A High-Performance-Liquid-Chromatography-Based Method for the Determination of Hydroxylated Testosterone Metabolites Formed In Vitro in Liver Microsomes from Gray Seal (*Halichoerus grypus*)

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Abstract

A reproducible and sensitive high-performance-liquid-chromatography (HPLC)-based method with UV–vis detection is developed and optimized for the determination of hydroxytestosterone compounds formed via the cytochrome P450 enzyme-mediated metabolism of testosterone. The method is used to characterize and quantitate hydroxytestosterone metabolites formed in vitro via testosterone incubation with hepatic microsomes from the liver of gray seals (*Halichoerus grypus*). The HPLC method employs a Zorbax Eclipse XDB-C18 column (5 µm, 250- × 4.6-mm i.d.) and a combination of step gradient and solvent systems of mixtures of acetonitrile, methanol, and water. Metabolites are detected at 254 nm. The eluted peaks of 10 testosterone metabolite standards are well-resolved and a flat baseline is maintained over the elution period of the entire chromatogram. The instrumental detection limits (signal-to-noise ratio = 3) of 6β-, 16β-, 16α-, and 7α-hydroxytestostone and androstenedione are 14, 3, 3, 14, and 3 pmol (20 µL injection), respectively. Eleven hydroxytestosterone metabolites are detected after in vitro testosterone incubation with hepatic microsomes of gray seals. Six are identified as 6β-, 7α-, 16α-, 16β-, and 2β-hydroxytestosterone and androstenedione. In order of abundance, the formation rates are 2100, 39.6, 12.8, 26.2, and 132 pmol/mg protein/min for 6β-, 7α-, 16α-, and 16β-hydroxytestosterone and androstenedione, respectively. The within-day precision (relative standard deviation) is less than 3% for testosterone metabolites. Five relatively substantial peaks are detected but not identified.

Introduction

Cytochrome P450 monooxygenase (CYP) enzymes are heme-thiolate proteins that mediate the biotransformation of a wide range of endogenous and exogenous chemicals (1). Considerable information on the isoforms and activities of CYP enzymes are available for laboratory animals such as rats, mice, and rabbits, but less is known about this important family of enzymes in wild, free-ranging animals such as seals (2). From a toxicological perspective, it is important to characterize and assess the catalytic activity of hepatic CYP isoenzymes in a free-ranging marine mammal species such as seals. Seals are exposed to and accumulate persistent and lipophilic, halogen-containing contaminants (e.g., polychlorinated biphenyls (PCBs) and organochlorine pesticides), which can induce CYP enzyme activity (3). The species differences and overlapping substrate specificities among different CYP enzymes result in varying patterns of contaminant accumulation and thus define the toxicologic potential of contaminants among species.

A number of catalytic bioassays have been developed to assess the specific activity of CYP isoenzymes in laboratory rodents and humans. Specific CYP activities based on alkoxyresorufin-*O*-dealkylase assays are good examples, such as ethoxyresorufin-*O*-deethylase (EROD, CYP1A-mediated) and pentoxyresorufin-*O*-depentylase (CYP2B-mediated) (4). Catalytic enzyme assays of this type have been applied to characterize CYP isoenzymes in marine mammals (2,5,6). In addition to immunologic analysis, information from catalytic assays have been used to characterize putative members of the CYP1A, CYP2B, CYP3A, and CYP4A subfamilies in marine mammal species such as seals, whales, and polar bears (7,8).

The regio- and stereoselective hydroxylation of testosterone is a commonly used catalytic assay in the assessment of the enzyme activity in vitro of different CYP isoenzymes in cells and microsomal preparations from tissues of humans and laboratory species (9–11). More recently, the testosterone hydroxylation assay has been applied to microsomal preparations from several species of seal including ringed, harp, and gray seal (2,5,6). Details of the optimization and validation of the assays and the profile and identity of hydroxylated testosterone metabolites that are formed in vitro are not usually described. For example, testosterone

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6β-hydroxylation (which, same as rodent studies, has been attributed to CYP3A-like activity) has been reported in these seal studies. However, in only one case (i.e., harp seals from Svalbard and the Barents Sea) has a more complete characterization of the profile of hydroxylated testosterone metabolites (i.e., 2β, 6β, 11α, 15α, 15β, and 16β) been reported (5,12).

A number of methods have been described for the analysis of hydroxylated testosterone metabolites. For many years the method of choice was the use of thin-layer chromatography on silica plates (13). This technique has the advantages of rapidity of analysis combined with simplicity of the setup needed for processing a large number of samples. However, attempts to quantitate the metabolites formed have been limited. Also, a radioactive substrate is typically required. Gas chromatography (GC)–mass spectrometry (MS) has been used to separate the testosterone metabolites, but a timely multistep derivatization of the hydroxylated metabolites is required. Testino et al. (14) determined testosterone and 6α-hydroxytestostone using a GC–MS method without sample derivatization, but other hydroxylated testosterone metabolites were not quantitated. The majority of hydroxylated testosterone metabolites possess similar or identical parent–product transitions, making time resolution an absolute requirement for the use of MS in the analysis and quantitation of these hydroxylated metabolites.

Several HPLC methods have been reported for the separation of testosterone and its hydroxylated metabolites (9,11,15–17). HPLC separation can be performed without a radioactive substrate and there is no need for derivatization. HPLC methods can also be used for the processing of large numbers of samples and the characterization of low concentrations of testosterone metabolites. However, most of the reported HPLC methods use a gradient elution and a complex mobile phase. Run times are generally excessively long, and the stability of the baseline fluctuates over the chromatographic elution period. The resolution of the various hydroxylated metabolites and the sensitivities are the main concerns of the HPLC method. Therefore, it is advantageous to develop an HPLC assay that significantly reduces the run time while maintaining baseline resolution to achieve greater efficiency in sample analysis.

In this study, a simple HPLC method has been developed for use in the separation, characterization, and quantitation of hydroxylated testosterone metabolites with application to metabolite determination resulting from testosterone incubation with liver microsomes from Baltic gray seal. To our knowledge, a detailed account of the determination and characterization of the hydroxytestosterone metabolite pattern has not been reported for gray seal. We have characterized the pattern and the rate of testosterone metabolites formed in vitro by hepatic microsomes from a single Baltic gray seal.

Experimental

Chemicals

Testosterone; 6α-, 6β-, 7α-, 15α-, 16α-, 16β-, 2α-, and 11β-hydroxytestosterone; and androstenedione were obtained from Steraloids (Wilton, NH). HPLC-grade methanol, dichloromethane, and acetonitrile were obtained from Merck (Darmstadt, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH) and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and solvents were of high analytical grade supplied by VWR Scientific Products (Suwanee, GA). Water was obtained from a Milli-Q (Millipore, San Jose, CA) filtration system equipped with a 0.22-µm filter.

Preparation of liver microsomes

The microsomes of the gray seals in this study originated from a healthy adult female taken from the Baltic Sea. These microsomes were kindly donated by Mrs. Madeleine Nyman (Finnish Game and Fisheries Research Institute, Helsinki, Finland). The liver samples were taken within 10 min of death from a hunted individual collected in April of 1996. The gray seal sampling occurred during the molting period and 1 month after the weaning of pups. The preparatory procedures used to obtain liver microsomes have been described elsewhere (18). EROD activity was determined as a prescreen for the enzymatic viability of the liver microsomes and found to be 254 pmol/mg protein/min based on the method of Eggans and Galgani (19). Total protein concentrations were determined with the Biorad assay based on the method developed by Bradford (20).

Microsomal testosterone hydroxylase assays

Testosterone, androstenedione, and the hydroxylated testosterone standards were dissolved in methanol to give a 1.0-mg/mL stock solution, which were stored at 4°C until further use. Working solutions for the generation of HPLC calibration curves were made by combining individual standards (6β-, 16β-, 16α-, and 7α-hydroxytestostone and androstenedione), followed by serial dilution of the standard mixture with methanol.

Testosterone (12.5mM, 20 µL) was incubated at 37°C in a total incubation mixture volume of 1.0 mL containing a potassium phosphate buffer (100mM, pH 7.6), $MgCl_2$ (3mM), EDTA (1mM), and gray seal liver microsomes (1.0 mg/mL) (3,9,21). The number of individual assays was limited by the availability of seal microsomes, but it was possible to perform each incubation in duplicate. Incubation mixtures and microsomes were mixed in a 25-mL Erlenmeyer flask while standing on ice. After gently mixing the flask contents on a vortex mixer, preincubation was carried out at ambient room temperature for 5 min. After 5 min of preincubation, the flasks were placed in a shaking water bath at 37°C, and the reaction was initiated by the addition of 0.1 mL of 11mM NADPH. Every 10 min, the same volume and concentration of NADPH was added to the incubation mixture to facilitate the continuation of the CYP enzyme-mediated catalytic reaction. In order to determine the optimum incubation time, the reaction was terminated after 10, 20, 30, 40, 50, and 60 min, respectively, by the addition of 10 mL dichloromethane to the incubation mixture. After incubation, the mixture was spiked with 50 µL of the internal standard (50µM of 6α-hydroxytestosterone, which was not metabolically formed in the microsomal incubation) and vortexed for 1 min before centrifugation. After centrifuging at 2000 rpm for 10 min, the top aqueous layer was aspirated and the organic phase was transferred to a clean tube and evaporated to

dryness in an analytical evaporator with the water bath set at 37°C under a gentle stream of nitrogen gas. The residue was redissolved in 0.3 mL of HPLC-grade methanol, vigorously vortexed, filtered with a syringe filter (0.45 µm), and transferred to injection vials. A 20-µL aliquot of this solution was analyzed by HPLC using a reverse-phase column.

The reference incubation assay contained the NaH_2PO_4 buffer, microsomal suspension, and testosterone substrate, but with an absence of NADPH, which is necessary in order to initialize and maintain the biotransformation reaction. The control assays received 0.1 mL of a 11mM NADPH solution every 10 min until the 20-min mark. The extraction efficiency (recovery) of the sample preparation procedure was estimated by comparing the peak-area ratios obtained from the chromatography of spiked extracted standards with those of nonextracted standards. Recovery was determined at concentrations of 2.5 nmol/mL for the hydroxylated metabolites and 275 nmol/mL for testosterone. In the control assays using the available standards, the method recoveries were 88–98% for all metabolites and testosterone.

HPLC

The HPLC used was a Beckman Gold Nouveau System consisting of a programmable solvent Module 126, a UV–vis photodiode-array detector Module 168, and an Autosampler 507. A Zorbax Eclipse XDB-C18 column (5 µm, 250- × 4.6-mm i.d.) protected by an analytical Eclipse XDB-C18 guard column (5 µm, 12.5- × 4.6-mm i.d.) was used. The analysis of testosterone metabolites was carried out as described by Sonderfan et al. (9) for rat liver microsomes with the following modifications. Gradient elution of the metabolites was achieved using a mobile phase consisting of a linear gradient from 100% solvent A (water–methanol–acetonitrile, 55:44:1) to 40% solvent B (water–methanol–acetonitrile, 25:70:5) in the first 20 min, followed by a second linear gradient to 55% solvent B from 20 to 25 min, then a third linear gradient to 100% solvent B from 25 to 26 min. Solvent B was held at 100% for 20 min, followed by a return to 100% A for a further 4 min. A flow rate of 0.6 mL/min was used, and the UV detector was set at 254 nm. All chromatography was performed at room temperature.

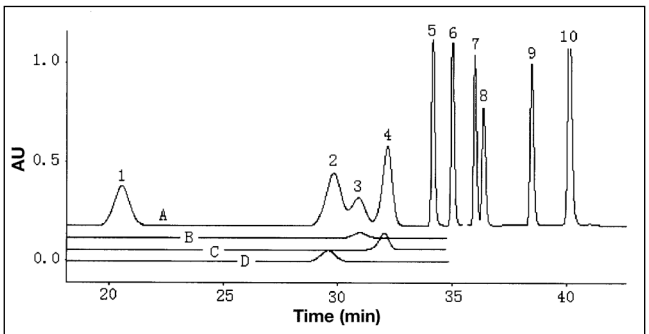

Figure 1. HPLC chromatogram showing the separation of (A) a mixture of testosterone and nine hydroxytestosterone authentic standards (i.e., (1) 6α-, (2) 15α-, (3) 6β-, (4) 7α-, (5) 16α-, (6) 16β-, (7) 2α-, and (8) 11β-hydroxytestosterone; (9) androstenedione; and (10) testosterone) and individual standards of (B) 6β-, (C) 7α-, and (D) 15α-hydroxytestosterone. For experimental details, see the Experimental section.

Replicate injections of the standard solution were made to verify the overall system equilibrium.

Results and Discussion

HPLC separation and detection of testosterone and hydroxytestosterone metabolites

Line A in Figure 1 shows a representative separation of nine commercially available testosterone metabolites and testosterone. Under the chromatographic conditions in this study, the hydroxytestosterone standards were well-resolved. The elution orders of 15α/6β and 11β/2α were reserved as compared with previous reports (9,11) using a similar C18 column. In order to further confirm the elution order of 6β, 7α, and 15α, the standard mixture and the individual 6β-, 7α-, and 15α-hydroxytestosterones were injected separately (lines B, C, and D, respectively). The retention times for 15α-, 6β-, and 7α-hydroxytestosterones were 29.62, 30.91, and 32.14 min, respectively. We compared the chromatograms of the mixture with and without 11β-hydroxytestosterone. Figure 2 demonstrates that peak 8 is 11β-hydroxytestosterone. These findings indicate that for a similar HPLC column, subtle changes in the chromatographic HPLC conditions can result in changes in the order of elution and the degree of separation for hydroxytestosterone compounds (16).

Testosterone metabolism by gray seal liver microsomes

The female Baltic gray seal from which the liver microsomes were prepared was from the heavily polluted Baltic Sea population. This population of seals contained high levels of CYP enzyme-inducing contaminants including polychlorinated dibenzo-*p*-dioxins and dibenzofurans, PCBs, and organochlorine pesticides (22). Testosterone is a useful substrate for distinguishing multiple forms of the CYP enzymes as a result of the unique and characteristic hydroxytestosterone metabolite patterns that are obtained (13). The optimized HPLC method for hydroxytestosterone determination was applied to testosterone metabolite fractions isolated from in vitro testosterone incubations with liver microsomes from gray seals. The rate of depletion of testosterone and the rate of formation of the hydroxytestosterone metabolites was examined for linearity with respect to time. The depletion of testosterone increased linearly up to 60

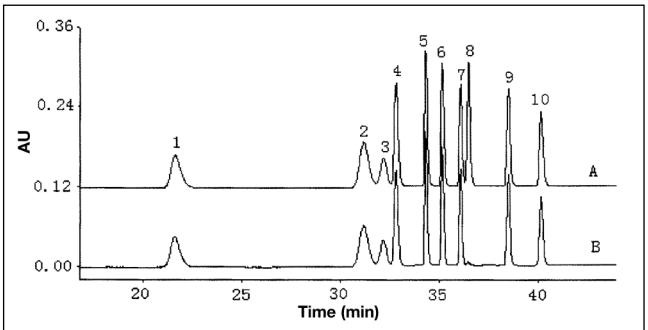

Figure 2. HPLC separation of testosterone and hydroxytestosterone standards (A) in the presence and (B) in the absence of 11β-hydroxytestosterone. The identity of the peaks is the same as indicated in Figure 1. For experimental details, see the Experimental section.

min to a maximum of approximately 60% (Figure 3A). However, the formation of the major 6β-, 16α-, 16β-, and 7α-hydroxytestosterone metabolites (as will be discussed) increased linearly with incubation time up to approximately 20 min (Figure 3B). After 20 min the amount of metabolite formed became nonlinear and appeared to be constant with further incubation time. This likely can be explained by the presence of high levels of steroid 5α-reductase activity, which occurs in mammalian female liver microsomes. This can interfere with the testosterone hydroxylase assay by rapidly metabolizing testosterone to dihydroxytestosterone (23,24). Therefore, testosterone is less available to the CYP isoenzymes as the incubation time increases. No attempt was made to reduce the steroid 5α-reductase activity in this study. For the gray seal assay a testosterone incubation time of 20 min was chosen as ideal because the metabolite formation rate remained linear and the maximum amount of metabolites were formed.

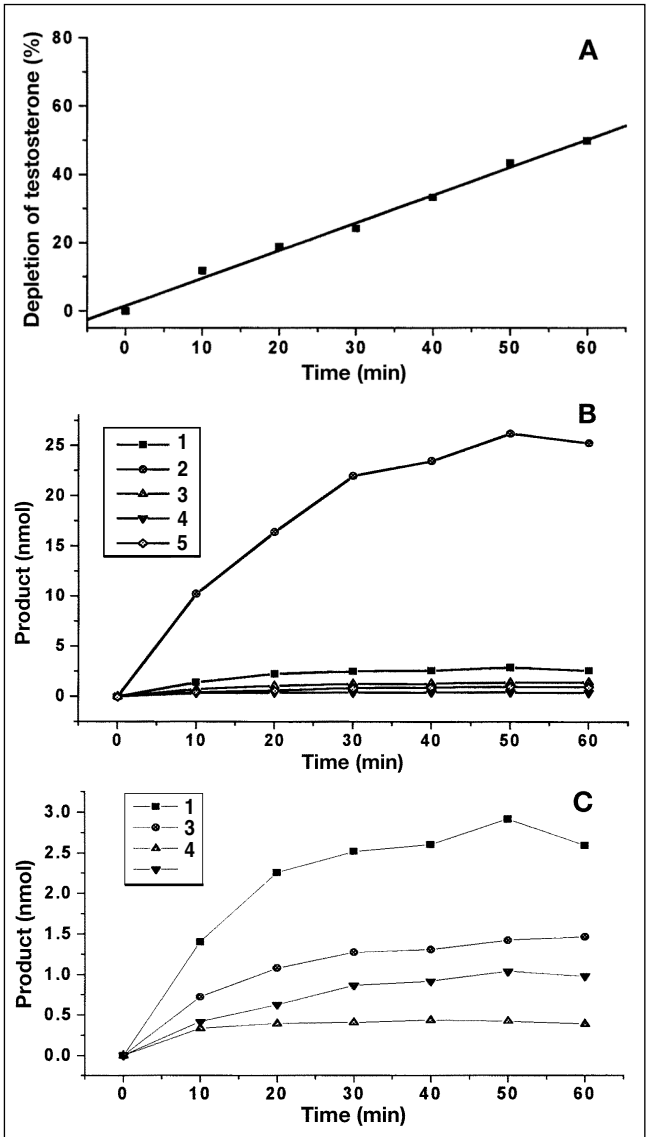

Figure 3. (A) The depletion of testosterone and (B) the formation of the testosterone metabolites (1) androstenedione and (2) 6α-, (3) 16α-, (4) 16β-, and (5) 7β-hydroxytestosterone after in vitro incubation with liver microsomes from Baltic gray seals. Figure 3C is the magnification of Figure 3B. For experimental details, see the Experimental section.

This incubation time was similar to that used in studies on testosterone hydroxylation in liver microsomes from other seal species (2,5).

In addition to testosterone, at least 12 chromatographic peaks were detected in the isolated testosterone metabolite fractions from microsomal incubations (line B in Figure 4). Five of the peaks were identified as 6β-, 7α-, 16α-, and 16β-OH-testosterone and androstendione by the direct comparison of retention times to the hydroxytestosterone compounds in the standard mixture (line A in Figure 4). Similar to major testosterone metabolites identified when incubated with liver microsomes of male and female ringed seals from Svalbard (5) and recently by Nyman et al. (2) with the same gray seal microsomes, 6β-hydroxytestosterone was a major metabolite (see also Figure 3B).

The 11β-hydroxytestosterone compound is often used as the internal standard in testosterone hydroxylase assays with liver microsomes of humans and laboratory rodents (9,13). 11β-Hydroxytestosterone has been shown not to be formed metabolically in humans and laboratory rodents (16). In this study, 11β-hydroxytestosterone was found to elute at the same time as one major hydroxytestosterone metabolite (compound X) formed in the gray seal microsomes (Figure 4). It has been previously reported that it is difficult to baseline separate 11β-hydroxytestosterone from 2β-hydroxytestosterone using reversed-phase HPLC (17). Based on the significant production of compound X in this study's gray seal microsomes and a comparable relative retention time with HPLC elution profiles reported elsewhere for laboratory rodents (16), we concluded that compound X was 2β-hydroxytestosterone. 6α-Hydroxytestosterone (which was not formed in this metabolism) was therefore used as the internal standard. The lack of formation of 6α-hydroxytestosterone in the seal microsomes was consistent with studies on testosterone in other species including humans, rodents, and birds (5,25).

To our knowledge, the presence of 7α- and 16α-hydroxytestosterone and androstendione metabolites formed in this study by gray seal microsomes (Figure 4) or any other seal species has not been previously reported. In rat liver microsome studies, a major

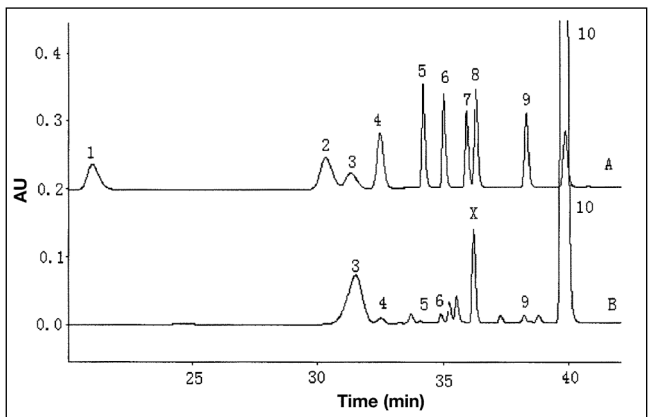

Figure 4. HPLC elution profile of (A) a mixture of testosterone and nine hydroxytestosterone authentic standards and (B) testosterone metabolites formed as a result of incubation (20 min) with liver microsomes from gray seals. The peak numbers in A correspond with the hydroxytestosterone metabolite labeling in Figure 1. The peak identities for B are (3) 6β-, (4) 7α-, (5) 16α-, (6) 16β-, and (X) 2β-hydroxytestosterone; (9) androstenedione; and (10) testosterone. For experimental details, see the Experimental section.

metabolite of (*a*) CYP2A1/2 is 7α-OH-testosterone, (*b*) CYP2B1 and CYP2C11 is 16α-OH-testosterone, and (*c*) CYP2B1 and CYP2C11 is androstendione (10). Nyman et al. (2) recently showed for these microsomes the presence of multiple forms of CYP enzymes including CYP1A, CYP3A, CYP2A, CYP2D, and CYP2E. 7a-Hydroxytestosterone formation in this study was consistent with CYP2A protein characterization in Baltic gray seal, high coumarin 7-hydroxylation (COH) activities, and COH inhibition by an anti-CYP2A5 antibody in gray seal (2). Androstendione formation in this study's gray seals may be via CYP2A and CYP3A mediation. CYP2A and CYP3A activity in rat liver are known to be minor contributors of androstendione formation (13). CYP2B and CYP2C enzymes were not found in Baltic gray seals from immunoblot studies with human and rodent antibodies (2). 16α-Hydroxytestosterone formation is known to be mediated by CYP2C and CYP2B activity in rats; however, these enzymes have not been detected via catalytic assays or immunologically in gray seal liver (2).

An additional six unidentified metabolite peaks were also detected in the microsomal testosterone incubation (line B in Figure 4). The two peaks eluting just after 16β-hydroxytestosterone were relatively major. The retention times for these metabolite peaks did not match any of the compounds for which authentic standards were available (line A in Figure 4). Purdon et al. (14) identified and quantitated the testosterone metabolites 1α-, 1β-, and 18-hydroxytestosterone, which elute between 16β- and 2α-hydroxytestosterone in the chromatogram using methanol–water as the mobile phase after testosterone incubation with liver microsomes from phenobarbital-induced rats. 1β-Hydroxytestosterone is a lone metabolite formed via CYP3A enzyme mediation in rat liver microsomes (26). Previously, a CYP3A isoenzyme was detected immunochemically in liver microsomes from the gray seal used in this study (2).

Quantitation of testosterone metabolites formed by gray seal microsomes

The quantitation of hydroxytestosterone metabolite formation in gray seal microsomes was determined by comparison with calibration curves obtained by spiking the incubation mixture (no microsomes) with five testosterone metabolites (6β-, 16β-, 16α-, and 7α-hydroxytestostone and androstenedione) and 6α-hydroxytestostone as the internal standard. Concentrations ranging from 0.5 to 5 nmol/mL were used for the calibration curves. Linear regression plots of peak-area ratios versus concentration were constructed and concentrations of hydroxytestosterone determined from the peak-area ratio relative to the calibration graph. A summary of a statistical analysis of the calibration curves is shown in Table I. The correlation coefficients were consistently > 0.999 for the metabolites. Because the concentration of testosterone in the incubation mixture was much higher than the metabolites and the depletion of the testosterone was small, the calibration standard mixture did not contain testosterone. The amount of testosterone in the incubation mixture was calculated by comparing it with the reference assay, in which all reagents were added in the reference samples with the exception of NADPH, and thus the enzyme-mediated reactions could not proceed. The precision of the method was determined by performing four repeated analyses of a spiked incubation mixture (no microsomes) containing 2.5 nmol/mL of five metabolites and internal standards. The relative standard deviations for repeatability assessed from peak-area ratio measurements were 2–5% for all of the analytes. The detection limits (signal-to-noise ratio = 3) of 6β-, 16β-, 16α-, and 7α-hydroxytestostone and androstenedione were 14, 3, 3, 14, and 3 pmol (20 µL injection), respectively.

Table I. Results of a Statistical Analysis of Calibration Response Curves Generated for Five Hydroxytestosterone Standards Spiked to Incubation Media Used for the In Vitro Testosterone Metabolism Assay

Analyte	Calibration curve	Calibration coefficient	Linear range (nmol)	Detection limit* (pmol)
6β	$y = -0.046 + 0.212x$	0.9992	0.5–5	14
7α	$y = 0.127 + 0.426x$	0.9996	0.5–5	14
16α	$y = 0.320 + 0.411x$	0.9990	0.5–5	3
16β	$y = 0.051 + 0.401x$	0.9999	0.5–5	3
Androstenedione	$y = 0.040 + 0.354x$	0.9999	0.5–5	3

* Signal-to-noise ratio = 3.

The rates of formation of the identified hydroxytestosterone metabolites in seal liver microsomes were 2100 pmol/mg/min for 6β-hydroxylation, 26.2 pmol/mg/min for 16β-hydroxylation, 132 pmol/mg/min for androstenedione, 12.8 pmol/mg/min for 16α-hydroxylation, and 39.6 pmol/mg/min for 7α-hydroxylation. Although a large peak was identified as 2β-hydroxytestosterone, it was not quantitated because of a lack of authentic standard. The formation rate of 6β-hydroxylation in ringed seals was 906 pmol/min/mg protein (5). In rat liver microsomes, the rates of formation of 6β-hydroxylation was reported as 5.87 nmol/min/mg protein (27) or 6.2 nmol/min/mg protein (28).

In conclusion, an HPLC-based method has been developed and optimized for the separation and determination of testosterone and nine hydroxytestosterone metabolites. The method was successfully applied for the characterization and quantitation of six identified and five unidentified hydroxytestosterone metabolites formed via in vitro testosterone metabolism in liver microsomes from gray seals.

Acknowledgments

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