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Determination of testosterone and 6β-hydroxytestosterone by gas chromatography–selected ion monitoring–mass spectrometry for the characterization of cytochrome p450 3A activity

Samuel A. Testino Jr.[a,*], Justyna Ozarowski[a], Archie W. Thurston[a], Richard F. Arrendale[a], Gabor Patonay[b]

[a]*Preclinical Research and Development Division, Department of Drug Disposition and Safety, Solvay Pharmaceuticals, Inc., 901 Sawyer Road, Marietta, GA 30062, USA*

[b]*Department of Chemistry, Georgia State University, Atlanta, GA 30303, USA*

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Abstract

A method for the determination of testosterone and its metabolite, 6β-hydroxytestosterone, in liver microsomal incubates employing gas chromatography with selected ion monitoring mass spectrometric detection (GC–SIM–MS) has been developed. The method is more rapid than previously reported methods. Testosterone and its metabolites are extracted from the incubation mixture in a single step with methylene chloride. The method does not require derivatization and testosterone and its metabolites are separated on a HP-5MS fused-silica capillary column in less than 15 min. The retention times of testosterone (*m*/*z* 288), methyltestosterone (*m*/*z* 302), and 6β-hydroxytestosterone (*m*/*z* 304) are approximately 12.7, 12.8, and 13.4 min, respectively. There are no interferences from other known CYP450 metabolites of testosterone. In addition, the selectivity and specificity of the mass spectrometer helps eliminate possible interferences from drugs and new chemical entities evaluated using this methodology. Calibration curves for testosterone and 6β-hydroxytestosterone are linear from 0.25 to 100 μ*M*. Extraction recoveries are better than 92% for both analytes and the internal standard, methyltestosterone. Over the course of five separate runs, within-day and inter-day precision (expressed as relative standard deviation) was less than 5% for all concentrations of testosterone and 6β-hydroxytestosterone. Accuracies ranged from 95.8 to 105.8% for testosterone and 94.6 to 104.2% for 6β-hydroxytestosterone. The assay has been used to characterize the CYP3A metabolic activity of multiple preparations of human, rat, and dog liver microsomes.

Keywords: Testosterone; 6β-Hydroxytestosterone; Cytochromes

1. Introduction

Cytochrome P450 (CYP) enzymes are heme-thiolate proteins that are responsible for the oxidative metabolism of a wide variety of xenobiotics [1]. They comprise a superfamily of related enzymes that are grouped into families and subfamilies based on similarities in amino acid sequences. The determination of the CYP enzymes responsible for the metabolism of new chemical entities (NCE) and the identification of interactions with a specific CYP isozyme (e.g. inhibition of that isozyme) can aid in

*Corresponding author.

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predicting clinical drug interactions [2]. The CYP family of enzymes has been shown to be responsible for the hydroxylation of steroids and related compounds. Specifically, the 6β-hydroxylation of testosterone is known to be mediated by the CYP3A family of CYP enzymes in a variety of species [3]. By measuring the rate of formation of the testosterone metabolite, 6β-hydroxytestosterone, the CYP3A activity of a preparation of hepatic microsomes can be determined.

Several HPLC methods have been reported for the separation of testosterone and its many hydroxylated metabolites [3–9]. Most use gradient elution with run times greater than 30 min followed by an additional between-run reequilibration. Some of these methods require complex gradient systems that often cause significant changes in the baseline that affect resolution and detection of later-eluting peaks. In an attempt to alleviate some of these problems, an isocratic HPLC method has been developed [8], however, the total run time for this method is still greater than 30 min. A slightly faster capillary electrophoretic method has recently been reported [10] with a run time of 20 min and two between-run rinses totaling 5 min. This method, however, suffers from poor sensitivity and poor inter-day migration time reproducibility. Watanabe et al. have reported a more rapid gas chromatography–mass spectrometry (GC–MS) method [11], but it requires an extraction step and a timely multi-step derivatization of the hydroxylated metabolites. In addition, separable *syn*- and *anti*-isomers of some of the derivatized metabolites are formed. A rapid non-chromatographic procedure employing tritium-labeled testosterone was also recently published [12]. Safety and cost considerations make this method far from ideal.

Herein is described a method for the determination of testosterone and 6β-hydroxytestosterone employing gas chromatography with selected ion monitoring mass spectrometric detection (GC–SIM–MS). The method does not require derivatization and testosterone and its metabolites are separated in less than 15 min. In addition, the selectivity and specificity of the mass spectrometer helps eliminate interferences from compounds being evaluated using this methodology which is occasionally a problem when using the HPLC–UV or CE–UV methods previously reported.

2. Experimental

2.1. Chemicals

Testosterone, 17α-methyltestosterone, androstenedione, 2β-hydroxytestosterone phenylmethylsulfonyl fluoride (PMSF), butylated hydroxytoluene (BHT), and Sigma Ultra grade potassium chloride, EDTA, HEPES, glycerol, and magnesium chloride were obtained from Sigma Chemical Co. (St.Louis, MO, USA). 2α-, 6β-, 7α-, 16α-, and 16β-hydroxytestosterone were obtained from Steraloids, Inc. (Wilton, NH, USA). HPLC-grade methylene chloride (J.T. Baker), GC–MS-grade methanol (Burdick and Jackson), and UV-grade acetonitrile (Burdick and Jackson) were purchased from VWR Scientific Products (Suwanee, GA, USA). Bovine serum albumin and other materials for determining microsomal protein content were obtained from BioRad (Hercules, CA, USA).

2.2. Isolation of hepatic microsomes

Human liver microsomes were prepared using a differential centrifugation method as follows: Human livers rejected for transplant were received fresh from the International Institute for the Advancement of Medicine (Exton, PA, USA) in less than 36 h post-clamp time or frozen from the Association of Human Tissue Users (Tucson, AZ, USA). The liver tissue was minced and rinsed in a 1.15% (w/v) KCl solution. The tissue was weighed, washed in three volumes of a homogenization buffer (100 m*M* Tris acetate, 1 m*M* EDTA, 100 m*M* KCl, 20 μ*M* BHT, pH 7.4) and then homogenized in a motorized homogenizer (Omni International, Atlanta, GA, USA) with two 20 s bursts. The homogenate was centrifuged at approximately 10 000 *g* for 30 min at 4°C. The supernatant (s9) was transferred to fresh centrifuge tubes and then centrifuged at approximately 100 000 *g* for 70 min at 4°C. The pellet was washed once in 1.5 volumes of a buffer containing 100 m*M* tetrapotassium pyrophosphate, 1 m*M* EDTA, and 20 μ*M* BHT (adjusted to pH 7.4 with 6 *M* HCl) by resuspending and homogenizing in a glass dounce homogenizer. The resuspended pellet was centrifuged again at 100 000 *g* for 70 min at 4°C. The resulting pellet was resuspended in a

storage buffer (10 m*M* Tris acetate, 1 m*M* EDTA, 100 μ*M* PMSF, and 20% glycerol, pH 7.4) using the glass dounce homogenizer, aliquoted (1 ml) into screw-cap cryovials, and stored at −80°C until use. Microsomal protein content was determined using the Bradford method [13] and total CYP content was determined spectrophotometrically using the method of Omura and Sato [14].

2.3. Microsomal incubations

A total incubation volume of 500 μl of HEPES buffer (50 m*M* HEPES, 15 m*M* $MgCl_2$, 0.1 m*M* EDTA, pH 7.6) containing an NADPH regeneration system (1 m*M* $NADP^+$, 10 m*M* glucose 6-phosphate, and 1 IU glucose 6-phosphate dehydrogenase) and hepatic microsomes (0.5 mg protein) was pre-incubated at 37°C for 3 min. The enzymatic reaction was initiated upon addition of testosterone (100 μ*M*, from 10 m*M* stock in MeOH). Blank incubations were prepared in the absence of the NADPH-regeneration system. Following a 30 min incubation at 37°C, the reaction was terminated with 3 ml of methylene chloride. After the addition of 50 μl of methyltestosterone internal standard solution (100 μ*M*), the samples were vortexed for about 30 s and then centrifuged at 1400 *g* for 2 min. The top aqueous layer and any precipitate at the phase interface was aspirated. The remaining methylene chloride layer was evaporated to dryness under a gentle stream of nitrogen. Acetonitrile (0.5 ml) was added, and each tube was vortexed briefly. Finally, the content of each tube was transferred to an autosampler vial for GC–SIM–MS analysis.

2.4. Calibration standard preparation

A stock solution containing testosterone (1 m*M*) and 6β-hydroxytestosterone (1 m*M*) was prepared in methanol and stored at 4°C. Working solutions of varying concentrations down to 2.5 μ*M* were prepared from dilutions of the stock solution with methanol. Calibration standard samples (0.25 μ*M*, 0.50 μ*M*, 1.0 μ*M*, 5.0 μ*M*, 10 μ*M*, 50 μ*M*, and 100 μ*M*) covering a calibration range of 0.25 to 100 μ*M* were prepared by adding 50 μl of each working solution to 400 μl water and 50 μl of HEPES buffer. The samples were spiked with 50 μl of methyltestosterone internal standard solution (100 μ*M*) and extracted as described above for the microsomal incubates.

2.5. GC–MS conditions

The samples were analyzed on a HP5890 Series II Gas Chromatograph interfaced to a HP5971A Mass Selective Detector (Hewlett-Packard, Palo Alto, CA, USA). Testosterone, its metabolites, and the internal standard were separated on a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness) that was introduced directly into the source of the mass spectrometer. The capillary column flow-rate (He) was kept constant at approximately 1 ml/min. using electronic pressure control. A 1 μl splitless injection was made using a purge activation time of 1 min. The injection port temperature was 280°C. The oven was held at 80°C for 1 min and then ramped at 20°C per min to a final temperature of 300°C and held for 3 min. Positive-ion electron ionization mass spectra were acquired at an electron energy of 70 eV with an ion source temperature of 170°C and an interface temperature of 280°C. For quantitation, the mass spectrometer was operated in the selected ion monitoring mode and set to monitor ions at *m/z* 288, 304, and 302 (100 ms per ion) for testosterone, 6β-hydroxytestosterone, and methyltestosterone, respectively.

3. Results and discussion

3.1. Method development

Full scan positive ion electron impact mass spectra (50–350 amu) of testosterone, methyltestosterone, and 6β-hydroxytestosterone were acquired to determine the ions to monitor in the SIM mode. Representative spectra are shown in Fig. 1. The base peak in the mass spectrum of testosterone was a common steroid fragment, *m/z* 124. Therefore, the molecular ion (*m/z* 288), which is less abundant but more specific, was chosen for testosterone. The base peaks for methyltestosterone and 6β-hydroxytestosterone were the molecular ions, *m/z* 302 and *m/z* 304, respectively. These ions were selected for the GC–SIM–MS method.

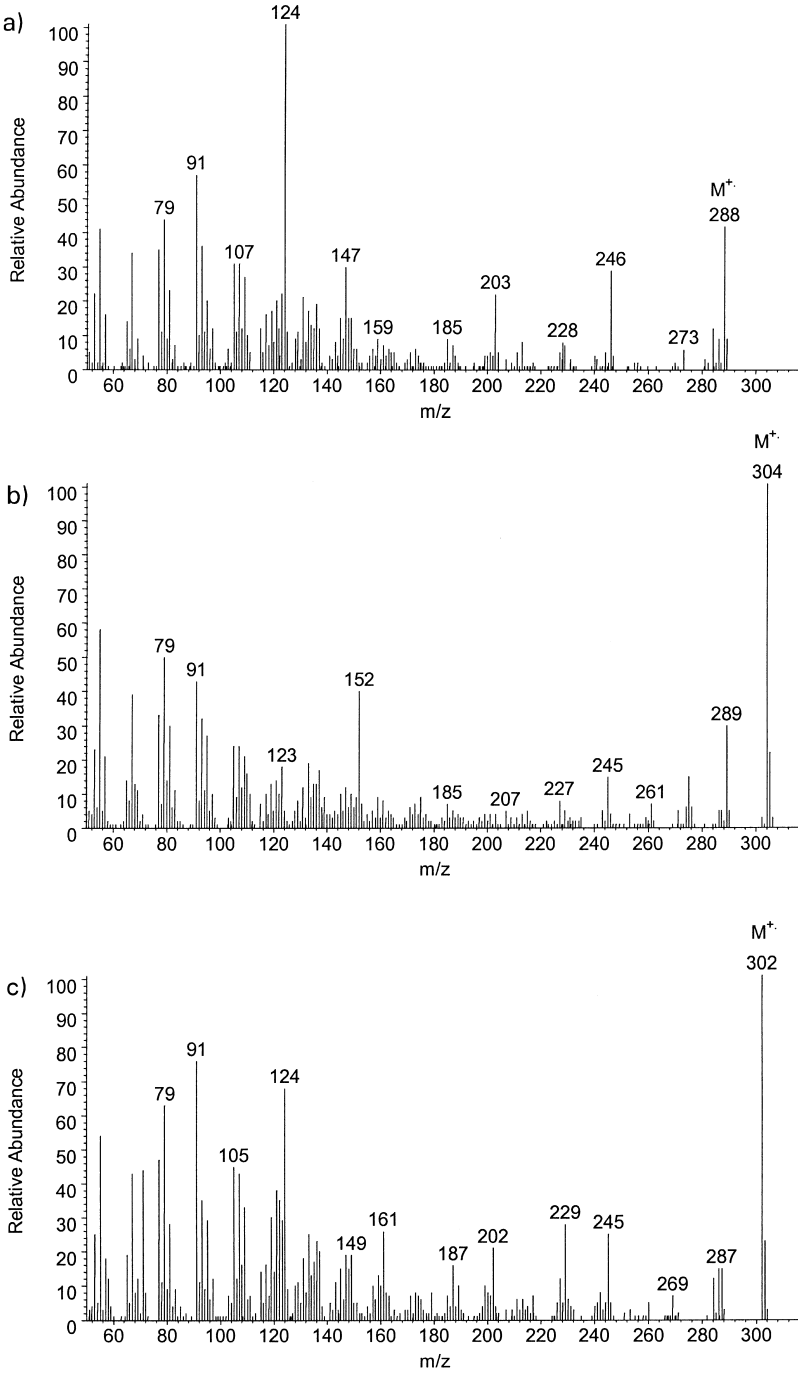

Fig. 1. Positive ion EI mass spectra of (a) testosterone, (b) 6β-hydroxytestosterone, and (c) methyltestosterone.

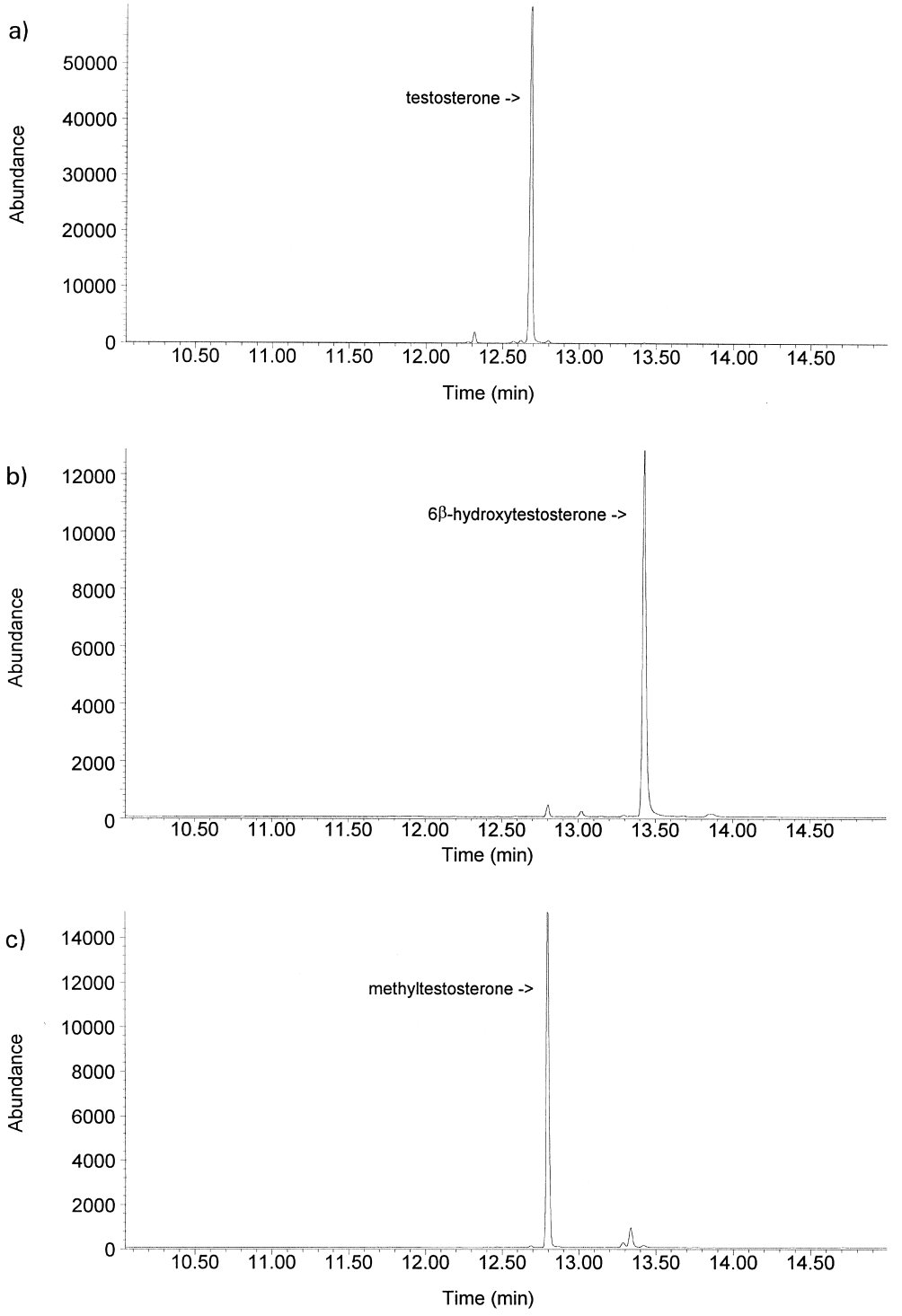

Fig. 2. Representative chromatogram of a sample of testosterone (100 μ*M*) incubated with human liver microsomes. Testosterone, 6β-hydroxytestosterone, and methyltestosterone are identified in the selected ion chromatograms of (a) *m*/*z* 288, (b) *m*/*z* 304, and (c) *m*/*z* 302, respectively.

A representative chromatogram from a sample containing testosterone after incubation with human liver microsomes is shown in Fig. 2. The retention times of testosterone, methyltestosterone, and 6β-hydroxytestosterone were approximately 12.7, 12.8, and 13.4 min, respectively. Even without derivatization, no significant tailing of the chromatographic peaks occurred. In addition, no thermal degradation of the steroids was observed.

3.2. Specificity

The method was evaluated for possible interferences from other known CYP450 metabolites of testosterone. Androstenedione (12.6 min), 2α-hydroxy- (13.1 min), 2β-hydroxy- (13.0 min), 7α-hydroxy- (13.6 min), 16α-hydroxy- (13.8 min), and 16β-hydroxytestosterone (13.9 min), were fully resolved from testosterone, 6β-hydroxytestosterone, and methyltestosterone. In addition, the inherent selectivity and specificity of the mass spectrometric detector has eliminated interferences from compounds evaluated using this methodology.

3.3. Linearity

Seven calibration standards ranging in concentration from 0.25 to 100 μ*M* for both testosterone and 6β-hydroxytestosterone were interspersed with samples for each analytical run. Weighted $(1/x^2)$ linear regressions of peak area ratios of testosterone and 6β-hydroxytestosterone to the internal standard, methyltestosterone, as a function of concentration were performed to obtain a standard curve from which unknowns were quantified. Weighted regressions were used to provide homogeneity of variance across the calibration range. The method was linear from 0.25 to 100 μ*M* for both compounds. Regression data (slope, intercept, and correlation coefficient) from five successive analytical runs are shown in Table 1. Excellent correlation and slope reproducibility was observed for each compound.

3.4. Precision and accuracy

Inter-day precision and accuracy of the method were determined from the back-calculated results for the seven calibration standards for testosterone and 6β-hydroxytestosterone from the five runs listed in Table 1. Within-day precision was determined by assaying six replicates at each of three different concentrations of testosterone and 6β-hydroxytestosterone in one analytical run. The results are shown in Table 2. Within-day and inter-day precision (expressed as relative standard deviation) was less than 5% for all concentrations of testosterone and 6β-hydroxytestosterone. Accuracies ranged from 95.8 to 105.8% for testosterone and 94.6 to 104.2% for 6β-hydroxytestosterone.

3.5. Extraction recovery

The extraction recovery of the analytes and internal standard was determined by comparing the peak areas of spiked microsomal samples extracted with methylene chloride to the peak areas of standard

Table 1
Regression parameters and statistics for testosterone and 6β-hydroxytestosterone calibration curves

Run	Testosterone			6β-Hydroxytestosterone		
	Slope	Intercept	Correlation coefficient (r^2)	Slope	Intercept	Correlation coefficient (r^2)
1	0.611	0.00228	0.997	1.17	0.00849	0.996
2	0.609	0.00085	0.998	1.22	0.00458	0.999
3	0.641	0.00148	0.999	1.23	0.00538	0.998
4	0.650	0.00149	0.999	1.37	0.01180	0.998
5	0.651	0.00215	0.999	1.30	0.00873	0.997
Mean	0.63	0.0017	0.998	1.26	0.008	0.998
SD	0.02	0.0006	0.001	0.08	0.003	0.001
RSD(%)	3.2	35.3	0.10	6.3	37.5	0.10

Table 2
Precision and accuracy of calibration standards

Standard concentration (μ*M*)	Mean calculated concentration (μ*M*)	Accuracy (%)	Inter-day ($n=5$) RSD (%)	Within-day ($n=6$) RSD (%)
Testosterone				
0.250	0.245	97.8	0.94	
0.500	0.508	101.7	0.85	2.5
1.00	1.06	105.8	2.4	
5.00	4.79	95.8	0.89	1.4
10.0	9.99	99.9	1.3	
50.0	50.0	100.1	1.6	1.6
100	98.7	98.7	1.0	
6β-hydroxytestosterone				
0.250	0.251	100.2	1.8	
0.500	0.500	100.0	4.1	5.0
1.00	1.00	100.2	2.0	
5.00	4.73	94.6	3.4	3.3
10.0	9.77	97.7	1.2	
50.0	52.1	104.2	3.0	4.8
100	103	103.2	1.3	

solutions at the same concentration not carried through the extraction procedure. Recovery was determined at three concentrations (0.5 μ*M*, 5 μ*M*, and 50 μ*M*) across the concentration range for testosterone and 6β-hydroxytestosterone, and at 10 μ*M* for methyltestosterone. Mean percentage recoveries for testosterone and 6β-hydroxytestosterone were 95%±2% ($n=18$) and 98%±7% ($n=18$), respectively. Mean percentage recovery for methyltestosterone was 98%±3% ($n=6$).

3.6. Applications

The method has been used to analyze over 2500 samples for 6β-hydroxytestosterone and testosterone. It has been used to characterize the CYP3A metabolic activity of multiple preparations of human, rat, and dog hepatic microsomes, precision-cut slices, and tissue homogenates.

The method has also been used to screen drugs and NCEs for inhibitory effects on human CYP3A4.

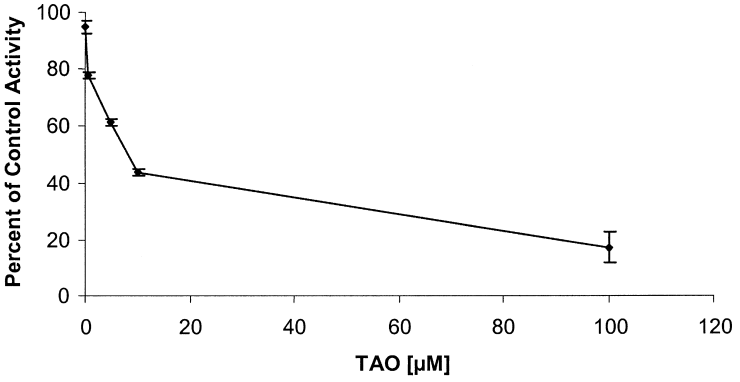

Fig. 3. Inhibition of testosterone 6β-hydroxylation by troleandomycin (TAO). The concentration of 6β-hydroxytestosterone in samples with varying concentrations of TAO (0.01–100 μ*M*, $n=3$ at each concentration) was divided by the concentration of 6β-hydroxytestosterone in samples with no TAO added to calculate ''Percent of Control Activity.''

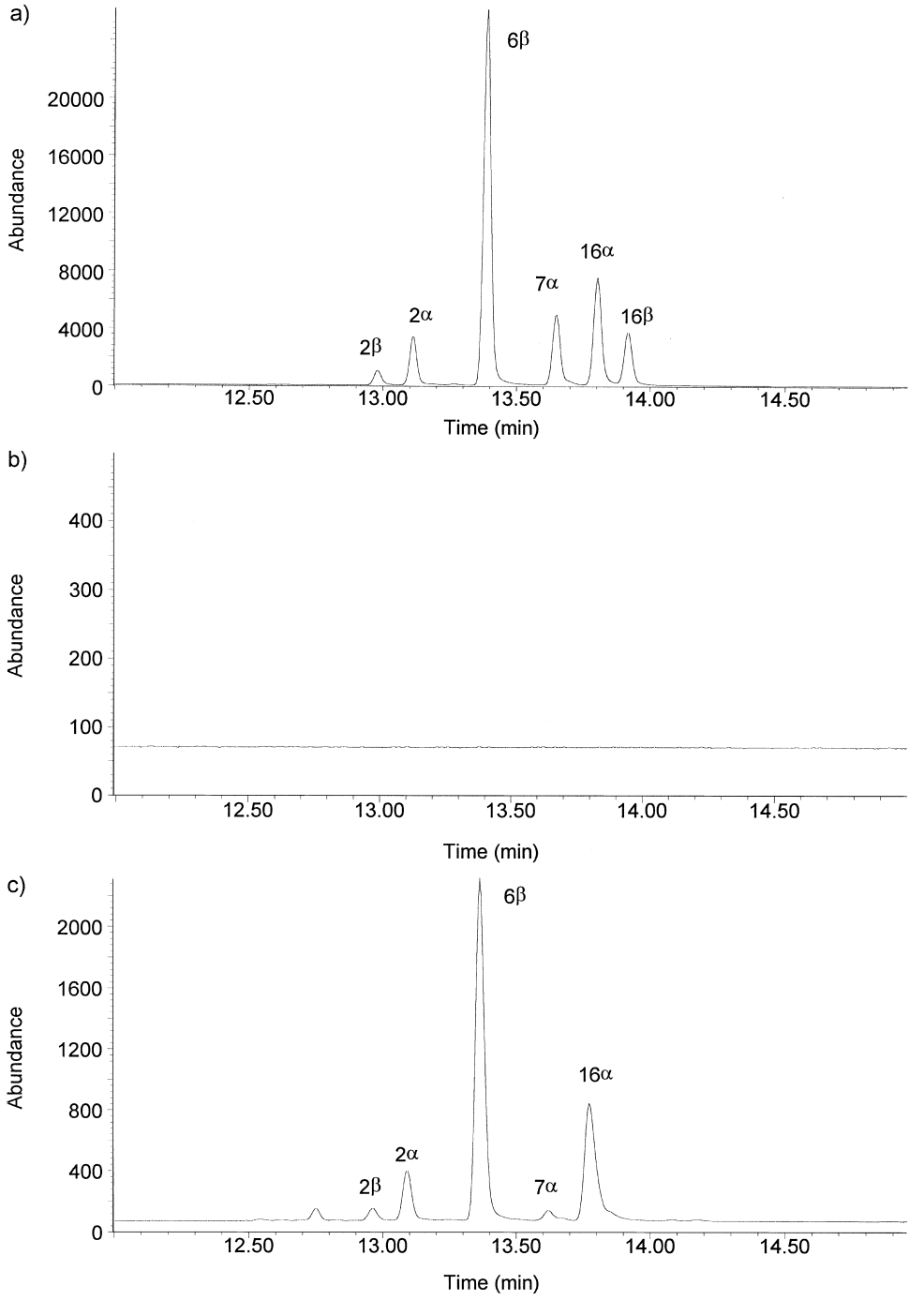

Fig. 4. Selected ion chromatograms (m/z 304) of (a) a mixture of testosterone and hydroxylated testosterone standards, (b) a sample of testosterone (100 μM) incubated with rat liver microsomes without NADPH, and (c) a sample of testosterone (100 μM) incubated with rat liver microsomes with NADPH. The various hydroxylated testosterone metabolites are identified.

The effect of troleandomycin (TAO), a known CYP3A4 inhibitor, on testosterone 6β-hydroxylation has been determined using this method. The percent inhibition of testosterone 6β-hydroxylation (as compared to control samples with no TAO present) was plotted as a function of TAO concentration (Fig. 3). The inhibition of testosterone 6β-hydroxylation by TAO determined using this method compares favorably to results reported previously [15].

Because other testosterone metabolites are resolved using this method, it can also be used to monitor the activity of other CYP enzymes that have been shown to produce specific hydroxylated testosterone metabolites [3]. Chromatograms from incubations of testosterone with rat liver microsomes with and without NADPH are shown in Fig. 4. The reconstructed ion chromatogram (m/z 304) from the sample of testosterone incubated with rat liver microsomes and NADPH shows evidence for 2α-, 2β-, 6β-, 7α-, and 16α-hydroxytestosterone. The method could be further optimized for these additional metabolites by monitoring the most abundant ions in the full scan mass spectra of these compounds.

4. Conclusions

A method has been developed for the determination of testosterone and 6β-hydroxytestosterone using GC–SIM–MS. The run time of the method (15 min) is half that of previously reported HPLC and CE methods. The method displays excellent precision and accuracy over a linear range of 0.25 to 100 μM for testosterone and 6β-hydroxytestosterone. The selectivity of the mass spectrometric detector helps eliminate possible interferences from compounds evaluated using this methodology. The assay has been used to characterize the CYP3A activity of preparations of hepatic microsomes, precision-cut slices, and tissue homogenates from a variety of species.

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