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# Determination of 3-keto-4-ene steroids and their hydroxylated metabolites catalyzed by recombinant human cytochrome P450 1B1 enzyme using gas chromatography–mass spectrometry with trimethylsilyl derivatization

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**Abstract**

A protocol utilizing gas chromatography with selected ion monitoring mass spectrometric detection (GC–SIM-MS) using a simplified trimethylsilyl (TMS) derivatization protocol was developed and validated for the determination of hydroxylated metabolites of 3-keto-4-ene steroids such as testosterone, progesterone and androstenedione. Hydroxylated metabolites catalyzed by human CYP1B1 were extracted with methylene chloride and derivatized with BSTFA–10% TMCS. To get an optimal derivatizing condition, the effect of various incubation times and temperatures was evaluated. When the incubation temperature and time in the presence of the TMS derivatizing agent were increased, the 3-keto group became derivatized with TMS to form a 3-TMS derivative. To minimize the formation of the TMS ether on the 3-keto group, a reaction condition of 56 °C for 10 min was used for the routine measurement of the steroids and their hydroxylated metabolite. Performance studies including linearity of calibration curves, extraction efficiency and precision were performed. Linearity of the calibration curves was satisfactory from 0.125 to 5 μ*M* for most compounds except 21-hydroxyprogesterone and 16α-hydroxyandrostenedione which deviated from linearity at the lower concentrations. Mean percentage extraction recoveries were greater than 80% for all compounds. Most compounds showed good precisions with C.V.s of within-day precision of less than 5% and C.V.s of between-day precision of less than 10%. The selected ion chromatograms from the recombinant human CYP1B1 incubations with testosterone, progesterone and androstenedione showed evidence of 6β-, 16α-, 2α-, and 15α-hydroxytestosterone, 6α- and 16α-hydroxyprogesterone and 6α- and 16α-hydroxyandrostenedione, respectively. There was no significant interference associated with *Escherichia coli* membrane extracts in detecting hydroxylated metabolites. This procedure provides a rapid and sensitive method for the evaluation of steroid hydroxylation by CYP isoenzymes.
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## 1. Introduction

Cytochrome P450 (CYP) enzymes play an im-

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portant role in the oxidation of both xenobiotic compounds, such as drugs, and endogenous substrates, such as steroids. CYP1B1 is a relatively recently identified member of the CYP1 gene family [1]. CYP1B1 can metabolize a range of toxic and carcinogenic chemicals in vitro with a unique stereoselectivity in some cases. Estradiol 4-hydroxylation appears to be a characteristic reaction catalyzed by human CYP1B1 [2]. Interest in CYP1B1 is particularly high in the field of cancer research because of this enzyme's ability to metabolize a variety of putative human carcinogens and its increased expression in a wide range of human tumors [3,4]. Thus, CYP1B1 appears to have potentially important roles in tumor development and progression, as a potential target for anticancer drugs and as a tumor biomarker.

The presence of CYP1B1 mRNA in a variety of fetal tissues has led to the suggestion that CYP1B1 may have an important role in normal fetal development. This hypothesis received recent support when mutations in CYP1B1 were linked to the development of primary congenital glaucoma (PCG), an autosomal recessive condition [5,6]. The mechanism by which abnormal CYP1B1 plays a role in the development of this form of glaucoma is not yet established. Some variants of CYP1B1 have been expressed in *Escherichia coli*, along with NADPH cytochrome P450 reductase, and the metabolic activity of the different variants on several steroid hormones, including estradiol, testosterone, and progesterone were assessed [7–10].

Determination of hydroxylated metabolites of steroids is important for the study of the association of CYP1B1 with tumor development and progression, and the pathogenesis of the genetic disorders such as PCG. A number of thin-layer chromatography (TLC) [9,11,12] and liquid chromatography [13–18] methods currently exist for the measurement of hydroxylated metabolites of steroids. Several high-performance liquid chromatography (HPLC) methods have been reported for the separation of testosterone and progesterone and their hydroxylated metabolites. Most HPLC methods, owing to the subtle changes in the physico-chemical properties of the hydroxylated products, require typically long complex gradients to achieve resolution of the various hydroxylated metabolites. The use of radioactively labeled substrates in TLC makes these methods inconvenient and cumbersome.

A gas chromatography–mass spectrometry (GC–MS) method with trimethylsilyl (TMS) derivatization has been reported for the measurement of hydroxylated metabolites of estradiol [10,18]. Lee et al. [18] reported that TMS derivatives of most hydroxylated metabolites of estradiol were successfully identified by GC–MS analysis. For the measurement of the 3-keto-4-ene steroids, most researchers have used the *O*-methyl oximes (MO) for the derivatization of the ketonic functions and the TMS ethers for hydroxyl functions [19]. However, the combination of MO–TMS ethers for derivatization requires a timely multi-step derivatization, which makes this method cumbersome. Recently Testino et al. [20] reported a method for the determination of testosterone and 6β-hydroxytestosterone by gas chromatography with selected ion monitoring mass spectrometric detection (GC–SIM-MS) without derivatization. Rendic et al. [21] reported GC–MS with TMS derivatization to measure 6α-hydroxytestosterone, but did not use this method for the measurement of other hydroxylated metabolites.

In the present study, we evaluated the GC–SIM-MS detection method using TMS derivatization for the determination of hydroxylated metabolites of 3-keto-4-ene steroids such as testosterone, progesterone and androstenedione and applied the method for the analysis of the hydroxylated metabolites formed following the reaction of these steroids with recombinant human CYP1B1 enzyme.

## 2. Experimental

### 2.1. Chemicals

Progesterone, testosterone, 4-androstene-3,17-dione, 17β-estradiol, L-ascorbic acid, L-α-dilauroyl phosphatidyl choline (DLPC), NADP sodium salt, and glucose-6-phosphate were purchased from Sigma (St. Louis, MO, USA). 2α-, 2β-, 6β-, 7α-, 15α-, 16α- and 16β-hydroxytestosterone, 6α-, 16α-, and 21-hydroxyprogesterone, and 6β- and 16α-hydroxyandrostenedione were purchased from Steraloids

(Wilton, NH, USA). Glucose-6-phosphate dehydrogenase was purchased from Roche Diagnostic (Indianapolis, IN, USA). Deuterium-labeled 4-androstene-3,17-dione-2,2,4,6,6,16,16-$d_7$, and progesterone-2,2,4,6,6,17α, 21,21,21-$d_9$ were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada), while *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 10% trimethylchlorosilane (TMCS) was obtained from Regis Technologies (Morton Grove, IL, USA). All the organic solvents used in the present study were of HPLC or GC grade.

### *2.2. Assay of recombinant human CYP1B1 hydroxylation activity*

Hydroxylation of testosterone, progesterone, and androstenedione was carried out in 1-ml assay volume. Human CYP1B1 and cytochrome P450 reductase were co-expressed in *E. coli*, and the bacterial membrane was prepared as described elsewhere with some modification [7,9,10]. Bacterial extracts from the *E. coli* containing active CYP1B1 and P450 reductase were reconstituted with 50 m*M* sodium phosphate, pH 7.4, 10 m*M* $MgCl_2$, 0.01% Na cholate and 20 μ*M* DLPC. The levels of substrate used were 100 μ*M* for testosterone, progesterone and androstenedione. Reaction was initiated by addition of NADPH generating system consisting of 0.5 m*M* $NADP^+$, 5 m*M* glucose 6-phosphate and 0.5 U of glucose-6-phosphate dehydrogenase. Following a 15–60-min incubation at 37 °C, the reaction was terminated by addition of 4 ml of methylene chloride. After stopping the reaction, 100 μl of internal standard solution was added. The samples were mixed on a vortex mixer for 30 s and then centrifuged at 1400 *g* for 5 min. The upper aqueous layer and any precipitate at the interface were removed by aspiration. The remaining methylene chloride layer was evaporated to dryness under a stream of nitrogen. Dried residue was derivatized with 50 μl of BSTFA–10% TMCS and 50 μl of acetonitrile. We evaluated the effect of incubation time and temperature on the derivatization process using the following conditions: 56 °C for 10 min; 56 °C for 30 min; 56 °C for 60 min; 65 °C for 30 min; and 90 °C for 60 min.

### *2.3. Gas chromatography–mass spectrometry*

A 2-μl sample of the derivatized mixture was delivered by automatic injector to a HP 6890 Series GC System (Agilent Technologies, Wilmington, DE, USA) splitless injection port connected to a 5% diphenyl–95% dimethylsiloxane capillary column (30 m×0.25 mm I.D.×0.25 μm film thickness, HP-5MS, Agilent Technologies). The carrier gas was helium at 1 ml/min. The initial temperature of the column was held at 160 °C for 0.5 min, programmed to 250 °C at 6 °C/min, held for 8 min at 250 °C, increased to 300 °C at 8 °C/min and held for 1 min at 300 °C. The temperature of injector and detector were 250 and 290 °C, respectively. An Agilent 5973 Mass Selective Detector was operated in the electron impact mode at 70 eV with an ion source temperature of 230 °C. Identification was performed in the full scan mode in a $m/z$ range of 50–550. Quantification was done in the selective-ion monitoring (SIM) mode.

### *2.4. Performance study*

#### *2.4.1. Calibration curves*

A stock solution containing 1 m*M* of testosterone and its hydroxylated metabolites was prepared in methanol and stored at −20 °C. Working standard solutions of varying concentrations (2.5–100 μ*M*) were prepared from the stock solution. Calibration standard samples (0.125, 0.25, 0.5, 2.5 and 5 μ*M*) were prepared by adding 50 μl of each working solution to 1 ml of sodium phosphate buffer and spiked with 100 μl of 100 μ*M* $d_7$-androstenedione as an internal standard (I.S.) solution. The mixtures were extracted as described above for bacterial membrane incubates. The calibration standards for progesterone, androstenedione and their hydroxylated metabolites were prepared in the same manner as testosterone and spiked with 100 μl of 100 μ*M* $d_9$-progesterone and 100 μl of 100 μ*M* $d_7$-androstenedione, respectively. The calibration curves for steroids and their hydroxylated metabolites were obtained by least-squares linear regression of the peak area ratios of the standard to the I.S. versus the concentrations of each standard.

*2.4.2. Extraction efficiency*

The extraction recovery was determined by comparing the peak areas of spiked bacterial membrane preparations extracted with methylene chloride to the peak areas of standard solutions at the same concentration not carried through the extraction procedure.

*2.4.3. Precision*

Within-day assay precision was determined by replicated analyses ($n=5$) of the quality control samples (0.5, 5 and 50 $\mu M$ for each metabolite). Between day precision was determined by assaying the QC samples on 4 different days. Precision was evaluated from the calculated coefficient of variation (C.V.).

## 3. Results and discussion

*3.1. Method development*

In the literature on the quantification of the 3-keto-4-ene steroids, *O*-methyl oximes (MO) for derivatization of ketonic functions and TMS ethers for hydroxyl functions is a still commonly used method [19]. The formation of methyl oximes serves two purposes: (i) the derivatives have greater thermal stability than the underivatised steroids; and (ii) the formation of enol-ethers, as in 4-en-3-oxo steroids, is prevented during the subsequent silylation procedure. However, the combination of MO–TMS ethers for derivatization requires timely multi-step derivatization and evaporation. A report by Testino et al. [20] reported a method of GC–SIM-MS without derivatization for the determination of testosterone and 6β-hydroxytestosterone, however in our hands, the method produced broad peaks and made quantification difficult in the lower concentration range of products. Therefore, we developed a simplified and rapid GC–SIM-MS detection method using TMS derivatization.

Full scan positive ion electron ionization mass spectra of testosterone, progesterone and androstenedione and their hydroxylated metabolite were acquired to optimize the TMS derivatization conditions. There have been several reports of the measurement of the hydroxylated estrogen metabolites using GC–MS with TMS derivatization. Hanna et al. [10] prepared TMS derivatives by heating the residue with BSTFA–1% TMCS and acetonitrile (1:1, v/v) at 56 °C for 30 min, and Lee et al. [18] prepared them with BSTFA–1% TMCS at 60–65 °C for 30 min, which showed reliable results. However, there have been few reports [21] of the TMS derivatization of hydroxylated metabolites of 3-keto-4-ene steroids because of the problem of subsequent silylation of 3-keto group. We explored the effect of silylation of 3-keto group at various incubation times and temperatures in order to optimize the TMS derivatization conditions.

Initially we used the same incubation condition for the measurement of 3-keto-4-ene steroids and their hydroxylated metabolites as for estradiol. However, the result was not satisfactory, because several TMS derivative peaks (usually three peaks) were detected in most compounds. To explore the reason for this derivative formation we evaluated the effect of various incubation times and temperatures during the derivatization process. TMS ether derivatives are usually formed on the hydroxyl groups of steroids. However when the incubation temperature and time with TMS derivatizing agent are increased, the 3-keto group of testosterone, progesterone and androstenedione may become derivatized with TMS to form a 3-TMS derivative. For example, both the 15α- and 20-hydroxyl groups of 15α-hydroxytestosterone were derivatized at 56 °C for 10 min to form bis-TMS (15α-, 20-TMS) derivatives which gave $m/z$ of 448 for $M^+$, 433 for M-$CH_3$ and 358 for M-TMSOH (Fig. 1). However, when temperature and time are increased, the 3-keto group becomes derivatized with TMS to form a tri-TMS (3-, 15α-, 20-TMS) derivative which gave $m/z$ of 520 for $M^+$, 505 for M-$CH_3$ and 430 for M-TMSOH (Fig. 1). The percentages of the peak area of tri-TMS (3-, 15α-, 20-TMS) derivative to the peak areas of total TMS derivatives (bis- and tri-TMS derivatives) of 15α-hydroxytestosterone produced at the different incubation conditions such as without heating, 56 °C for 10 min, 56 °C for 30 min, 56 °C for 60 min, 65 °C for 30 min, and 90 °C for 60 min were 0.6, 3.1, 6.4, 12.0, 13.6 and 79.9%, respectively.

The comparisons (in percent) of the peak area of 3-TMS derivative to the peak area of total TMS derivatives according to the incubation condition of

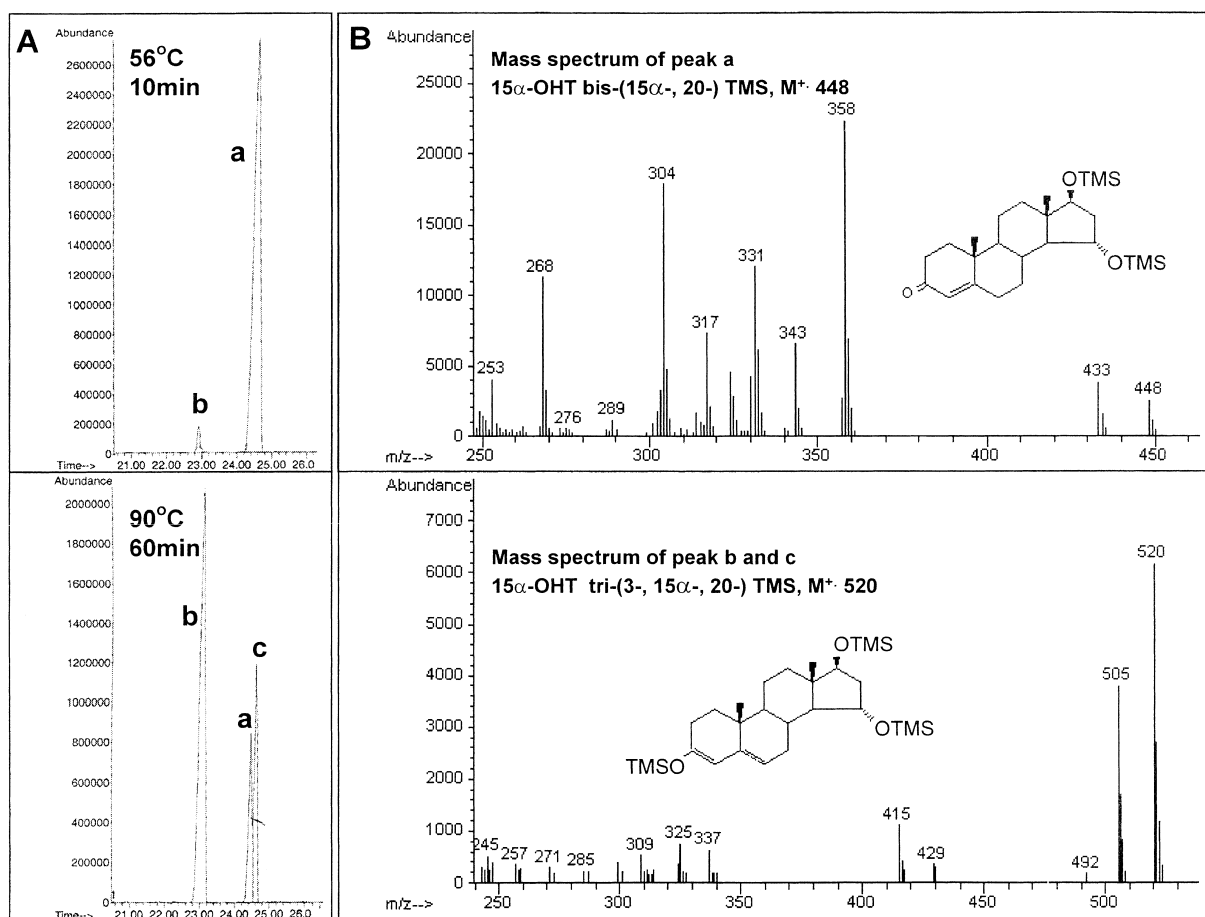


Fig. 1. Total ion chromatograms (TIC) and mass spectra of TMS derivatives of 15α-hydroxytestosterone (OHT). (A) TIC of TMS derivatives of 15α-OHT with different incubation conditions of 560 °C for 10 min and 90 °C for 60 min. Peak a is bis- (15α-, 20-) TMS derivative and peaks b and c are tri- (3-, 15α-, 20-) TMS derivatives. Peaks b and c are considered to be 3,5 dienol and 2,4 dienol isomers, respectively. (B) Mass spectra of bis- (peak a) and tri- (peaks b and c) TMS derivatives of 15α-OHT.

56 °C for 10 min and 90 °C for 60 min are summarized for all other steroids and their hydroxylated metabolites in Table 1. For most compounds, more than 80% of 3-keto groups were derivatized to form the 3-TMS derivative at 90 °C for 60-min incubation. However, the 3-TMS derivatives were barely formed in 2α-hydroxytestosterone and 2β-hydroxytestosterone. And only 30% were changed into 3-TMS derivative in case of 6β-hydroxytestosterone, 6β-hydroxyprogesterone and 6β-hydroxyandrostenedione. This may be due to the presence of a hydroxyl group close to the 3-keto group leading to steric hindrance of the formation of 3-TMS derivative.

Liere et al. [22] reported that progesterone with its 3-keto group reacts similarly to 3-hydroxyl steroids in the derivatization reaction with heptafluorobutyric anhydride (HFBA). Two dienol 3-HFB isomers (2,4- and 3,5-dienols) were formed with the major isomer being the 3,5 dienol 3-HFB. Likewise, two 3-TMS derivatives can be formed with the major isomer being the 3,5 dienol 3-TMS derivative. As mentioned above, in the incubation condition with higher temperature and longer time, usually three peaks were detected: one main peak (TMS derivative with intact, underivatized, 3-keto group), a smaller peak with earlier retention time (3,5-dienol 3-TMS derivative)

Table 1
Percentages of the peak area of 3-TMS derivative relative to the peak areas of the total TMS derivatives according to the different incubation conditions (55 °C for 10 min and 90 °C for 60 min)

| Compounds | Relative percentages | |
|---|---|---|
| | 56 °C, 10 min | 90 °C, 60 min |
| *Testosterone* | 8.3 | 95.1 |
| α-OHT | 1.6 | 1.6 |
| 2β-OHT | 0.2 | 4.9 |
| 6β-OHT | 1.8 | 31.9 |
| 7α-OHT | 8.5 | 84.8 |
| 15α-OHT | 3.1 | 83.5 |
| 16α-OHT | 4.5 | 97.0 |
| 16β-OHT | 7.3 | 85.4 |
| *Progesterone* | 10.2 | 74.8 |
| 6β-OHP | 2.7 | 30.3 |
| 16α-OHP | 10.6 | 88.1 |
| 21-OHP | 13.9 | 90.9 |
| *Androstenedione* | 16.3 | 91.6 |
| 6β-OHA | 4.7 | 33.6 |
| 16α-OHA | 14.9 | 92.1 |

OHA, hydroxyandrostenedione; OHP, hydroxyprogesterone; OHT, hydroxytestosterone.

and the smallest peak having a similar retention time to the main peak (2,4-dienol 3-TMS derivative) (Fig. 1).

To minimize the formation of TMS ether on the 3-keto group, 56 °C for 10 min was used for the routine measurement of the steroids and their hydroxylated metabolite. The retention time and quantified mass ion for SIM mode are shown in Table 2. The 15α-hydroxytestosterone and 16β-hydroxytestosterone were not separated well in the chromatographic conditions used and had similar fragmented mass ions patterns, and hence we were unable to quantify them.

### 3.2. Validation of the analytical method

#### 3.2.1. Linearity

Weighted ($1/x^2$) linear regressions of peak area ratios of compounds to the internal standard were performed to obtain a standard curve with the range from 0.125 to 5 μ*M*. Weighted regressions were used to provide homogeneity of variance across the calibration range. Slope and correlation coefficient

Table 2
Retention times and characteristic mass fragments of TMS derivatives of steroids and their hydroxylated metabolites

| Compounds | Retention time (min) | Characteristic mass fragments (*m/z*) |
|---|---|---|
| *Testosterone* | 20.43 | 129, $\underline{360}$, 73, 270, 345 |
| 2α-OHT | 24.84 | $\underline{433}$, 73 |
| 2β-OHT | 23.38 | $\underline{433}$, 73, 332 |
| 6β-OHT | 21.85 | 73, 129, $\underline{392}$, 433, 448 |
| 7α-OHT | 21.69 | 73, $\underline{358}$, 317, 433 |
| 15α-OHT | 24.28 | 73, 217, $\underline{358}$, 191, 304 |
| 16α-OHT | 25.98 | 73, 147, 191, $\underline{358}$, 448, 343 |
| 16β-OHT | 24.28 | 73, 147, 191, $\underline{358}$, 448, 343 |
| *Progesterone* | 24.04 | 124, 272, $\underline{314}$, 229 |
| $d_9$-progesterone | 23.86 | 129, 279, $\underline{323}$, 233 |
| 6β-OHP | 25.27 | $\underline{346}$, 387, 73, 402, 331 |
| 16α-OHP | 27.19 | 73, 157, $\underline{312}$, 269, 297, 387, 402 |
| 21-OHP | 29.48 | $\underline{299}$, 73, 271, 253, 387 |
| *Androstenedione* | 19.52 | $\underline{286}$, 124, 244 |
| $d_7$-androstenedione | 19.39 | $\underline{293}$, 129, 249 |
| 6β-OHA | 20.63 | $\underline{359}$, 318, 73, 374 |
| 16α-OHA | 24.07 | $\underline{303}$, 116, 73, 359 |

Underlined fragments were used for quantification. OHA, hydroxyandrostenedione; OHP, hydroxyprogesterone; OHT, hydroxytestosterone.

Table 3
Between-day variation of calibration curves (weighting factor: $1/conc^2$)

| Compounds | Slope | | Correlation coefficient ($r^2$) | |
|---|---|---|---|---|
| | Mean±SD | C.V. (%) | Mean±SD | C.V. (%) |
| *Testosterone* | 2.44±2.43 | 9.97 | 0.997±0.016 | 1.66 |
| 2α-OHT | 2.70±0.12 | 4.34 | 0.977±0.006 | 0.66 |
| 2β-OHT | 3.28±0.12 | 3.79 | 0.968±0.014 | 1.39 |
| 6β-OHT | 5.24±0.17 | 3.20 | 0.976±0.018 | 1.83 |
| 7α-OHT | 9.46±0.55 | 5.77 | 0.968±0.020 | 2.03 |
| 15α- and 16β-OHT | 7.74±0.47 | 6.11 | 0.967±0.020 | 2.34 |
| 16α-OHT | 2.58±0.18 | 6.90 | 0.957±0.020 | 2.53 |
| *Progesterone* | 1.58±0.02 | 1.41 | 0.983±0.010 | 1.28 |
| 6β-OHP | 1.03±0.01 | 1.12 | 0.971±0.010 | 0.91 |
| 16α-OHP | 1.99±0.07 | 3.44 | 0.936±0.020 | 1.83 |
| 21-OHP | 2.00±0.04 | 2.02 | 0.905±0.030 | 3.35 |
| *Androstenedione* | 5.27±0.41 | 7.73 | 0.984±0.003 | 0.34 |
| 6β-OHA | 1.45±0.13 | 9.20 | 0.975±0.012 | 1.24 |
| 16α-OHA | 1.43±0.07 | 4.71 | 0.936±0.009 | 0.91 |

Weighted ($1/\chi^2$) linear regressions were performed to obtain a standard curve with the range from 0.125 to 5 μ*M*. Slope and correlation coefficient values of regression data were calculated from four successive analytical runs. OHA, hydroxyandrostenedione; OHP, hydroxyprogesterone; OHT, hydroxytestosterone.

values of regression data from four successive analytical runs are summarized in Table 3. Linearity was satisfactory for all steroids and their hydroxylated metabolites except 21-hydroxyprogesterone and 16α-hydroxyandrostenedione which deviated from linearity at the lower concentrations. These slight deviations from the linearity of the 21-hydroxyprogesterone and 16α-hydroxyandrostenedione may be due to the imprecision in the between-day assay at the lower concentration (Table 4). However according to some reports [12,13], the ranges of 21-hydroxyprogesterone and 16α-hydroxyandrostenedione produced by liver microsome or recombinant human P450 enzyme incubates are between 0.5 and 9 μ*M* which are within the assay range (0.125–5 μ*M*). Even though these P450 enzymes were not CYP1B1, the deviation of the linearity of the 21-hydroxyprogesterone and 16α-hydroxyandrostenedione at the extremely low concentration may not be problematic for the determination of the analytes catalyzed by recombinant human CYP1B1 enzyme.

#### *3.2.2. Extraction recovery*

Extraction efficiency was estimated by comparing the peak area of spiked extracted samples to those non-extracted standards. Recovery was determined at three concentrations (0.5, 5 and 50 μ*M*). Mean percentage extraction recoveries were greater than 80% for all compounds (Table 4).

#### *3.2.3. Precision*

Within-day precision was determined by assaying five replicates of three different concentrations (Table 4). Most compounds showed good precision with C.V.s of less than 5%. Between-day precision was determined by assaying three different concentrations on 4 consecutive days. All compounds showed satisfactory between-day precision with C.V.s of less than 10%, even though imprecision was greater for the lower concentrations of some compounds.

### *3.3. Applications for recombinant human CYP1B1 hydroxylation activity*

The selected ion chromatogram from the recombinant CYP1B1 incubations with testosterone, progesterone and androstenedione showed evidence of 6β-, 16α-, 2α-, and 15α-hydroxytestosterone, 6β- and 16α-hydroxyprogesterone, and 6β- and 16α-hy-

Table 4
Within-day and between-day precision and mean extraction recovery

| Compounds | Within-day precision (C.V., %) | | | Between-day precision (C.V., %) | | | Mean recovery (%) |
|---|---|---|---|---|---|---|---|
| | 0.5 μ*M* | 5 μ*M* | 50 μ*M* | 0.5 μ*M* | 5 μ*M* | 50 μ*M* | |
| *Testosterone* | 4.56 | 4.74 | 3.75 | 1.97 | 2.15 | 4.85 | 97.1 |
| 2α-OHT | 4.48 | 1.93 | 2.19 | 2.63 | 0.37 | 2.57 | 87.4 |
| 2β-OHT | 4.88 | 2.00 | 1.83 | 3.32 | 1.10 | 3.31 | 89.3 |
| 6β-OHT | 3.37 | 2.89 | 1.66 | 7.93 | 0.28 | 3.40 | 89.2 |
| 7α-OHT | 8.54 | 4.18 | 1.43 | 7.06 | 2.66 | 2.29 | 84.4 |
| 15α- and 16β-OHT | 2.16 | 3.90 | 2.08 | 6.22 | 3.38 | 3.67 | 84.9 |
| 16α-OHT | 4.63 | 4.83 | 2.16 | 5.46 | 5.15 | 2.46 | 83.2 |
| *Progesterone* | 3.16 | 0.83 | 1.91 | 7.62 | 2.41 | 3.33 | 96.7 |
| 6β-OHP | 2.41 | 5.56 | 2.42 | 3.52 | 2.49 | 4.35 | 89.9 |
| 16α-OHP | 2.87 | 3.31 | 0.16 | 1.56 | 8.97 | 2.03 | 93.3 |
| 21-OHP | 4.44 | 1.28 | 0.87 | 8.22 | 5.52 | 4.81 | 96.3 |
| *Androstenedione* | 1.15 | 1.65 | 2.56 | 2.12 | 2.52 | 3.22 | 96.4 |
| 6β-OHA | 8.02 | 1.75 | 1.59 | 3.54 | 4.59 | 6.53 | 91.7 |
| 16α-OHA | 4.22 | 3.51 | 3.13 | 8.56 | 2.99 | 1.68 | 93.6 |

Within-day precision was determined by assaying five replicates of three different concentrations. Between-day precision was determined by assaying three different concentrations on 4 consecutive days. Extraction efficiency was estimated by comparing peak area of spiked extracted samples to those non-extracted standards of three different concentrations. OHA, hydroxyandrostenedione; OHP, hydroxyprogesterone; OHT, hydroxytestosterone.

droxyandrostenedione, respectively. There was no significant interference associated with the *E. coli* membrane preparations in detecting hydroxylated metabolites, which gave sensitivity and specificity similar to that obtained from performance studies using authentic standard mixture.

## 4. Conclusions

We have developed a rapid, reliable protocol for the determination of various 3-keto-4-ene steroids (testosterone, progesterone, and androstendione) and their hydroxylated derivatives formed upon catalysis by P450 enzymes. The method utilizes TMS derivatization and separation by gas chromatography with MS-SIM detection. The method allowed for the analysis of the various metabolites formed by the reaction of human CYP1B1 with several steroids and should prove to be of great value in the analysis of other P450 enzymes.

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