except glucose-6-phosphate dehydrogenase whereas the sample cuvette contains all components except NADP.

6. Place the cuvettes in the sample and reference holders in the spectrophotometer and turn on the high voltage current to the photomultiplier tube.
7. Using the beam balance, adjust the absorbance to 0.02 to 0.2 and record a baseline for 3-5 seconds.
8. Add 10  $\mu\text{l}$  of 100 mM NADP to sample cuvette. **Note:** This can be added through the cuvette compartment port to avoid switching off the photomultiplier tube.
9. Record the increase in absorbance with time for at least 6 seconds (which corresponds to 3 inches on the horizontal scale).

**Calculations**

The rate of NADPH formation is calculated from the initial rate of absorbance change at 340 nm ( $\Delta E/\text{min}$ ). If the initial slope of absorbance *versus* time is not linear for 6 seconds, draw a tangent to initial part (*i.e.*, the steepest part) of the curve.

Measure the vertical rise over a 6-sec period (which corresponds to 3 inches on the horizontal scale) The vertical component of this slope represents the absorbance increase in 0.1 min.

$\Delta A/0.1 \text{ min} = \text{absorbance increase in 6 seconds}$ , hence,  $\Delta A/\text{min} = \Delta E/0.1 \text{ min} \times 10$

Note:  $\Delta A = \text{fraction of full scale deflection} \times \text{Full Scale Absorbance}$

$$\text{Rate of NADP reduction } (\mu\text{mol NADPH formed/ml/min}) = \frac{\Delta A/\text{min}}{\epsilon} = \frac{\Delta A/\text{min}}{6.22}$$

where  $\epsilon = \text{extinction coefficient of NADPH} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$  (which indicates that a 1 mM solution of NADPH, which equals 1  $\mu\text{mol/ml}$ , would have an absorbance value of 6.22 in a cuvette with a 1 cm light path).

Inasmuch as the sample cuvette contains 1 Unit of glucose-6-phosphate dehydrogenase in 1 ml, the rate of NADPH production can theoretically equal 1.0  $\mu\text{mol/ml/min}$ . However, because the rate of NADPH production is measured in the absence of  $\text{MgCl}_2$  at room temperature, not 25°C, the observed rate of NADPH production is usually less than 1.0  $\mu\text{mol/ml/min}$ . Rates ranging from 0.2 to 1.0  $\mu\text{mol/ml/min}$  are acceptable.

**Procedure to verify NADPH production by frozen components of the NADPH-generating system**

NADPH production is examined each time the NADPH-generating system is prepared from frozen aliquots. The concentration of NADPH is measured to ascertain whether at least 20% of the NADP is reduced to NADPH prior to its addition to microsomal incubation (which verifies that the concentration of NADPH in the microsomal incubations is at least 0.2 mM). The concentration of NADPH in the NADPH-generating system is determined from the absorbance at 340 nm, which is determined with a DW 2C dual beam spectrophotometer (SLM Aminco, Urbana, Illinois). The instrument is used as described by the manufacturer with the following settings:

Lamp	UV (5 minute warm-up required)
Wavelength	220 to 380 nm
Beam Chopper Speed	Low (15 times/sec)
Time Base	Off
Wavelength Scan	5 nm/sec
Beam Selector	Sample and Reference
Filter	Open
Full Scale Absorbance	2.0

**Procedure to measure the concentration of NADPH**

Most microsomal reactions are started by adding 50  $\mu$ l of the NADPH-generating system to a 950- $\mu$ l incubation mixture (to give a final volume of 1 ml). The 50- $\mu$ l sample of NADPH-generating system consists of 20  $\mu$ l of water plus 10  $\mu$ l each of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The final concentration of NADPH in the incubation mixture is 1 mM. At 340 nm, a 1 mM solution of NADPH would give an absorbance reading of 6.22 in a cuvette with a 1-cm light path ( $\epsilon = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$ ). This absorbance value is too great to be measured spectrophotometrically because the maximum Full Scale Absorbance is 2.0. Consequently, the NADPH-generating system is diluted 200 fold to give a final concentration of NADPH of 0.1 mM (which would give an absorbance reading of 0.622 in a cuvette with a 1-cm light path).

**Procedure to measure the concentration of NADPH (continued)**

1. Dispense 995  $\mu$ l of 100 mM potassium phosphate buffer (pH 7.4) into a 1-ml sample cuvette at room temperature.
2. Dispense 1.0 ml of the same buffer into a 1-ml matched reference cuvette at room temperature.
3. Place the cuvettes in the sample and reference holders in the spectrophotometer and turn on the high voltage current to the photomultiplier tube.
4. Record a baseline absorbance from 220 to 380 nm.
5. Add 5  $\mu$ l of NADPH-generating system to the sample cuvette.
6. Record the absorbance from 220 to 380 nm.

**Calculations**

The concentration of NADPH is calculated from the absorbance peak at 340 nm.

Note: Absorbance = fraction of full scale deflection x Full Scale Absorbance

$$\text{Concentration of NADPH in the sample cuvette (mM)} = \frac{\text{Absorbance}}{\epsilon} = \frac{\text{Absorbance}}{6.22}$$

where  $\epsilon$  = extinction coefficient of NADPH = 6.22  $\text{mM}^{-1} \text{cm}^{-1}$  (which indicates that a 1 mM solution of NADPH would have an absorbance value of 6.22 in a cuvette with a 1 cm light path).

$$[\text{NADPH}] \text{ in microsomal incubation} = [\text{NADPH}] \text{ in cuvette} \times \frac{50 \mu\text{l/ml}}{5 \mu\text{l/ml}}$$

where 50  $\mu$ l is the volume of NADPH-generating system added per ml of microsomal incubation mixture, and 5  $\mu$ l is the volume added to the 1-ml sample cuvette.

Acceptable values for the concentration of NADPH in the microsomal incubation range from 0.2 to 1.0 mM (i.e., 20 to 100% of the theoretical value of 1 mM)

The final equation must be modified if the volume of NADPH-generating system added per ml of incubation mixture is not 50  $\mu$ l, or if the volume added to the 1-ml sample cuvette is not 5  $\mu$ l. For example, in the assay of lauric acid hydroxylation, the volume of NADPH-generating system added per ml of incubation mixture is 80  $\mu$ l. In this case, the volume of NADPH-generating system added to the 1-ml sample cuvette can be either 5  $\mu$ l (the usual amount) or 8  $\mu$ l (which represents 10% of the volume added to the incubation mixture).

**NADPH-GENERATING SYSTEM WORKSHEET**

Date new components were prepared: \_\_\_\_\_

Which component or components of the NADPH-generating system were prepared on this date?

1. NADP (100 mM)
2. Glucose-6-phosphate (500 mM)
3. Glucose-6-phosphate dehydrogenase (100 Units/ml)

**DETAILS OF THE NEW COMPONENTS**

Chemical Name	Catalog No.	Batch No.	Date received	Expiration date
1. NADP (100 mM)	N-3386			
2. Glucose-6-phosphate (500 mM)	G-7879			
3. Glucose-6-phosphate dehydrogenase (100 Units/ml)	G8878			

Did the new components generate NADPH faster than 0.2µmol/ml/min?      Yes      No

If no, what action was taken?

\_\_\_\_\_

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**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

## **SOP 07.00 PIERCE BCA PROTEIN ASSAY**

**DATE:** 09.17.92

### **STOCK REAGENT: PIERCE BCA KIT**

This kit contains a standard protein solution and Reagents A and B.

**Source:** Pierce Chemical Company, Rockford, Illinois

**Catalog:** #23225

**Storage:** Store at 4°C. Should either reagent A or B precipitate upon long-term storage, warm on stir plate until precipitate redissolves.

**Expiration:** One year. Note: Reagents A and B are stable at room temperature for 12 months.

**Protein Standard:** Bovine serum albumin (BSA). Supplied in 10 x 1-ml glass ampules containing 2 mg/ml of BSA in 0.9% sodium chloride containing 0.05% sodium azide as preservative.

**Reagent A:** 1000 ml of base reagent containing sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.2 N sodium hydroxide.

**Reagent B:** 25 ml of 4% copper sulfate.

### **WORKING REAGENT**

Mix 50 parts Reagent A with 1 part reagent B.

For example, mix 200 ml Reagent A with 4 ml Reagent B.

Initially while adding reagent B to reagent A, the solution will appear slightly turbid. When mixed it will clear to a light green color. The working reagent is stable at room temperature for 12-24 hours.

**PROTOCOL**

The concentration of protein is determined according to the manufacturer's instructions (supplied with each kit).

Volume of test sample or standard: 500  $\mu$ l containing up to 200  $\mu$ g protein

Volume of working reagent: 2.5 ml

Assay the test samples and BSA standards in duplicate.

**BSA standards**

The standard solution of BSA (2 mg/ml) is added from zero to 100  $\mu$ l (in 10  $\mu$ l increments) to clean tubes that contain 400-500  $\mu$ l of water as shown in the following table. The volume of each standard is 500  $\mu$ l.

Assay each standard in duplicate.

Volume of water	Volume of BSA standard (2 mg/ml)	Amount of BSA
500 $\mu$ l	0	zero
490 $\mu$ l	10 $\mu$ l	20 $\mu$ g/tube
480 $\mu$ l	20 $\mu$ l	40 $\mu$ g/tube
470 $\mu$ l	30 $\mu$ l	60 $\mu$ g/tube
460 $\mu$ l	40 $\mu$ l	80 $\mu$ g/tube
450 $\mu$ l	50 $\mu$ l	100 $\mu$ g/tube
440 $\mu$ l	60 $\mu$ l	120 $\mu$ g/tube
430 $\mu$ l	70 $\mu$ l	140 $\mu$ g/tube
420 $\mu$ l	80 $\mu$ l	160 $\mu$ g/tube
410 $\mu$ l	90 $\mu$ l	180 $\mu$ g/tube
400 $\mu$ l	100 $\mu$ l	200 $\mu$ g/tube

Dispense the water first and add the BSA solution second.

For a large number of test samples, repeat the 100  $\mu$ g standard (50  $\mu$ l of 2 mg/ml BSA) after each set of 10-20 samples to check intrabatch drift.

### Test Samples

If the protein concentration is known or can be reasonably estimated, dilute the sample to a protein concentration of approximately 1 mg/ml. Add approximately 75 - 100 µg of protein to tubes that contain sufficient water to bring the volume to 500 µl.

If the protein concentration cannot be estimated with reasonable accuracy, assay a constant volume of multiple dilutions (*e.g.*, 100 µl of a 5-, 10- and 20-fold dilution) or various amounts of the same diluted sample (*e.g.*, 50, 100 and 200 µl of a 10-fold dilution).

The sample volume should be adjusted to 500 µl with water. Dispense the water first and add the diluted sample second.

Assay each test sample in duplicate.

### Solubilization and color development

To solubilize the protein and initiate color development, add 2.5 ml of working reagent to each tube and mix on a vortex mixer. Incubate at 37° for 30 min or at room temperature for 2 hours. For greater sensitivity incubate at 60° for 30 minutes.

Cool the samples to room temperature (this can be achieved quickly by immersing the tubes in room temperature water).

Transfer the samples to glass or plastic cuvettes (1- or 3-ml cuvettes can be used).

Determine the absorbance of each standard and test sample at 562 nm. A split-beam or a single beam spectrophotometer operated in the visible mode can be used.

Set the absorbance to zero with water in the sample cuvette. For a split-beam spectrophotometer the reference cuvette can also be filled with water.

Replace the water in the sample cuvette with the *Zero Protein Blank* and record the absorbance.

Reset the absorbance to zero.

Record the absorbance of each standard and test sample at 562 nm.

### Data analysis

Average the absorbance values of the duplicate determinations.

If necessary, subtract the blank value (this will not be necessary if the spectrophotometer were set to zero with the *Zero Protein Blank* in the sample cuvette).

For the standards, plot the average of the blank-corrected absorbances at 562 nm *versus* the amount of BSA protein per tube (normal calibration curve). In addition, plot the amount of BSA protein per tube *versus* the average of the blank-corrected absorbances at 562 nm (reverse calibration curve)

Determine the equation of the lines for the normal and reverse calibration curves by linear or polynomial regression analysis. The equation from linear regression analysis will of the type:

$$y = mx + c$$

where  $m$  is the slope of the line and  $c$  is a constant corresponding to the  $y$ -axis intercept. For the normal calibration curve (absorbance *versus* protein),  $y$  = absorbance and  $x$  = amount of protein/tube. This form of the equation is inconvenient for analyzing the data from the test samples because  $x$  is the value to be determined from the experimental absorbance values. A more convenient equation to analyze the data is that derived from the reverse calibration curve (protein *versus* absorbance), where  $y$  = amount of protein per tube and  $x$  = absorbance.

Use the equation for the reverse calibration curve (protein *versus* absorbance) to determine the amount of protein in the test samples from the average of the absorbance values.

The concentration of protein in the diluted test sample can be calculated as the quotient of the amount of protein per tube and the volume of test sample added to the tube:

$$\text{Concentration of protein in the diluted sample } (\mu\text{g}/\mu\text{l or mg/ml}) = \frac{\text{Amount of protein in the tube } (\mu\text{g})}{\text{Volume of diluted sample added } (\mu\text{l})}$$

The concentration of protein in the undiluted (neat) test sample can be calculated as the product of the concentration of protein in the diluted sample and the dilution factor:

$$\text{Concentration of protein in the undiluted (neat) sample (mg/ml)} = \text{Concentration of protein in the diluted sample (mg/ml)} \times \text{Dilution factor}$$

**Protocol checks and actions**

1. If the absorbance of the *Zero Protein Blank* relative to water exceeds 0.1 absorbance units, repeat the assay with a freshly prepared working reagent. If the problem persists, use a new kit.
2. If the duplicate absorbance values for a test sample differ from each other by more than 10%, the sample should be reanalyzed (unless the same sample was also assayed at a different dilution or volume with values that agree within 10% of each other).
3. If the absorbance of the test sample falls outside the range of absorbances covered by the standard BSA solution, the sample should be reanalyzed (unless the same sample was also assayed at a different dilution or volume with values that fall within the range of standard absorbances).
4. If the standard BSA solutions assayed after each 10-20 samples consistently differ by more than 15% of their theoretical value, correct the test sample absorbances for intrabatch drift or repeat the assay.

**WORKSHEET FOR PIERCE BCA PROTEIN DETERMINATION - PAGE 1**

**BSA STANDARDS**

Absorbance (562 nm) of *Zero Protein Blank* versus water: \_\_\_\_\_ absorbance units

Amount of BSA standard (µg/tube)	Absorbance at 562 nm	
	Value 1	Value 2
0 µg		
20 µg		
40 µg		
60 µg		
80 µg		
100 µg		
120 µg		
140 µg		
160 µg		
180 µg		
200 µg		

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**WORKSHEET FOR PIERCE BCA PROTEIN DETERMINATION - PAGE 2a**

**TEST SAMPLES**

Tube	Sample	Dilution factor	Volume Assayed	Absorbance at 562 nm	
				Value 1	Value 2
1,2					
3,4					
5,6					
7,8					
9,10					
<hr/>					
11,12					
13,14					
15,16					
17,18					
19,20					
<hr/>					
21,22					
23,24					
25,26					
27,28					
29,30					
<hr/>					
31,32					
33,34					
35,36					
37,38					
39,40					
<hr/>					
41,42					
43,44					
45,46					
47,48					
49,50					
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**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**WORKSHEET FOR PIERCE BCA PROTEIN DETERMINATION - PAGE 2b****TEST SAMPLES**

Tube	Sample	Dilution factor	Volume Assayed	Absorbance at 562 nm	
				Value 1	Value 2
51,52					
53,54					
55,56					
57,58					
59,60					
61,62					
63,64					
65,66					
67,68					
69,70					
71,72					
73,74					
75,76					
77,78					
79,80					
81,82					
83,84					
85,86					
87,88					
89,90					
91,92					
93,94					
95,96					
97,98					
99,100					

**Analyst****Date**

**WORKSHEET FOR PIERCE BCA PROTEIN DETERMINATION - PAGE 3**

**Project or notebook number:** \_\_\_\_\_

**Date:** \_\_\_\_\_

**Pipet identification numbers:**

Pipetman P20 \_\_\_\_\_

Pipetman P200 \_\_\_\_\_

Pipetman P1000 \_\_\_\_\_

Pipetman P5000 \_\_\_\_\_

**Samples (short description): e.g., Rat liver microsomes**

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**Sample dilution:**      **Fold dilution:** \_\_\_\_\_

**Reagents:**              **Expiration date of BCA kit:** \_\_\_\_\_

**Working reagent:**      **Volume of Reagent A:** \_\_\_\_\_

**Volume of Reagent B:** \_\_\_\_\_

**Protocol:**              **Incubation temperature:** \_\_\_\_\_

**Incubation time:** \_\_\_\_\_

**Spectrophotometer:**

SLM-Aminco DW2C \_\_\_\_\_

Other (specify) \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**WORKSHEET FOR PIERCE BCA PROTEIN DETERMINATION - PAGE 4**

**Protocol Checks**

- 1. Did the absorbance of the *Zero Protein Blank* against water exceed 0.1 absorbance units? \_\_\_\_\_
  
- 2a. For any test samples, did the absorbance values for duplicate determinations differ from each other by more than 10%? \_\_\_\_\_
  
- 2b. If the answer to question 2a was YES, which samples were affected? \_\_\_\_\_  
\_\_\_\_\_
  
- 3a. Did the average absorbance value of any test sample fall outside the range of absorbances for the BSA standards? \_\_\_\_\_
  
- 3b. If the answer to question 3a was YES, which samples were affected? \_\_\_\_\_  
\_\_\_\_\_
  
- 4. Did the BSA standards included to check for intrabatch drift differ from their theoretical value by more than 15%? \_\_\_\_\_
  
- 5. Did the procedure to measure the concentration of protein deviate from the Standard Operating Procedure? \_\_\_\_\_

If the answer to any of the above questions was YES, explain how the procedure deviated from Standard Operating Procedure, what action was taken and what impact this would have on the results obtained.

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**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

## SOP 08.00. CYTOCHROME b<sub>5</sub> and P450 CONCENTRATION

**DATE:** 04.19.92

### SPECIAL REAGENTS

#### Sodium hydrosulfite (sodium dithionite)

Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>

**Source:** Aldrich Chemical Company, Milwaukee, Wisconsin  
**Catalog number:** 15,795-3  
**Molecular weight:** 174.1 g/mol  
**Storage:** Room temperature  
**Expiration date:** Two years

#### Carbon monoxide

CO

**Source:** Matheson Gas Products, St. Louis, Missouri  
**Catalog number:** UN1016 (Research purity)  
**Molecular weight:** 28.0 g/mol  
**Storage:** Room temperature (gas cylinder)  
**Expiration date:** None

#### β-Nicotinamide adenine nucleotide, reduced form

β-NADH•4 H<sub>2</sub>O

**Source** Sigma Chemical Co, St. Louis, Missouri  
**Catalog No.** 340-102  
**Formula weight** 781 g/mol (This may vary from batch-to-batch depending the manufacturer's analysis of the water content).  
**Storage** Store the solid material at room temperature  
**Expiration date** Specified by the manufacturer

## 20 mM β-NADH

A 20 mM solution of NADH is prepared by dissolving NADH in potassium phosphate buffer (100 mM, pH 7.4). NADH is supplied in pre-weighed vials each containing 2 mg β-NADH (approximately 2.56 μmol depending on the lot analysis). A 20 mM solution, which is equivalent to 0.020 μmol/μl, is prepared according to the following formula:

$$\text{Volume of buffer } (\mu\text{l}) \text{ added to the pre-weighed vial} = \frac{\mu\text{mol NADH in pre-weighed vial}}{0.020 \mu\text{mol}/\mu\text{l}}$$

The number of μmol NADH in the pre-weighed vial is specified by the manufacturer. For example, if the pre-weighed vial contains 2.56 μmol NADH, 128 μl of potassium phosphate buffer (100 mM, pH 7.4) would be added to prepare a 20 mM solution.

**Storage:** The 20 mM solution of NADH is prepared fresh and used within 8 hours. The solution is not stored.

**Note:** In contrast to NADPH, NADH is not strongly acidic

## COMMON REAGENTS

### Potassium Phosphate Buffer, 100 mM pH 7.4

This working buffer is prepared by diluting the 1.0 M stock potassium phosphate buffer 10 fold with de-ionized water. The 1.0 M potassium phosphate buffer, pH 7.4, is prepared as described in SOP 01.00.

1. Add 100 ml of 1.0 M potassium phosphate stock buffer to 900 ml of ice-cold de-ionized water (room temperature water can be used if the buffer will be stored at 4°C for at least 24 hours).
2. Filter through a 0.45 μm nylon membrane to remove any particulate matter.

**Storage:** Store in a polypropylene or polyethylene container at 4°C.

**Expiration date:** Two months from date of preparation

### **CYTOCHROME $b_5$**

The concentration of cytochrome  $b_5$  is determined from the difference spectrum between NADH-reduced and oxidized microsomes, as described by Omura and Sato (1). Liver microsomes are diluted to ~1 mg/ml in 100 mM potassium phosphate buffer, pH 7.4, and divided between two 1-ml cuvettes. After a baseline of equal light absorbance is obtained between 400 and 500 nm, 5  $\mu$ l of 20 mM NADH is added to the 1-ml sample cuvette, and the difference spectrum is recorded. The concentration of cytochrome  $b_5$  is determined from the absorbance difference between 410 nm (trough) and 425 nm (peak), based on an extinction coefficient of 185 mM<sup>-1</sup> cm<sup>-1</sup> (1).

### **Cytochrome P450**

The same samples used to determine the concentration of cytochrome  $b_5$  can be used to determine the concentration of cytochrome P450 by the method of Omura and Sato (1). After the cytochrome  $b_5$  spectrum is recorded, 5  $\mu$ l of 20 mM NADH is added to the reference cuvette (to balance the NADH previously added to the sample cuvette), and a few grains of solid sodium dithionite (0.2 - 2 mg) are added to both cuvettes (slightly more is added to the sample cuvette to give a positive absorbance reading at 400 nm). Immediately following the addition of dithionite, the contents of the sample cuvette are saturated with carbon monoxide (30-40 bubbles over a 15- to 20-sec period), and the carbon monoxide-difference spectrum of reduced microsomes is repeatedly recorded between 400 and 500 nm until two coincidental spectra are obtained (NB: In contrast to the cytochrome  $b_5$  spectrum, which develops in seconds, the cytochrome P450 spectrum may take up to 2 min to develop fully). The concentration of cytochrome P450 is determined from the absorbance difference between 450 nm (peak) and 490 nm (isosbestic point), based on an extinction coefficient of 91 mM<sup>-1</sup> cm<sup>-1</sup> (1).

### **Reference**

1. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. Evidence for its hemoprotein nature. *J Biol Chem* 1964;239:2379-2385.

**Procedure to measure the concentration of cytochrome  $b_5$  and cytochrome P450**

The concentration of cytochrome  $b_5$  and cytochrome P450 is determined with a DW 2C dual beam spectrophotometer (SLM Aminco, Urbana, Illinois). The instrument is used as described by the manufacturer with the following settings:

Lamp	VIS
Wavelength	400 to 500 nm
Beam Chopper Speed	Low (15 times/sec)
Time Base	Off
Wavelength Scan	5 nm/sec
Beam Selector	Sample and Reference
Filter	Open
Full Scale Absorbance	0.002 - 2.0 (depending on the concentration of cytochrome $b_5$ and cytochrome P450)

1. Prepare at least 2 ml of microsomes diluted to ~1.0 mg/ml in potassium phosphate buffer (100 mM, pH 7.4). Keep the samples at 4°C (*i.e.*, on ice). For multiple samples, a constant dilution may be made (*e.g.*, each sample may be diluted 20 fold) or each sample may be diluted to a constant protein concentration. A wide range of protein concentrations can be accommodated simply by changing the Full Scale Absorbance on the spectrophotometer.
2. Add 1 ml of the diluted microsomes to the 1-ml sample and reference cuvettes.
3. Record a baseline of equal light absorbance between 400 and 500 nm. This baseline should have an absolute absorbance value approximately equal to 40% of the Full-Scale Absorbance so that positive and negative absorbance changes can be monitored. On a vertical axis of 10 inches, the baseline should appear approximately 4 inches from the bottom.
4. Add 5  $\mu$ l of 20 mM NADH to the 1-ml sample cuvette and record the difference spectrum. Note: The final concentration of NADH is 100  $\mu$ M. Cytochrome  $b_5$  is reduced so rapidly that the spectrum can be recorded without delay. The spectrum of reduced *versus* oxidized cytochrome  $b_5$  is characterized by an absorbance peak at 425 nm and a trough at 410 nm. The concentration of cytochrome  $b_5$  is determined from the absorbance difference between 425 nm (peak) and 410 nm (trough), based on an extinction coefficient of 185 mM<sup>-1</sup>cm<sup>-1</sup> (see below).
5. After recording the cytochrome  $b_5$  spectrum, add 5  $\mu$ l of 20 mM NADH to the reference cuvette to balance the NADH previously added to the sample cuvette.
6. Add a few grains of solid sodium dithionite to the reference cuvette. The amount of dithionite is not weighed but is sufficient to cover the tip of a small spatula. The actual weight is between 0.2 and 1 mg.
7. Add a few grains of solid sodium dithionite to the sample cuvette. Slightly more dithionite is added to sample cuvette to give a positive absorbance reading at 400 nm.

8. Immediately saturate the contents of sample cuvette with 30-40 bubbles of carbon monoxide. The flow rate should be approximately 2 bubbles/sec, hence, this procedure should take 15-20 seconds.
9. Record the CO-difference spectrum of reduced microsomes from 400 - 500 nm repeatedly until two identical spectra are obtained. The spectrum may take up 2 min to develop fully. The spectrum of carboxyferrocyanochrome P450 *versus* ferricytochrome P450 is characterized by an absorbance peak at 450 nm, an isosbestic point at 490 nm, a trough at 405-410 nm, possibly with a shoulder or second absorbance peak at 420 nm. Absorbance at 420 nm is due to the presence of hemoglobin or cytochrome P420, which is the inactive form of cytochrome P450. Hemoglobin contamination can be assessed as described in SOP 09.00. The concentration of cytochrome P450 is determined from the absorbance difference between 450 nm (peak) and 490 nm (isosbestic point), based on an extinction coefficient of  $91 \text{ mM}^{-1}\text{cm}^{-1}$ , as described below.

**Calculation of cytochrome b<sub>5</sub> concentration**

The concentration of cytochrome b<sub>5</sub> in the sample cuvette is determined from the absorbance difference between 410 nm (trough) and 425 nm (peak) based on an extinction coefficient of 185 mM<sup>-1</sup>cm<sup>-1</sup> (which is the same as 0.185 μM<sup>-1</sup>cm<sup>-1</sup>).

$$[\text{Cytochrome } b_5] \text{ (nmol/ml)} = A_{425} - A_{410} \times 1 \text{ cm lightpath} \times \frac{1}{0.185 \mu\text{M}^{-1}\text{cm}^{-1}}$$

$$A_{425} - A_{410} = \text{absorbance difference between 425 nm (peak) and 410 nm (trough)}$$

$$= \text{fraction of full scale} \times \text{Full Scale Absorbance}$$

$$\text{lightpath} = \text{distance that light travels through the cuvettes (1 cm)}$$

$$0.185 \mu\text{M}^{-1}\text{cm}^{-1} = \text{extinction coefficient of reduced versus oxidized cytochrome } b_5.$$

This calculation determines the concentration of cytochrome b<sub>5</sub> in the sample cuvette (in units of nmol/ml). To calculate the concentration of cytochrome b<sub>5</sub> in the original sample, this value must be multiplied by the dilution factor.

In cuvettes with a 1-cm lightpath, a sample containing 1 μM cytochrome b<sub>5</sub> would produce an absorbance value of 0.185 between 425 and 410 nm. On a Full Scale Absorbance of 0.2 with a 10-inch vertical scale, the peak-to-trough distance would be 9.25 inches. As a fraction of full scale, this corresponds to 0.925.

$$A_{425} - A_{410} = \text{absorbance difference between 425 nm (peak) and 410 nm (trough)}$$

$$= \text{fraction of full scale} \times \text{Full Scale Absorbance}$$

$$= 0.925 \times 0.2$$

$$= 0.185$$

If the same sample were analyzed on a Full Scale Absorbance of 0.5, the peak-to-tough distance would 3.7 inches. As a fraction of full scale, this corresponds to 0.37.

$$A_{425} - A_{410} = \text{absorbance difference between 425 nm (peak) and 410 nm (trough)}$$

$$= \text{fraction of full scale} \times \text{Full Scale Absorbance}$$

$$= 0.37 \times 0.5$$

$$= 0.185$$

The above calculations illustrate how the choice of Full Scale Absorbance influences the peak-to-trough distance (9.25 inches *versus* 3.7 inches) but it does alter the calculated value  $A_{425} - A_{410}$  (which in both cases was determined to be 0.185).

### Calculation of cytochrome P450 concentration

The concentration of cytochrome P450 in the sample cuvette is determined from the absorbance difference between 450 nm (peak) and 490 nm (isosbestic point) based on an extinction coefficient of  $91 \text{ mM}^{-1}\text{cm}^{-1}$  (which is the same as  $0.091 \text{ }\mu\text{M}^{-1}\text{cm}^{-1}$ ).

$$[\text{Cytochrome P450}] \text{ (nmol/ml)} = A_{450} - A_{490} \times 1 \text{ cm lightpath} \times \frac{1}{0.091 \text{ }\mu\text{M}^{-1}\text{cm}^{-1}}$$

$$A_{450} - A_{490} = \text{absorbance difference between 425 nm (peak) and 410 nm (trough)} \\ = \text{fraction of full scale} \times \text{Full Scale Absorbance}$$

$$\text{lightpath} = \text{distance that light travels through the cuvettes (1 cm)}$$

$$0.091 \text{ }\mu\text{M}^{-1}\text{cm}^{-1} = \text{extinction coefficient of reduced versus oxidized cytochrome } b_5.$$

This calculation determines the concentration of cytochrome P450 in the sample cuvette (in units of nmol/ml). To calculate the concentration of cytochrome P450 in the original sample, this value must be multiplied by the dilution factor.

In cuvettes with a 1-cm lightpath, a sample containing  $1 \text{ }\mu\text{M}$  cytochrome P450 would produce an absorbance value of 0.091 between 450 and 490 nm. On a Full Scale Absorbance of 0.1 with a 10-inch vertical scale, the peak-to-trough distance would be 9.1 inches. As a fraction of full scale, this corresponds to 0.91.

$$A_{425} - A_{410} = \text{absorbance difference between 425 nm (peak) and 410 nm (trough)} \\ = \text{fraction of full scale} \times \text{Full Scale Absorbance} \\ = 0.91 \times 0.1 \\ = 0.091$$

If the same sample were analyzed on a Full Scale Absorbance of 0.2, the peak-to-trough distance would be 4.55 inches. As a fraction of full scale, this corresponds to 0.455.

$$A_{425} - A_{410} = \text{absorbance difference between 425 nm (peak) and 410 nm (trough)} \\ = \text{fraction of full scale} \times \text{Full Scale Absorbance} \\ = 0.455 \times 0.2 \\ = 0.091$$

The above calculations illustrate how the choice of Full Scale Absorbance influences the distance between the peak and isosbestic point (9.1 inches *versus* 4.55 inches) but it does not alter the calculated value  $A_{450} - A_{490}$  (which in both cases was determined to be 0.091).

### Cytochrome P420

The spectrum of carboxyferrocycytochrome P450 *versus* ferricytochrome P450 is characterized by an absorbance peak at 450 nm, an isosbestic point at 490 nm, a trough at 405-410 nm, possibly with a shoulder or second absorbance peak at 420 nm. Absorbance at 420 nm is due to the presence of hemoglobin or cytochrome P420, which is the inactive form of cytochrome P450. If the sample is not contaminated with hemoglobin, the concentration of cytochrome P420 can be calculated from the absorbance at 420 nm, as described by Guengerich (2).

1. Determine a *corrected isosbestic point* by multiplying the absorbance difference between 490 and 450 nm by 41/91, ( $\Delta A \times 0.45$ ) and subtracting this *correction value* from the original absorbance at 490 nm. To do this, measure the height of the peak at 450 nm relative to 490 nm and multiply this value by 0.45. For example, if the 450 nm peak were 5.0 inches above the baseline at 490 nm, the *correction value* would be 2.25 inches (5 inches  $\times$  0.45). A point 2.25 inches below the original absorbance value at 490 nm corresponds to the *corrected isosbestic point*.

2. Calculate the absorbance at 420 nm relative to the *corrected isosbestic point* at 490 nm.

$$A_{420} - A_{490} = \text{absorbance difference between 420 nm and 490 nm (corrected)}$$

$$= \text{fraction of full scale} \times \text{Full Scale Absorbance}$$

3. The concentration of cytochrome P420 in the sample cuvette is determined from the absorbance difference between 420 nm and 490 nm (corrected isosbestic point) based on an extinction coefficient of 110 mM<sup>-1</sup>cm<sup>-1</sup> (which is the same as 0.11 μM<sup>-1</sup>cm<sup>-1</sup>).

$$[\text{Cytochrome P420}] = A_{420} - A_{490} \times 1 \text{ cm lightpath} \times \frac{1}{0.11 \mu\text{M}^{-1}\text{cm}^{-1}}$$

(nmol/ml)

In hemoglobin-free microsomes, the absorbance at 420 nm can be used to calculate the amount of cytochrome P420. However, when the concentration of cytochrome P450 is determined by the method of Omura and Sato, any hemoglobin in the microsomal sample will contribute to the absorbance at 420 nm. Hemoglobin contamination can be assessed as described in SOP 09.00.

### Reference

2. Guengerich, FP, Analysis and characterization of enzymes. In: *Principles and Methods of Toxicology*. Second Edition. pp. 777-814. Raven Press, Ltd., New York, 1989.

**WORKSHEET FOR CYTOCHROME b<sub>5</sub> AND CYTOCHROME P450**

Project notebook number: \_\_\_\_\_

Date: \_\_\_\_\_

Samples (short description): \_\_\_\_\_  
*E.g., Rat liver microsomes*

Sample dilution:      Fold dilution: \_\_\_\_\_

Protein concentration after dilution: \_\_\_\_\_

**REAGENT DETAILS**

Chemical Name	Catalog No.	Batch No.	Date received	Expiration date
1. NADH	340-102			
2. Sodium dithionite	15,795-3			

Did the procedure to measure the concentration of cytochrome b<sub>5</sub> and cytochrome P450 conform to the Standard Operating Procedure?

Yes

No

If not, explain how the procedure deviated from standard operating procedure and what impact this would have on the results obtained.

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**SOP 16:00. TESTOSTERONE OXIDATION - HPLC ASSAY**

DATE: 05.26.92

**SOURCES OF SPECIAL REAGENTS****Testosterone metabolites**

	<b><u>Source</u></b>
Androstenedione	Sigma Chemical Co. (St. Louis, MO)
1 $\alpha$ -Hydroxytestosterone	G.D. Searle & Co. (Skokie, IL)
1 $\beta$ -Hydroxytestosterone	Dr.W. Levin, Hoffmann-La Roche, Inc. (Nutley, NJ)
2 $\alpha$ -Hydroxytestosterone	Steroid Reference Collection, MRC (London, England)
2 $\beta$ -hydroxytestosterone	Steroid Reference Collection, MRC (London, England)
6 $\alpha$ -hydroxytestosterone	Steroid Reference Collection, MRC (London, England)
6 $\beta$ -Hydroxytestosterone	Steraloids, In. (Wilton, NH)
6-dehydrotestosterone	Steraloids, In. (Wilton, NH)
7 $\alpha$ -hydroxytestosterone	Steraloids, In. (Wilton, NH)
7 $\beta$ -hydroxytestosterone	Dr.W. Levin, Hoffmann-La Roche, Inc. (Nutley, NJ)
11 $\alpha$ -hydroxytestosterone	Steraloids, In. (Wilton, NH)
11 $\beta$ -hydroxytestosterone	Sigma Chemical Co. (St. Louis, MO)
14 $\alpha$ -hydroxytestosterone	Steroid Reference Collection, MRC (London, England)
15 $\alpha$ -hydroxytestosterone	Steroid Reference Collection, MRC (London, England)
15 $\beta$ -hydroxytestosterone	G.D. Searle & Co. (Skokie, IL)
16 $\alpha$ -hydroxytestosterone	Sigma Chemical Co. (St. Louis, MO)
16 $\beta$ -hydroxytestosterone	Steraloids, In. (Wilton, NH)
16-ketotestosterone	Dr.W. Levin, Hoffmann-La Roche, Inc. (Nutley, NJ)
18-hydroxytestosterone	G.D. Searle & Co. (Skokie, IL).
19-hydroxytestosterone	Steraloids, In. (Wilton, NH) or Steroid Reference Collection, MRC (London, England)
Testosterone	Sigma Chemical Co. (St. Louis, MO)
17 $\beta$ -N,N-diethylcarbamoyl-4-methyl -4-aza-5 $\alpha$ -androstan-3-one (4-MA)	Dr. G. Rasmusson (Merck Sharp & Dohme, Rahway, NJ).

### **Purification of testosterone**

All reagents are used as supplied by the manufacturer with the exception of testosterone, which is purified by preparative, reverse-phase HPLC as described in Notebook 3029, page 24 (Kathleen Fitzgerald). The column is a Preparative Supelcosil C18 column (25 x 2.12 cm, 12  $\mu$ m particle size) from Supelco, Bellefonte, PA (Catalog No. 5-9185, column No. 157009AA).

Approximately 100 mg of testosterone is dissolved in methanol to give a solution of approximately 20 mg/ml. Aliquots (500  $\mu$ l) of this solution are injected onto a preparative C18 column at room temperature. Testosterone is eluted isocratically with methanol (HPLC grade) and monitored at 254 nm (attenuation 8) (See Notebook 3021, page 36 for HPLC parameters). Column eluent containing purified testosterone is collected during the time the absorbance peak for testosterone goes off scale and returns back to scale (see Notebook 3021, page 38 for a diagram of this procedure). Testosterone (retention time = 12.5-14.5 min) is preceded by androstenedione (retention time = 11.5 min) and followed by an unknown contaminant (retention time 15-15.5 min). Fractions are collected to minimize inclusion of androstenedione and the unknown metabolite. After all the testosterone solution has been injected on the column (usually 10 injections), an aliquot (500  $\mu$ l) of the pooled eluent fraction is re-injected on the column to check the purity of testosterone (this testosterone sample is also collected and added to the column eluent). If necessary, this purification procedure is repeated to obtain highly purified testosterone. The column eluent containing purified testosterone is evaporated to dryness in a Speed-Vac concentrator. The residue is weighed and redissolved in methanol to produce a 12.5 mM solution of testosterone (see below).

## **COMMON REAGENTS**

Unless indicated otherwise, de-ionized water is used. The following *Common Reagents* are prepared according to SOP 01.00 or SOP 02.00

Potassium phosphate buffer (1.0 M, pH 7.4)  
Magnesium chloride (100 mM)  
EDTA (100 mM, pH 7.4)  
Sucrose (250 mM)  
Glucose-6-phosphate (500 mM, pH 7.4)  
Glucose-6-phosphate dehydrogenase (100 Units/ml)  
NADP (100 mM, pH 7.4)

The following reagents are HPLC grade solvents, and are used as provided by the manufacturer.

Dichloromethane (methylene chloride)  
Methanol  
Acetonitrile  
Water (for HPLC mobile phases only)

## **PREPARATION OF SPECIAL REAGENTS**

### **Testosterone (MW = 288.4)**

Source: Sigma Chemical Company, St. Louis, MO (purified as described on page 1)  
Catalog No.: T-1500  
Storage conditions: -20°C in desiccated container  
Expiration date: None

### **Working solution**

Concentration: 12.5 mM (3.61 mg/ml)  
Solvent: Methanol  
Storage conditions: -20°C in desiccated container  
Expiration date: 5 years

### **Example**

Weigh out ~15 mg of testosterone and add methanol to give 3.61 mg/ml. If 13.8 mg of testosterone were weighed out, 3.82 ml of methanol would be added to give 3.61 mg/ml.

## SPECIAL REAGENTS

### Steroid 5 $\alpha$ -reductase inhibitor, 4-MA (MW = 388)

Proper name: (17 $\beta$ -*N,N*-diethylcarbamoyl-4-methyl-4-aza-5 $\alpha$ -androstan-3-one)  
Source: Dr. Gary Rasmusson, Merck, Sharp and Dohme, Rahway NJ  
Batch: L-636,028-000J021  
Storage conditions: -20°C in desiccated container  
Expiration date: None

**CAUTION:** 4-MA is a potent teratogen. Wear gloves and a mask when weighing out solid material.

### Working solution

Concentration: 1 mM (0.388 mg/ml)  
Solvent: Acetone (from Burdick & Jackson)  
Storage conditions: -20°C in desiccated container  
Expiration date: 1 year

### Example

Weigh out ~1 mg of 4-MA and add acetone to give 0.388 mg/ml. If 1.1 mg of 4-MA were weighed out, 2.84 ml of acetone would be added to give 0.388 mg/ml.

### HPLC Mobile Phases

In preparing the HPLC phases, each solvent is measured in a separate measuring cylinder and combined in a 2000 ml mixing cylinder. Each mobile phase is mixed in the mixing cylinder and transferred to a dark brown, narrow neck bottle (4-liter capacity). The narrow neck minimizes evaporation of the solvents. To remove dissolved gases, each mobile phase is sonicated for 5 min at room temperature under reduced pressure (by means of a vacuum pump).

<u>Solvent</u>	<u>Mobile phase A</u>	<u>Mobile phase B</u>
Water:	1280 ml	360 ml
Methanol:	700 ml	1600 ml
Acetonitrile:	20 ml	40 ml

## SPECIAL REAGENTS

### 11 $\beta$ -Hydroxytestosterone: Internal Standard

Unlike all other standards, the internal standard is dissolved in dichloromethane (methylene chloride). A 2.5 mM stock solution is prepared by dissolving 11 $\beta$ -hydroxytestosterone in dichloromethane at a concentration of 0.761 mg/ml. A 30  $\mu$ M working solution is prepared by adding 1.0 ml of 2.5 mM stock solution to 82.3 ml dichloromethane.

100  $\mu$ l of 30  $\mu$ M 11 $\beta$ -hydroxytestosterone is dispensed with a Hamilton repetitive syringe into each incubation mixture, after the reaction is stopped with 6 ml dichloromethane, i.e., each sample is spiked with 3,000 pmol of internal standard.

If 4 of the 6 ml dichloromethane is evaporated to dryness, the residue re-dissolved in 200  $\mu$ l of HPLC Solvent A, and 50  $\mu$ l injected on the column, then the amount of 11 $\beta$ -hydroxytestosterone injected on the column will be 500 pmol.

100% recovery is determined by comparing the peak area of the internal standard with the peak area of 11 $\beta$ -hydroxytestosterone that was dispensed into empty culture tubes and taken through the evaporation/reconstitution process. This is done by dispensing 100  $\mu$ l of 30  $\mu$ M internal standard into 4 empty culture tubes, evaporating the dichloromethane under nitrogen at 37°C, and re-dissolving the residue in 200  $\mu$ l HPLC Solvent A. If 50  $\mu$ l is injected then 750 pmol of 11 $\beta$ -hydroxytestosterone will be injected on the column. The different amounts of 11 $\beta$ -hydroxytestosterone injected on the column (500 *versus* 750 pmol) must be taken into account when comparing the peak areas.

**NOTE:** Before using 11 $\beta$ -hydroxytestosterone as an internal standard, it must first be verified that this potential metabolite is not formed from testosterone during an incubation with biological samples. This is an important consideration when analyzing testosterone oxidation by new sources of microsomes (e.g., microsomes from different tissues or different species to those normally tested).

## SPECIAL REAGENTS

### Testosterone and metabolites for HPLC

All standards are dissolved in HPLC-grade methanol. Concentration of each stock solution = 2.5 mM. Each stock solution is sealed and stored at -20°C in a desiccated container.

MW Testosterone = 288.4	2.5 mM = 0.721 mg/ml
MW Androstenedione = 286.4	2.5 mM = 0.716 mg/ml
MW 6-Dehydrotestosterone = 286.4	2.5 mM = 0.716 mg/ml
MW Monohydroxytestosterone = 304.4	2.5 mM = 0.761 mg/ml
MW Dihydrotestosterone = 290.4	2.5 mM = 0.726 mg/ml

### Mixture of standards

The routine mixture of standards consists of 15 steroids, and is prepared by mixing 25 µl of each standard and adjusting the volume to 5 ml. This is divided into five 1-ml aliquots, which are stored in a dry, sealed container at -20°C.

The 15 standards are:

		<b>Notebook</b>
(1)	1α-hydroxytestosterone	5000-50
(2)	2α-	5000-50
(3)	2β-	4003-12
(4)	6α-	5000-50
(5)	6β-	4003-63
(6)	7α-	5000-50
(7)	11α-	5000-50
(8)	15α-	5000-50
(9)	15β-	4002-83
(10)	16α-	5000-50
(11)	16β-	5000-50
(12)	18-hydroxytestosterone	4002-83
(13)	6-Dehydrotestosterone	4007-74
(14)	Androstenedione	4003-63
(15)	Testosterone	4003-63

The concentration of each standard in the mix is 12.5 µM. Add 40 µl of standard mix to 168.3 µl of HPLC Solvent A. Inject 50 µl. Amount of each standard injected = 120 pmol.

Four or more mixtures of standards are included in each HPLC analysis.

### RARE OR DUPLICATE STANDARDS

Methanolic solutions of  $1\beta$ -hydroxytestosterone,  $7\beta$ -hydroxytestosterone and 16-ketotestosterone are stored in a separate container at  $-20^{\circ}\text{C}$ . The amounts of these rare standards were too small to permit accurate weighing, but their concentration is approximately 2.5 mM, based on their absorbance at 254 nm (see book 4003-40 for details).

These "rare" standards are to be used sparingly.

This same container also contains 2.5 mM methanolic solutions of  $5\alpha$ - and  $5\beta$ -dihydrotestosterone (see book 5000-50 for details).

Other steroids include:

11 $\alpha$ -hydroxytestosterone (2.5 mM)

19-hydroxytestosterone from Steraloids (2.5 mM)

19-hydroxytestosterone from the Steroid Reference Collection (2.5 mM).

A mixture of  $5\alpha$ -dihydrotestosterone, testosterone and androstenedione (see book 1004-22 for details).

## **PROTOCOL**

### **Incubation conditions**

The pathways of testosterone oxidation catalyzed by liver microsomes are determined by reverse-phase HPLC, essentially as described by Wood *et al.* (1) and Sonderfan *et al.* (2,3). Liver microsomes (0.1 to 1.0 mg) are incubated at 37°C in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl<sub>2</sub> (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/ml), testosterone (250 μM) and the steroid 5α-reductase inhibitor, 4-MA (1 μM), at the final concentrations indicated. Testosterone (12.5 mM) and 4-MA (1 mM) are added to each 1-ml incubation in 20 μl methanol and 1 μl acetone, respectively. Reactions are started by addition of the NADPH-generating system, and are stopped after 1-20 min by addition of 6 ml of dichloromethane. Zero-time incubations serve as blanks. If possible, a selected microsomal sample should be incubated for half and twice the regular incubation period (to verify metabolite formation is proportional to incubation time), and at half and twice the regular protein concentration (to ensure that metabolite formation is proportional to enzyme concentration). The sample selected for these additional incubation should be in plentiful supply and should have a relatively high capacity to oxidize testosterone.

1. Liver microsomes are typically diluted to 1 to 10 mg protein/ml with 250 mM sucrose.
2. An aliquot (100 or 200 μl) is dispensed with a Pipetman P200 into 18 x 150 mm test tubes on ice.
3. An aliquot (750 or 850 μl) of freshly prepared *substrate solution* (see below) is added to each tube.
4. Reactions are started at 10 or 15 second intervals by addition of an NADPH-generating system.
5. Each tube is incubated at 37°C in a Dubanoff shaking water bath for 1-20 min.
6. Reactions are stopped by addition of 6 ml of dichloromethane (added with a dispenser).
7. Each sample is spiked with 3 nmol of 11β-hydroxytestosterone (in 100 μl dichloromethane, dispensed with a Hamilton Repeating Syringe) and vigorously mixed on a batch vortexer (3 x 1 min). The internal standard is also added to each of four culture tubes (12 x 75 mm), and the dichloromethane is allowed to evaporate at room temperature. These four samples will be used to determine the 100% 11β value (see below).
8. After the two phases are separated by low-speed centrifugation (2,000 g for 10 min), the aqueous (upper) phase is aspirated and discarded.
9. An aliquot (4 ml) of the organic phase is transferred to a culture tube (12 x 75 mm) and evaporated in a Speed-Vac concentrator (Savant Instruments, Farmingdale, NY).

10. The residue is redissolved in 200  $\mu$ l mobile phase A (see below), and a 50- $\mu$ l aliquot is analyzed by HPLC (see below). The residue in the four tubes set up to determine the 100% 11 $\beta$  value (see step 7) is redissolved in 300  $\mu$ l of mobile phase A, and a 50- $\mu$ l aliquot is analyzed by HPLC. Note: The residue from each incubated sample is redissolved in 200  $\mu$ l of mobile phase A, whereas the residue from the four tubes set up to determine the 100% 11 $\beta$  value is redissolved in 300  $\mu$ l of mobile phase A. This difference compensates for the fact that the residue from each incubated sample was derived from only two thirds of the organic phase (4 of 6 ml), hence, it contained only two thirds of the added 11 $\beta$ -hydroxytestosterone). In contrast, the residue from the tubes set up to determine the 100% 11 $\beta$  value contained all of the added 11 $\beta$ -hydroxytestosterone.
11. Either before or after the samples have been incubated, the NADPH-generating system is analyzed spectrophotometrically to ensure production of NADPH, according to SOP 02.00.

**Contents of incubation mixture**

Incubation volume:	1 ml
Volume of microsomes:	100 or 200 $\mu$ l
Volume of substrate solution:	850 or 750 $\mu$ l
Volume of NADPH-generating system:	50 $\mu$ l

**Dilution of microsomes**

Microsomal samples are diluted to the desired protein concentration (1-10 mg/ml) with 250 mM sucrose. The amount of microsomal material should be slightly greater than the amount to be added to the incubation. For example, 250  $\mu$ l of diluted microsomal protein might be prepared to carry out two incubations each containing 100  $\mu$ l of microsomal material. The following equation is used to calculate the amount of 250 mM sucrose to add to each microsomal sample to achieve the desired protein concentration:

$$\text{Volume of 250 mM sucrose} = \left( \frac{\text{Initial [Protein]}}{\text{Final [Protein]}} \times \text{vol of microsomes} \right) - \text{vol of microsomes}$$

**Substrate solution**

The *substrate solution* is prepared as shown below. Approximately 10% more substrate solution is prepared than actually needed. For example, if the experiment involved 90 incubation tubes, the amount of substrate solution prepared would be calculated for 100 tubes, as shown below.

Stock reagent	Amount/tube	No. Tubes	Volume	Final conc.
Water	739 $\mu$ l/tube*	100	73.9 ml	
Potassium phosphate buffer (1 M, pH 7.4)	50 $\mu$ l/tube	100	5 ml	50 mM
EDTA (100 mM, pH 7.4)	10 $\mu$ l/tube	100	1 ml	1 mM
MgCl <sub>2</sub> (100 mM)	30 $\mu$ l/tube	100	3 ml	3 mM
Testosterone (12.5 mM dissolved in methanol)	20 $\mu$ l/tube	100	2 ml	250 $\mu$ M
4-MA (1 mM dissolved in acetone)	1 $\mu$ l/tube	100	0.1 ml	1 $\mu$ M

\*This volume of water is used if the microsomal protein is added in 100  $\mu$ l. If the volume of microsomes is increased from 100 to 200  $\mu$ l, the volume of water is decreased from 739 to 639  $\mu$ l.

## HPLC

The HPLC procedure for testosterone metabolites is carried out with a Shimadzu LC-6A binary gradient HPLC system equipped with a SIL-6A autosampler, SPD-6A variable wavelength UV detector and CTO-6A column heater (Shimadzu Scientific Instruments, Columbia, MD). The reverse-phase column is a Supelcosil LC-18 octyldecylsilane ( $C_{18}$ ) column (5  $\mu$ m particle size, 15 cm x 4.6 mm), which is preceded by a Supelcosil LC-18 guard column (40  $\mu$ m particle size, 2 cm x 4.6 mm) (Supelco, Bellefonte, PA). Metabolites are quantified by comparison of their peak areas (integrated by a Shimadzu C-R3A recording data processor) with those of authentic standards.

Testosterone, androstenedione, 6-dehydrotestosterone, and 12 hydroxytestosterone isomers are resolved by binary gradient HPLC on a Supelcosil LC-18 reverse-phase octyldecylsilane column (15 cm) preceded by a Supelcosil LC-18 guard column. The method is similar to that described by Sonderfan *et al.* (2), with slight changes in mobile phase A and the solvent program. The ratio of water:methanol:acetonitrile (v/v/v) is 64:35:1 for mobile phase A, and 18:80:2 for mobile phase B. The total flow rate is 2.0 ml/min and the column temperature is 40°C. The solvent program is as follows: 100% A from zero to 10 min, a linear gradient from zero to 45% B from 10-28 min, a linear gradient from 45% to 100% B from 28-29 min, 100% B from 29 to 31 min, a linear gradient to 100% A from 31-32 min followed by re-equilibration with 100% A from 32 to 34 min. Total analysis time is ~37 min per sample. The slight changes in mobile phase A and the gradient system enable 6 $\beta$ - and 15 $\alpha$ -hydroxytestosterone to be resolved, whereas they co-eluted in the original system described by Sonderfan *et al.* (2). Testosterone and its potential metabolites are monitored at 254 nm. The retention times for 6 $\alpha$ -, 15 $\beta$ -, 6 $\beta$ -, 15 $\alpha$ -, 7 $\alpha$ -, 16 $\alpha$ -, 16 $\beta$ -, 1 $\beta$ -, 18-, 11 $\beta$ -, 2 $\alpha$ -, 2 $\beta$ -hydroxytestosterone, androstenedione, 6-dehydrotestosterone and testosterone are approximately 6.5, 7.0, 9.5, 10.2, 11.4, 15.2, 16.3, 17.3, 17.8, 18.7, 19.6, 20.3, 24.0, 24.3 and 26.0 min, respectively, although these may change slightly one column to the next. Although not formed metabolically, 7 $\beta$ -, 14 $\alpha$ - and 19-hydroxytestosterone can also be resolved by this HPLC system (2). However, this HPLC system does not resolve 1 $\alpha$ - from 1 $\beta$ -hydroxytestosterone, nor does it resolve 18- from 12 $\alpha$ -hydroxytestosterone (4,5). Metabolites are quantified by comparison of their peak areas with those of authentic standards. The recovery of testosterone and its metabolites from incubation mixtures is essentially quantitative (>95%). Nevertheless, sample-to-sample variations in extraction efficiency are corrected based on recovery of the internal standard, 11 $\beta$ -hydroxytestosterone.

The samples typically analyzed by HPLC are as follows: Each test sample, 4 samples of internal standard used to calculate the 100% 11 $\beta$  value, and 4 samples of a mixture of authentic metabolites. The 4 internal standards and the 4 mixtures of standards are usually dispersed at regular intervals throughout the test samples, although this is not necessary.

## CALCULATIONS

### Recovery of 11 $\beta$ -hydroxytestosterone (internal standard)

Fractional recovery is calculated as the quotient of the area under the curve for 11 $\beta$ -hydroxytestosterone in each test sample (AUC 11 $\beta$  TEST) and the average area under the curve for the four tubes set up to determine the 100% 11 $\beta$  value (see step 7, page 8).

$$\text{Fractional recovery} = \frac{\text{AUC 11}\beta \text{ TEST}}{\text{Average 100\% 11}\beta \text{ value}}$$

This equation assumes that, for each test sample, 4 of 6 ml of organic phase was evaporated to dryness and the residue redissolved in 200  $\mu$ l of mobile phase, and that, for the 100% 11 $\beta$  samples, the residue was dissolved in 300  $\mu$ l of mobile phase. If the residue from the 100% 11 $\beta$  samples were redissolved in only 200  $\mu$ l of mobile phase, the average 100% 11 $\beta$  value must be multiplied by  $\frac{2}{3}$  (see part 10, page 9)

Most fractional recoveries fall in the 0.8 to 1.1 range (indicating that between 80 and 110% of the metabolites had been recovered). When values fall outside the range 0.5 to 1.5, the sample should be re-analyzed.

### Correcting AUC values

The AUC value for each metabolite in each test sample is adjusted for the fractional recovery of the internal standard as follows:

$$\text{Corrected AUC} = \frac{\text{AUC value}}{\text{Fractional recovery}}$$

For example, if only half of the metabolites and internal standard were recovered, the fractional recovery value would be 0.5. When divided by 0.5, each AUC value would double, which corrects for the fact that only half the metabolites were recovered.

### Calculating rates of testosterone oxidation

The rate of formation of each metabolite (pmol/mg protein/min) is calculated as follows:

$$\text{Rate} = \frac{\text{Corrected AUC}}{\text{Standard AUC}} \times 120 \text{ pmol} \times \frac{200 \mu\text{l}}{50 \mu\text{l}} \times \frac{6 \text{ ml}}{4 \text{ ml}} \times \frac{1}{\text{mg protein}} \times \frac{1}{\text{time (min)}}$$

where: **Corrected AUC** is the sample AUC of the metabolite corrected for recovery of the internal standard (see previous page),

**Standard AUC** is the average AUC of the same metabolite in the mixture of authentic standard-metabolites

**120 pmol** is the amount of each standard metabolite injected on the HPLC column

**200  $\mu\text{l}$**  is the volume of mobile phase used to redissolve the organic residue, of which only **50  $\mu\text{l}$**  was analyzed

**6 ml** is the volume of organic solvent used to extract the metabolites, of which only **4 ml** was evaporated to dryness.

**mg protein** is the amount of protein in the 1 ml incubation mixture

**time** is the incubation period (in minutes)

**REFERENCES**

1. Wood AW, Ryan DE, Thomas PE and Levin W, Regio- and stereoselective metabolism of two C<sub>19</sub> steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes. *J. Biol. Chem.* **258**: 8839-8847, 1983.
2. Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK and Parkinson A, Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P-450. *Arch. Biochem. Biophys.* **255**: 27-41, 1987.
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4. Arlotto MP, Greenway DJ and Parkinson A, Purification of two isozymes of rat liver microsomal cytochrome P-450 with testosterone 7 $\alpha$ -hydroxylase activity. *Arch. Biochem. Biophys.* **270**: 441-457, 1989.
5. Smith, S, Korzekwa, KR, Aoyama, T, Gonzalez, FJ, Darbyshire, JF, Sugiyama, K and Gillette, JR, 12 $\alpha$ -Hydroxytestosterone: A hitherto unidentified testosterone metabolite produced by cytochrome P-450 2A2. *Drug Metab. Dispos.* **20**: 566-571, 1992.

## TESTOSTERONE ASSAY WORK SHEETS

### **Instructions:**

1. Use either page 1a or 1b of the worksheets depending on the volume of microsomes to be added to the incubation.
2. Use pages 2a-2c of the worksheets as necessary (depending on the number of incubation samples).
3. Complete worksheet pages 3 and 4.

**TESTOSTERONE ASSAY WORK SHEET - PAGE 1a****Use this work sheet for 100  $\mu$ l microsomal samples**

DATE: \_\_\_\_\_

Notebook or protocol No: \_\_\_\_\_

**Microsomes (100  $\mu$ l per tube)**

Protein concentration: \_\_\_\_\_ mg/ml

Amount of protein per incubation: \_\_\_\_\_ mg

Source Species: \_\_\_\_\_

Sex: \_\_\_\_\_

Treatment: \_\_\_\_\_

Organ: \_\_\_\_\_

**Substrate mix (850  $\mu$ l per tube)**

<b>Stock reagent</b>	<b>Amount/tube</b>	<b>No. Tubes</b>	<b>Volume</b>	<b>Final conc.</b>
Water	739 $\mu$ l/tube			
Potassium phosphate buffer (1 M, pH 7.4)	50 $\mu$ l/tube			50 mM
EDTA (100 mM, pH 7.4)	10 $\mu$ l/tube			1 mM
MgCl <sub>2</sub> (100 mM)	30 $\mu$ l/tube			3 mM
Testosterone (12.5 mM dissolved in methanol)	20 $\mu$ l/tube			250 $\mu$ M
4-MA (1 mM dissolved in acetone)	1 $\mu$ l/tube			1 $\mu$ M

**NADPH-generating system (50  $\mu$ l per tube)**

<b>Stock reagent</b>	<b>Amount/tube</b>	<b>No. Tubes</b>	<b>Volume</b>	<b>Final conc.</b>
Water	20 $\mu$ l/tube			
Glucose-6-phosphate (500 mM, pH 7.4)	10 $\mu$ l/tube			5 mM
Glucose-6-phosphate dehydrogenase (100 Units/ml)	10 $\mu$ l/tube			1 Unit/ml
NADP (100 mM, pH 7.4)	10 $\mu$ l/tube			1 mM

Analyst \_\_\_\_\_ Date \_\_\_\_\_

**TESTOSTERONE ASSAY WORK SHEET - PAGE 1b**

**Use this work sheet for 200 µl microsomal samples**

**DATE:** \_\_\_\_\_

**Notebook or protocol No:** \_\_\_\_\_

**Microsomes (100 µl per tube)**

Protein concentration: \_\_\_\_\_ mg/ml  
 Amount of protein per incubation: \_\_\_\_\_ mg  
 Source Species: \_\_\_\_\_  
 Sex: \_\_\_\_\_  
 Treatment: \_\_\_\_\_  
 Organ: \_\_\_\_\_

**Substrate mix (750 µl per tube)**

<u>Stock reagent</u>	<u>Amount/tube</u>	<u>No. Tubes</u>	<u>Volume</u>	<u>Final conc.</u>
Water	639 µl/tube			
Potassium phosphate buffer (1 M, pH 7.4)	50 µl/tube			5 mM
EDTA (100 mM, pH 7.4)	10 µl/tube			1 mM
MgCl <sub>2</sub> (100 mM)	30 µl/tube			3 mM
Testosterone (12.5 mM dissolved in methanol)	20 µl/tube			250 µM
4-MA (1 mM dissolved in acetone)	1 µl/tube			1 µM

**NADPH-generating system (50 µl per tube)**

<u>Stock reagent</u>	<u>Amount/tube</u>	<u>No. Tubes</u>	<u>Volume</u>	<u>Final conc.</u>
Water	20 µl/tube			
Glucose-6-phosphate (500 mM, pH 7.4)	10 µl/tube			5 mM
Glucose-6-phosphate dehydrogenase (100 Units/ml)	10 µl/tube			1 Unit/ml
NADP (100 mM, pH 7.4)	10 µl/tube			1 mM

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**TESTOSTERONE ASSAY WORK SHEET - PAGE 2a**

Sample	Tube	Volume of microsomes	Volume of substrate solution	Volume of NADPH system	Incubation time
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	14				
	15				
	16				
	17				
	18				
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	26				
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	28				
	29				
	30				
	31				
	32				
	33				
	34				
	35				
	36				
	37				
	38				
	39				
	40				

**Analyst** \_\_\_\_\_

**Date** \_\_\_\_\_

**TESTOSTERONE ASSAY WORK SHEET - PAGE 2b**

Sample	Tube	Volume of microsomes	Volume of substrate solution	Volume of NADPH system	Incubation time
	41				
	42				
	43				
	44				
	45				
	46				
	47				
	48				
	49				
	50				
	51				
	52				
	53				
	54				
	55				
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	69				
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	71				
	72				
	73				
	74				
	75				
	76				
	77				
	78				
	79				
	80				

**Analyst** \_\_\_\_\_

**Date** \_\_\_\_\_

**TESTOSTERONE ASSAY WORK SHEET - PAGE 2c**

Sample	Tube	Volume of microsomes	Volume of substrate solution	Volume of NADPH system	Incubation time
	81				
	82				
	83				
	84				
	85				
	86				
	87				
	88				
	89				
	90				
	91				
	92				
	93				
	94				
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	112				
	113				
	114				
	115				
	116				
	117				
	118				
	119				
	120				

**Analyst**

**Date**

**TESTOSTERONE ASSAY WORK SHEET - PAGE 3**

**DATE** \_\_\_\_\_

**Notebook or Project No.** \_\_\_\_\_

**Pipette Identification No.:**

**Pipetman P5000:** \_\_\_\_\_

**Pipetman P1000:** \_\_\_\_\_

**Pipetman P200:** \_\_\_\_\_

**Pipetman P20:** \_\_\_\_\_

**Special Reagents**

**Testosterone (see page 2)**  
**(12.5 mM dissolved in methanol)**

**Catalog No.** Sigma T-1500

**Batch or lot No:** \_\_\_\_\_

**Date purified:** \_\_\_\_\_

**Notebook #:** \_\_\_\_\_

**Expiration date of working solution:** \_\_\_\_\_

**4-MA (see page 3)**  
**(1 mM dissolved in acetone)**

**Notebook #** \_\_\_\_\_

**Expiration date of working solution:** \_\_\_\_\_

**Common Reagents**

**Expiration Date of Working Solution**

**250 mM Sucrose** \_\_\_\_\_

**Potassium phosphate buffer (1 M, pH 7.4)** \_\_\_\_\_

**EDTA (100 mM, pH 7.4)** \_\_\_\_\_

**MgCl<sub>2</sub> (100 mM)** \_\_\_\_\_

**Glucose-6-phosphate (500 mM, pH 7.4)** \_\_\_\_\_

**Glucose-6-phosphate dehydrogenase (100 Units/ml)** \_\_\_\_\_

**NADP (100 mM, pH 7.4)** \_\_\_\_\_

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**TESTOSTERONE ASSAY WORK SHEET - PAGE 4**

<u>Solvent</u>	<u>Supplier</u>	<u>Catalog No.</u>	<u>Batch or Lot No.</u>
Dichloromethane			
Methanol			
Acetonitrile			
Water			

**HPLC SYSTEM**

HPLC Number (System 1 or 2): \_\_\_\_\_

File number: \_\_\_\_\_

Column number (from column tag): \_\_\_\_\_

Actual column oven reading: \_\_\_\_\_

Actual flow rate (ml/min): \_\_\_\_\_

Data storage disc: \_\_\_\_\_

**Protocol checks**

1. Did the concentration of NADPH in the assay exceed 0.2 mM? \_\_\_\_\_
2. Were any fractional recovery values less than 0.5 or greater than 1.5? \_\_\_\_\_
3. Was metabolite formation directly proportional to incubation time? \_\_\_\_\_
4. Was metabolite formation directly proportional to protein concentration? \_\_\_\_\_
5. Were there any deviations from the standard operating procedure? \_\_\_\_\_

If the answer to question 1, 2, 3 or 4 is no, what actions were taken? If the answer to question 5 is yes, describe the deviation(s) from standard protocol, its impact on the results obtained, and what actions, if any, were taken (use additional pages if necessary).

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

## **SOP 17.00. UDP-Glucuronosyltransferase (UDPGT) assay with testosterone as substrate**

**DATE:** 06.11.92

### **COMMON REAGENTS**

The following reagents are prepared according to SOP 01.00.:

1. Tris.HCl (1.0 M, pH 8.0 at room temperature [pH 7.7 at 38°C]).
2. Magnesium chloride (100 mM MgCl<sub>2</sub>)
3. EDTA (100 mM, pH 7.4).
4. Sucrose (250 mM)

### **SOURCES OF SPECIAL REAGENTS**

#### **3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate**

CHAPS (detergent)

**Source:** Calbiochem, San Diego, California  
**Catalog number:** 220201  
**Molecular weight:** 614.9 g/mol  
**Storage:** Room temperature  
**Expiration date:** Five years

#### **D-Saccharic acid-1,4-lactone**

β-glucuronidase inhibitor.

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog number:** S0375  
**Molecular weight:** 192 g/mol  
**Storage:** 0-5° C  
**Expiration date:** Five years

#### **UDP-glucuronic acid (sodium salt)**

UDPGA (cofactor)

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog number:** U6751  
**Molecular weight:** 580.3 g/mol  
**Storage:** < 0° C  
**Expiration date:** Five years

**Testosterone**

Non-radioactive substrate

**Source:** Sigma Chemical Company, St. Louis, Missouri  
**Catalog number:** T-1500  
**Molecular weight:** 288.4 g/mol  
**Storage:** -20°C in desiccated container  
**Expiration date:** None

Testosterone is purified by preparative HPLC as described in SOP 16.00.

**[<sup>14</sup>C] Testosterone**

Radioactive substrate

**Source:** Amersham, Arlington Heights, Illinois  
**Catalog number:** CFA.129  
**Specific activity:** 50-60 mCi/mmol (actual value may vary from lot-to-lot).  
**Concentration:** 0.05 mCi/ml in toluene  
**Amount/vial:** Each vial contains 50  $\mu$ Ci (1 ml), which is sufficient for ~200 incubations  
**Storage:** -20° C  
**Expiration date:** Five years

**PREPARATION OF STOCK/WORKING SOLUTIONS OF SPECIAL REAGENTS**

**10% CHAPS (160 mM)**

1. Dissolve 10 g of CHAPS in a final volume of 100 ml de-ionized water.
2. Add 100 µl of 100 mM EDTA, pH 7.4.

**Storage:** Store in polypropylene or polyethylene bottle at 4°C.  
**Expiration date:** Two years

**10 mM D-Saccharic acid-1,4-lactone (MW = 192)**

1. Weigh out ~5 mg the β-glucuronidase inhibitor.
2. Dissolve in water to give 1.92 mg/ml.

**Storage:** Store in glass, polypropylene or polyethylene bottle at -20°C.  
**Expiration date:** Six months

**40 mM UDP-glucuronic acid.**

1. Weigh out ~1.4 mg UDPGA per incubation tube.
2. Dissolve in de-ionized water to give 27.4 mg/ml.

**Storage:** Do not store the aqueous solution (*i.e.*, prepare a fresh solution on the day of the assay).  
**Expiration date:** One day.

**CHAPS-solubilization buffer.**

1. Prepare the solubilization buffer by combining the components listed in the Table below (combine in the order they are listed)

Reagent	Amount	Concentration
1 M Tris.HCl, pH 8.0.	8.00 ml	400 mM
Sucrose (solid)	1.71 g	250 mM
10% CHAPS (160 mM)	1.25 ml	0.625% (10 mM)
Water	Adjust volume to 20 ml	

**Storage:** This buffer can be stored at 4°C if it has been filtered through a 0.45 µm nylon filter to remove particulate matter  
**Expiration date:** One year

**12.5 mM Testosterone.**

1. This solution is prepared from purified testosterone as described in SOP 16.00 (*Testosterone Oxidation*). Note: 12.5 mM = 3.61 mg/ml. The solvent is methanol.

**Storage:** The solution is stored at -20°C.

**Expiration date:** 5 years.

**[<sup>14</sup>C]-Testosterone.**

This radioactive substance is supplied in 1-ml vials containing 50 μCi (0.05 mCi) of [<sup>14</sup>C]-testosterone with a specific activity of 50-60 mCi/mmol. The concentration of radioactive testosterone is 0.83 - 1.0 mM dissolved in toluene. The exact concentration can be calculated as follows:

$$(1) \quad \text{Concentration of } [^{14}\text{C}]\text{-testosterone (mM)} = \frac{0.05 \text{ mCi/ml}}{\text{Specific activity (mCi/mmol)}}$$

The concentration of radioactive (*hot*) testosterone must be calculated because it slightly influences the overall substrate concentration when mixed with non-radioactive (*cold*) testosterone, as indicated below.

**Working solution of [<sup>14</sup>C]-testosterone.**

1. Transfer the 1-ml solution of [<sup>14</sup>C]-testosterone (50 μCi) to a culture tube and evaporate the toluene in a Speed-Vac Concentrator.
2. Redissolve the residue of [<sup>14</sup>C]-testosterone in 1 ml of 12.5 mM testosterone (*i.e.*, the *cold* testosterone dissolved in methanol).
3. Calculate the overall concentration of testosterone from the following equation:

$$[\text{TESTOSTERONE}]_{\text{OVERALL}} = [\text{TESTOSTERONE}]_{\text{COLD}} + [\text{TESTOSTERONE}]_{\text{HOT}}$$

where  $[\text{TESTOSTERONE}]_{\text{COLD}}$  is 12.5 mM and  $[\text{TESTOSTERONE}]_{\text{HOT}}$  is 0.83 to 1.0 mM (the exact concentration is determined from equation 1 as described above).

Note: Because the volume of *cold* testosterone used to redissolve the residue of [<sup>14</sup>C]-testosterone is equal to the volume of *hot* testosterone evaporated, the concentration of [<sup>14</sup>C]-testosterone remains unchanged at 50 μCi/ml (0.05 mCi/ml).

**Storage:** The working solution is stored at -20°C.

**Expiration date:** 2 years.

**Glucuronidation of [<sup>14</sup>C]-testosterone by rat liver microsomes.**

The rate of testosterone glucuronidation by liver microsomes is determined by a solvent partition method, essentially as described by Jacobson *et al.* (1) and Arlotto *et al.* (2). Microsomal protein (5 mg/ml) is solubilized for 15 min with an equal volume of 10 mM CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane) in 250 mM sucrose and 400 mM Tris.HCl (pH 8.0 at room temperature) prior to its addition (in 50 µl aliquots) to incubation mixtures. The final incubation volume is 500 µl, and contains Tris.HCl buffer (200 mM, pH 8.0), MgCl<sub>2</sub> (10 mM), EDTA (1 mM), D-saccharic acid-1,4-lactone (0.1 mM), UDP-glucuronic acid (4 mM), CHAPS (0.5 mM), [<sup>14</sup>C]-testosterone (130 µM, 0.5 µCi/ml) and solubilized microsomal protein (0.25 mg/ml), at the final concentrations indicated. Reactions are started with UDP-glucuronic acid and stopped after a 15-min incubation at 37°C with 6 ml of dichloromethane, followed by 1.1 ml of water (to increase the volume of the aqueous phase to 1.6 ml). After vigorous mixing on a batch vortex mixer (3 x 1 min), the aqueous and organic phase are separated by centrifugation (2,000 g for 10 min). The extraction with 6 ml dichloromethane is repeated. An aliquot (400 µl = 25%) of the aqueous (upper) phase (containing testosterone glucuronide) is mixed with 5 ml of BioSafe Biodegradable Counting Cocktail (Research Products International Corporation, Mount Prospect, IL), and the amount of radioactivity is determined with a Packard 2000 CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL). Zero-time incubations serve as blanks.

Each 500-µl incubation consists of 50 µl of microsomes, 50 µl of UDP-glucuronic acid, and 400 µl of substrate solution (Tris.HCl, MgCl<sub>2</sub>, EDTA, D-saccharic acid-1,4-lactone, CHAPS and [<sup>14</sup>C]-testosterone). Aliquots of this substrate solution (100 µl = 25% of the amount added to each incubation) are also analyzed by scintillation spectrometry. The amount of radioactivity in 25% of the aqueous phase (400 µl) will equal the amount of radioactivity in 25% of the substrate solution (100 µl) only if 100% of the substrate (testosterone) is converted to water-soluble metabolites (testosterone glucuronide). Therefore, the ratio of the radioactivity in the aqueous phase to the radioactivity in the substrate solution represents the fraction of substrate converted to metabolites. This ratio can be used to determine the overall rate of testosterone glucuronidation, based on the following equation:

Rate of testosterone glucuronidation (nmol/mg protein/min)

$$= \frac{\text{dpm in 400 } \mu\text{l of aqueous phase}}{\text{dpm in 100 } \mu\text{l of substrate solution}} \times \frac{\text{nmol testosterone}}{\text{incubation}} \times \frac{1}{\text{mg protein/incubation}} \times \frac{1}{\text{min}}$$

where nmol testosterone/incubation = 67 - 68 depending on the overall concentration of [<sup>14</sup>C]-testosterone.

**PROTOCOL****Basic incubation conditions**

Incubation volume = 0.5 ml.	Microsomes:	50 $\mu$ l per tube
	Substrate mix:	400 $\mu$ l per tube
	Cofactor (40 mM UDPGA):	50 $\mu$ l per tube

The following protocol assumes that the specific activity of [ $^{14}$ C]-testosterone was originally 50 mCi/mmol. If 50  $\mu$ Ci of *hot* testosterone were evaporated to dryness and redissolved in 1 ml of 12.5 mM *cold* testosterone (as described above), the overall concentration of testosterone would be 13.5 mM (*i.e.*, the *hot* testosterone would increase the concentration of the *cold* testosterone by 1 mM). The concentration of [ $^{14}$ C]-testosterone would be 50  $\mu$ Ci/ml.

The *substrate solution* is prepared as shown below. Approximately 10% more substrate solution is prepared than actually needed. For example, if the experiment involved 90 incubation tubes, the amount of substrate solution prepared would be calculated for 100 tubes, as shown below. Prepare the substrate solution *ice*.

Stock reagent	Amount/tube	No. Tubes	Volume	Final conc.
Water	245 $\mu$ l/tube	100	24.5 ml	
Tris.HCl buffer (1.0 M, pH 7.4)	90 $\mu$ l/tube*	100	9.0 ml	200 mM*
MgCl <sub>2</sub> (100 mM)	50 $\mu$ l/tube	100	5.0 ml	10 mM
EDTA (100 mM, pH 7.4)	5 $\mu$ l/tube	100	0.5 ml	1 mM
10 mM saccharic acid lactone	5 $\mu$ l/tube	100	0.5 ml	100 $\mu$ M
[ $^{14}$ C]-Testosterone (13.5 mM, 50 $\mu$ Ci/ml)	5 $\mu$ l	100	0.5 ml	135 $\mu$ M 0.5 $\mu$ Ci/ml§

\*Addition of the microsomal sample effectively adds 10  $\mu$ l of this buffer per tube.

§Each 0.5-ml incubation contains 67.5 nmol testosterone and 0.25  $\mu$ Ci of radioactivity (~555,000 dpm per incubation)

1. Dilute 1 mg of microsomal protein to 5 mg/ml with 250 mM sucrose (*i.e.*, take 1 mg protein, and add sufficient 250 mM sucrose to give 100  $\mu$ l).
- Note:** To determine the effects of protein concentration, a selected microsomal sample is also diluted to 2.5 mg/ml (half as concentrated) and 10 mg/ml (twice as concentrated).
2. Add an equal volume (*i.e.*, 100  $\mu$ l) of CHAPS-solubilization buffer. Mix on a vortex mixer, and incubate at room temperature for 15 min. At this point, the concentration of protein is 2.5 mg/ml, and the concentration of CHAPS is 0.313% (5 mM).
  3. Add 50  $\mu$ l of solubilized microsomes to duplicate tubes and place them on ice. Discard the remaining 100  $\mu$ l or use it for blank incubations. The final concentration of protein in the incubation mixture is 0.25 mg/ml. The final concentration of CHAPS is 0.0313% (0.5 mM).
  4. Add 400  $\mu$ l of substrate solution to each tube (which already contains 50  $\mu$ l of solubilized microsomes). Do not discard the leftover mix because some will be added directly to scintillation vials.
  5. Prepare 2 blanks that contain no microsomal protein (replace solubilized microsomes with 50  $\mu$ l of a 1:1 mixture of 250 mM sucrose and CHAPS-solubilization buffer).
  6. Prepare 6 zero-time blanks (*i.e.*, incubations to which UDPGA is added *after* the reactions are stopped).
  7. Start reactions at 10 or 15 sec intervals by addition of 50  $\mu$ l of cofactor (40 mM UDPGA).
  8. Incubate at 37-38°C for up to 30 minutes.
  9. Stop reactions at 10- or 15-sec intervals by addition of 6 ml dichloromethane
  10. Add 1.1 ml water (to bring volume of aqueous phase to 1.6 ml).
  11. Mix on a batch vortex mixer for 2 x 1 min (speed setting ~5).
  12. If necessary, separate the organic and aqueous layers by centrifugation (2,000 g for 10 min).
  13. Aspirate and discard the organic (upper) phase.
  14. Repeat the extraction process with an additional 6 ml of dichloromethane. **Note:** It is necessary to extract >95% of the unreacted substrate from the aqueous phase.
  15. Transfer 400  $\mu$ l of the aqueous (lower) phase to 7 ml plastic scintillation vials. **Note:** 400  $\mu$ l represent 25% of the aqueous phase.
  16. To 3 of the 6 zero-time blanks, add 100  $\mu$ l of substrate mix. **THE RADIOACTIVITY IN THIS SAMPLE REPRESENTS 100% GLUCURONIDATION OF THE SUBSTRATE.**
- Note:** 100  $\mu$ l is one fourth of 400  $\mu$ l, which is the amount of mix added to each tube. One fourth is the fraction of aqueous phase that ends up in the scintillation counter (*i.e.*, 400  $\mu$ l out of 1.6 ml). A 400  $\mu$ l aliquot of the aqueous phase from the blank incubations is added for quench purposes. This aliquot contains little or no radioactivity because the unreacted substrate has been previously extracted with dichloromethane.
17. Add 100  $\mu$ l of substrate mix to 3 scintillation vials (without an aliquot of aqueous phase from the zero-time incubations). These vials will give an indication of the degree of quench introduced by the aqueous phase.

18. Add 400  $\mu$ l of substrate mix to 3 scintillation vials (again without any aqueous phase). The radioactivity in these vials represents the total amount of radioactivity added to each incubation tube.
19. Add 5 ml scintillation fluid (BioSafe Biodegradable Counting Cocktail from Research Products International Corporation, Mount Prospect, IL).
20. Determine the amount of radioactivity with a Packard 2000 CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL).

**Calculation of the rate of testosterone glucuronidation**

Each 500- $\mu$ l incubation consists of 50  $\mu$ l of solubilized microsomes, 50  $\mu$ l of UDP-glucuronic acid, and 400  $\mu$ l of substrate solution (Tris.HCl, MgCl<sub>2</sub>, EDTA, D-saccharic acid-1,4-lactone, CHAPS and [<sup>14</sup>C]-testosterone). Aliquots of this substrate solution (100  $\mu$ l, equivalent to 25% of the amount added to each incubation) are also analyzed by scintillation spectrometry. The amount of radioactivity in 25% of the aqueous phase (400  $\mu$ l) will equal the amount of radioactivity in 25% of the substrate solution (100  $\mu$ l) only if 100% of the substrate (testosterone) is converted to water-soluble metabolites (testosterone glucuronide). Therefore, the ratio of the radioactivity in the aqueous phase to the radioactivity in the substrate solution represents the fraction of substrate converted to metabolites. This ratio can be used to determine the overall rate of testosterone glucuronidation as follows.

Rate of testosterone glucuronidation (nmol/mg protein/min)

$$= \frac{\text{dpm in 400 } \mu\text{l of aqueous phase}}{\text{dpm in 100 } \mu\text{l of substrate solution}} \times \frac{\text{nmol testosterone}}{\text{incubation}} \times \frac{1}{\text{mg protein/incubation}} \times \frac{1}{\text{time}}$$

$$= \frac{\text{TEST dpm} - \text{BLANK dpm}}{100\% \text{ dpm} - \text{BLANK dpm}} \times \frac{67.5 \text{ nmol testosterone}}{\text{incubation}} \times \frac{1}{0.125 \text{ mg protein}} \times \frac{1}{15 \text{ min}}$$

TEST dpm	Average radioactivity in duplicates.
BLANK dpm	Average radioactivity in the 3 zero-time incubations.
100% dpm	Average radioactivity in the 3 zero-time incubations spiked with 100 $\mu$ l of substrate mix.
[Substrate]	135 $\mu$ M or 67.5 nmol/incubation (this may change depending on the specific activity of the [ <sup>14</sup> C]-testosterone)
[Protein]	Usually 0.25 mg/ml or 0.125 mg/incubation. Some incubations will contain 0.0625 or 0.25 mg ( <i>i.e.</i> , half and twice the usual protein concentration)
Time	Usually 15 min. Some incubations will be stopped after 7.5 or 30 min ( <i>i.e.</i> , half and twice the usual incubation time)

**EXAMPLE CALCULATION**

TEST dpm	=	25,500 dpm
BLANK dpm	=	500 dpm
100% dpm	=	137,500 dpm
TEST - BLANK	=	25,000 dpm
100% - BLANK	=	137,000 dpm

**Rate of testosterone glucuronidation (nmol/mg protein/min)**

$$\begin{aligned}
 &= \frac{\text{dpm in 400 } \mu\text{l of aqueous phase}}{\text{dpm in 100 } \mu\text{l of substrate solution}} \times \frac{\text{nmol testosterone}}{\text{incubation}} \times \frac{1}{\text{mg protein/incubation}} \times \frac{1}{\text{time}} \\
 &= \frac{\text{TEST dpm} - \text{BLANK dpm}}{100\% \text{ dpm} - \text{BLANK dpm}} \times \frac{67.5 \text{ nmol testosterone}}{\text{incubation}} \times \frac{1}{0.125 \text{ mg protein}} \times \frac{1}{15 \text{ min}} \\
 &= \frac{25,000 \text{ dpm}}{137,000 \text{ dpm}} \times 67.5 \text{ nmol} \times \frac{1}{0.125 \text{ mg}} \times \frac{1}{15 \text{ min}} \\
 &= 6.6 \text{ nmol/mg protein/min}
 \end{aligned}$$

$$\text{Amount of radioactivity/incubation} = \frac{400 \mu\text{l}}{100 \mu\text{l}} \times 137,000 \text{ dpm} = 548,000 \text{ dpm}$$

$$\text{Percent radioactivity in blank} = \frac{500 \text{ dpm}}{137,500 \text{ dpm}} \times 100 = -0.4\%$$

(i.e., >>95% of the radioactivity was extracted).

$$\text{Percent metabolism} = \frac{25,000 \text{ dpm}}{137,000 \text{ dpm}} \times 100 = 18\%$$

$$\text{Signal-to-noise ratio} = \frac{25,000 \text{ dpm}}{500 \text{ dpm}} = 50$$

**REFERENCES**

- Jacobson, MM, Levin, W and Conney, AH, Studies on bilirubin and steroid glucuronidation by rat liver microsomes. *Biochem. Pharmacol.* 24: 655-662, 1975.
- Arlotto, MP, Sonderfan, AJ, Klaassen, CD and Parkinson, A, Studies on the pregnenolone-16 $\alpha$ -carbonitrile-inducible form of rat liver microsomal cytochrome P-450 and UDP-glucuronosyltransferase. *Biochem. Pharmacol.* 36: 3859-3866, 1987.

## WORKSHEETS FOR SOP 17.00: TESTOSTERONE GLUCURONIDATION ASSAY

### INSTRUCTIONS:

1. Complete worksheet pages 1 and 2.
2. Use pages 3a-3c of the worksheets as necessary (depending on the number of incubation samples).
3. Complete worksheet pages 4 and 5.

### Step-by-step procedural guideline

Turn on Dubanoff shaking water bath and set temperature to 37-38°C.

Evaporate 50  $\mu$ Ci of [ $^{14}$ C]-testosterone and prepare working solution of [ $^{14}$ C]-testosterone.

Prepare substrate solution and place on ice.

Weigh out UDPGA (dissolve in water just before use)

Dilute and solubilize microsomes with CHAPS-solubilization buffer

Dispense microsomes and place tubes on ice.

Add substrate solution. Do not discard excess.

Add 6.0 ml of dichloromethane to the *blanks*, mix and add 50 $\mu$ l of 40 mM UDPGA

To start reactions, add 50 $\mu$ l of 40 mM UDPGA at 10- or 15-second intervals. Mix on a vortex mixer and incubate at 37-38°C in a Dubanoff shaking-water bath.

After 15 min (or designated time), stop reactions at 10- or 15-second intervals with 6.0 ml of dichloromethane.

Add 1.1 ml water to each tube.

Mix on a vortex mixer and discard organic phase.

Repeat the extraction with an additional 6 ml of dichloromethane.

Measure radioactivity in aqueous phase.

**TESTOSTERONE GLUCURONIDATION ASSAY WORK SHEET - PAGE 1**

**DATE:** \_\_\_\_\_

**Notebook or protocol No:** \_\_\_\_\_

**Microsomes (50 µl/tube after solubilization)**

Protein concentration after solubilization: \_\_\_\_\_ mg/ml

Amount of protein per incubation: \_\_\_\_\_ mg

Source (e.g, liver microsomes from male rats):

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**40 mM UDP-Glucuronic acid.**

Prepare 5 -10% more cofactor than is actually needed.

Amount of UDPGA required = -1.4 mg/tube x number of incubation tubes.

Dissolve in de-ionized water to give 27.4 mg/ml.

Amount of UDPGA weighed out: \_\_\_\_\_

Volume of water added: \_\_\_\_\_

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**TESTOSTERONE GLUCURONIDATION ASSAY WORK SHEET - PAGE 2****Working solution of radioactive testosterone**

For reasons outlined on page 3, the concentration of testosterone in the working solution ranges from 13.3 to 13.5 mM depending in the specific activity of the radioactive (*hot*) testosterone (which ranges from 50 to 60 mCi/mmol). The exact concentration can be determined as follows

Specific activity of *hot* testosterone ( $[^{14}\text{C}]$ -testosterone): .....mCi/mmol

$$[\text{TESTOSTERONE}]_{\text{HOT}} = \frac{0.05 \text{ mCi/ml}}{\text{Specific activity (mCi/mmol)}} = \text{.....mM}$$

$$\begin{aligned} [\text{TESTOSTERONE}]_{\text{OVERALL}} &= [\text{TESTOSTERONE}]_{\text{COLD}} + [\text{TESTOSTERONE}]_{\text{HOT}} \\ &= 12.5 \text{ mM} + [\text{TESTOSTERONE}]_{\text{HOT}} = \text{.....mM} \end{aligned}$$

**Substrate solution (400  $\mu\text{l}$  per tube)**

Prepare approximately 10% more substrate solution than is actually needed.

Stock reagent	Amount/tube	No. Tubes	Volume	Final conc.
Water	245 $\mu\text{l}$ /tube			
Tris.HCl buffer (1.0 M, pH 7.4)	90 $\mu\text{l}$ /tube*			200 mM*
MgCl <sub>2</sub> (100 mM)	50 $\mu\text{l}$ /tube			10 mM
EDTA (100 mM, pH 7.4)	5 $\mu\text{l}$ /tube			1 mM
10 mM saccharic acid lactone	5 $\mu\text{l}$ /tube			100 $\mu\text{M}$
$[^{14}\text{C}]$ -Testosterone (13.3-13.5 mM, 50 $\mu\text{Ci/ml}$ )	5 $\mu\text{l}$			133-135 $\mu\text{M}$ 0.5 $\mu\text{Ci/ml}$

\*Addition of the microsomal sample effectively adds 10  $\mu\text{l}$  of this buffer per tube.

Analyst \_\_\_\_\_

Date \_\_\_\_\_

**TESTOSTERONE GLUCURONIDATION ASSAY WORK SHEET - PAGE 3a**

Sample	Tube	Volume of microsomes	Volume of substrate solution	Volume of UDPGA	Incubation time
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	14				
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	16				
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	32				
	33				
	34				
	35				
	36				
	37				
	38				
	39				
	40				

**Analyst**

**Date**

**TESTOSTERONE GLUCURONIDATION ASSAY WORK SHEET - PAGE 3c**

Sample	Tube	Volume of microsomes	Volume of substrate solution	Volume of UDPGA	Incubation time
	81				
	82				
	83				
	84				
	85				
	86				
	87				
	88				
	89				
	90				
	91				
	92				
	93				
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	114				
	115				
	116				
	117				
	118				
	119				
	120				

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_

**TESTOSTERONE GLUCURONIDATION ASSAY WORK SHEET - PAGE 4**

**DATE** \_\_\_\_\_

**Notebook of Project No.** \_\_\_\_\_

**Pipette Identification No.:**

Pipetman P5000: \_\_\_\_\_

Pipetman P1000: \_\_\_\_\_

Pipetman P200: \_\_\_\_\_

Pipetman P20: \_\_\_\_\_

**Reagents**

**Expiration Date**

- Testosterone (12.5 mM in methanol) \_\_\_\_\_
- [<sup>14</sup>C]-Testosterone (50 μCi/ml in toluene) \_\_\_\_\_
- Working [<sup>14</sup>C]-testosterone solution \_\_\_\_\_
- Saccharic acid-1,4-lactone (10 mM) \_\_\_\_\_
- UDPGA (40 mM) Prepared fresh \_\_\_\_\_
- 10% CHAPS (160 mM) \_\_\_\_\_
- Solubilization buffer \_\_\_\_\_
- 250 mM Sucrose \_\_\_\_\_
- Tris.HCl buffer (1.0 M, pH 8.0 at room temperature) \_\_\_\_\_
- EDTA (100 mM, pH 7.4) \_\_\_\_\_
- MgCl<sub>2</sub> (100 mM) \_\_\_\_\_

<u>Specialty Reagent</u>	<u>Supplier</u>	<u>Catalog No.</u>	<u>Batch or Lot No.</u>
CHAPS	Calbiochem	220201	
Saccharic acid-1,4-lactone	Sigma	S0375	
UDP-glucuronic acid	Sigma	U6751	
[ <sup>14</sup> C]-Testosterone	Amersham	CFA.129	
Testosterone	Sigma	T-1500	

Date purified: \_\_\_\_\_

Notebook No. \_\_\_\_\_

**Analyst** \_\_\_\_\_ **Date** \_\_\_\_\_



## Triage of 8(e) Submissions

Date sent to triage: \_\_\_\_\_

NON-CAP

CAP

Submission number: 12809 A

TSCA Inventory: Y    N    D

Study type (circle appropriate):

Group 1 - Gordon Cash (1 copy total)

ECO            AQUATO

Group 2 - Ernie Falke (1 copy total)

ATOX    ~~SBTOX~~    SEN            w/NEUR   

Group 3 - HERD (1 copy each)

STOX                    CTOX                    EPI            ~~RTOX~~                    GTOX  
 STOX/ONCO            CTOX/ONCO            IMMUNO            CYTO                    NEUR

Other (FATE, EXPO, MET, etc.): \_\_\_\_\_

Notes: re-classified - no SBTOX study

- This is the **original** 8(e) submission; refile after triage evaluation.
- This **original** submission has been **split**; rejoin after triage evaluation.
- Other: *Send to RTOX then send to Ernie  
Received RTOX ↗*

**Photocopies Needed for Triage Evaluation**

entire document:	0	1	2	3
front section and CECATS:	0	1	2	3
Initials: _____	Date: _____			

CECATS DATA:

Submission # BEHQ: 12-13-13-007

SEQ. A

TYPE: INT SUPP FLWP

SUBMITTER NAME: Allied-Signal Inc.

CECATS/TRIAGE TRACKING DBASE ENTRY FORM

INFORMATION REQUESTED: FLWP DATE:

- 0501 NO INFO REQUESTED
- 0502 INFO REQUESTED (TECH)
- 0503 INFO REQUESTED (VOL ACTIONS)
- 0504 INFO REQUESTED (REPORTING RATIONALE)

DISPOSITION:

- 0639 REFER TO CHEMICAL SCREENING
- 0678 CAP NOTICE

VOLUNTARY ACTIONS:

- 0401 NO ACTION REPORTED
- 0402 STUDIES PLANNED/IN PROGRESS
- 0403 NOTIFICATION OF WORKERS/STUDIES
- 0404 LABEL/MSDS CHANGES
- 0405 PROCESS/HANDLING CHANGES
- 0406 APP/USE DISCONTINUED
- 0407 PRODUCTION DISCONTINUED
- 0408 CONFIDENTIAL

SUB. DATE: 12/14/93

OTS DATE: 12/22/93

CSRAD DATE: 06/27/96

CHEMICAL NAME:

HFC 134A

CAS#

811-97-2

INFORMATION TYPE:

P F C

0201	ONCO (HUMAN)	01 02 04
0202	ONCO (ANIMAL)	01 02 04
0203	CELL TRANS (IN VITRO)	01 02 04
0204	MUTA (IN VITRO)	01 02 04
0205	MUTA (IN VIVO)	01 02 04
0206	REPRO/TERATO (HUMAN)	01 02 04
0207	REPRO/TERATO (ANIMAL)	01 02 04
0208	NEURO (HUMAN)	01 02 04
0209	NEURO (ANIMAL)	01 02 04
0210	ACUTE TOX. (HUMAN)	01 02 04
0211	CHR. TOX. (HUMAN)	01 02 04
0212	ACUTE TOX. (ANIMAL)	01 02 04
0213	SUB ACUTE TOX (ANIMAL)	01 02 04
0214	SUB CHRONIC TOX (ANIMAL)	01 02 04
0215	CHRONIC TOX (ANIMAL)	01 02 04

INFORMATION TYPE:

P F C

0216	EPI/CLIN	01 02 04
0217	HUMAN EXPOS (PROD CONTAM)	01 02 04
0218	HUMAN EXPOS (ACCIDENTAL)	01 02 04
0219	HUMAN EXPOS (MONITORING)	01 02 04
0220	ECO/AQUA TOX	01 02 04
0221	ENV. OCC/REL/FATE	01 02 04
0222	EMER INCI OF ENV CONTAM	01 02 04
0223	RESPONSE REQEST DELAY	01 02 04
0224	PROD/COMP/CHEM ID	01 02 04
0225	REPORTING RATIONALE	01 02 04
0226	CONFIDENTIAL	01 02 04
0227	ALLERG (HUMAN)	01 02 04
0228	ALLERG (ANIMAL)	01 02 04
<u>0239</u>	METAB/PHARMACO (ANIMAL)	01 02 04
0240	METAB/PHARMACO (HUMAN)	01 02 04

INFORMATION TYPE:

P F C

0241	IMMUNO (ANIMAL)	01 02 04
0242	IMMUNO (HUMAN)	01 02 04
0243	CHEM/PHYS PROP	01 02 04
0244	CLASTO (IN VITRO)	01 02 04
0245	CLASTO (ANIMAL)	01 02 04
0246	CLASTO (HUMAN)	01 02 04
0247	DNA DAM/REPAIR	01 02 04
0248	PROD/USE/PROC	01 02 04
0251	MSDS	01 02 04
0299	OTHER	01 02 04

TRIAJE DATA:

NON-CBI INVENTORY

ONGOING REVIEW

SPECIES

TOXICOLOGICAL CONCERN:

USE:

PRODUCTION:

YES

YES (DROP/REFER)

RAT

LOW

CAS SR

NO

NO (CONTINUE)

MED

IN TERMINI

REFER

HIGH

COMMENTS:

Testosterone metabolism by liver microsomes by inhalation exposure 6 hrs/day, 5 days/wk for 28-days. 0, 2500, 10,000, 50,000 ppm

No effect on metabolism of testosterone by liver microsomal Cyt. P450 & UDP glucuronosyl-transferase upon exposure to 2,500 - 50,000 ppm.

CECATS/TRIAGE TRACKING DBASE ENTRY FORM

CECATS DATA:

Submission # BEHQ-1293-12809 SEQ. A

TYPE: INT. SUPP FLWP

SUBMITTER NAME: Allied-Signature

INFORMATION REQUESTED: FLWP DATE: \_\_\_\_\_

- 0501 NO INFO REQUESTED
- 0502 INFO REQUESTED (TECH)
- 0503 INFO REQUESTED (VOL ACTIONS)
- 0504 INFO REQUESTED (REPORTING RATIONALE)

DISPOSITION:

- 0539 REFER TO CHEMICAL SCREENING
- 0678 CAP NOTICE

VOLUNTARY ACTIONS:

- 0401 NO ACTION REPORTED
- 0402 STUDIES PLANNED/IN PROGRESS
- 0403 NOTIFICATION OF WORK RATIONALE
- 0404 LABEL/MSDS CHANGES
- 0405 PROCESS/HANDLING CHANGES
- 0406 APP/USE DISCONTINUED
- 0407 PRODUCTION DISCONTINUED
- 0408 CONFIDENTIAL

SUB. DATE: 12/16/93 OTS DATE: 12/22/93 CSRAD DATE: 6/27/96

CHEMICAL NAME:

HFC 134A

CASE#

811-97-2

INFORMATION TYPE:	P F C	INFORMATION TYPE:	P F C	INFORMATION TYPE:	P F C
0201 ONCO (HUMAN)	01 02 04	0216 EPI/CLIN	01 02 04	0241 IMMUNO (ANIMAL)	01 02 04
0202 ONCO (ANIMAL)	01 02 04	0217 HUMAN EXPOS (PROD CONTAM)	01 02 04	0242 IMMUNO (HUMAN)	01 02 04
0203 CELL TRANS (IN VITRO)	01 02 04	0218 HUMAN EXPOS (ACCIDENTAL)	01 02 04	0243 CHEM/PHYS PROP	01 02 04
0204 MUTA (IN VITRO)	01 02 04	0219 HUMAN EXPOS (MONITORING)	01 02 04	0244 CLASTO (IN VITRO)	01 02 04
0205 MUTA (IN VIVO)	01 02 04	0220 ECO/AQUA TOX	01 02 04	0245 CLASTO (ANIMAL)	01 02 04
<u>0206</u> REPRO/TERATO (HUMAN)	<u>01</u> 02 04	0221 ENV. OCC/REL/FATE	01 02 04	0246 CLASTO (HUMAN)	01 02 04
0207 REPRO/TERATO (ANIMAL)	01 02 04	0222 EMER INCI OF ENV CONTAM	01 02 04	0247 DNA DAM/REPAIR	01 02 04
0208 NEURO (HUMAN)	01 02 04	0223 RESPONSE REQUEST DELAY	01 02 04	0248 PROD/USE/PROC	01 02 04
0209 NEURO (ANIMAL)	01 02 04	0224 PROD/COMP/CHEM ID	01 02 04	0251 MSDS	01 02 04
0210 ACUTE TOX. (HUMAN)	01 02 04	0225 REPORTING RATIONALE	01 02 04	0299 OTHER	01 02 04
0211 CHR. TOX. (HUMAN)	01 02 04	0226 CONFIDENTIAL	01 02 04		
0212 ACUTE TOX. (ANIMAL)	01 02 04	0227 ALLERG (HUMAN)	01 02 04		
<u>0213</u> SUB ACUTE TOX (ANIMAL)	<u>01</u> 02 04	0228 ALLERG (ANIMAL)	01 02 04		
0214 SUB CHRONIC TOX (ANIMAL)	01 02 04	<u>0239</u> METAB/PHARMACO (ANIMAL)	01 02 04		
0215 CHRONIC TOX (ANIMAL)	01 02 04	0240 METAB/PHARMACO (HUMAN)	01 02 04		

TRIAGE DATA:	NON-CBI INVENTORY	ONGOING REVIEW	SPECIES	TOXICOLOGICAL CONCERN:	USE:	PRODUCTION:
	<u>YES</u>	YES (DROP/REFER)	<u>RAT</u>	<u>SBTOX</u> LOW		
CAS SR	NO	NO (CONTINUE)		MED		
	IN PLANNING	REFER		HIGH		

(199912)