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Isocratic high-performance liquid chromatographic method for the separation of testosterone metabolites

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic (HPLC) method using an Ultrasphere IP column has been developed for the determination of testosterone and its metabolites after incubation of 4-^{14}C-labelled or unlabelled testosterone with rat liver microsomes. Compounds were eluted with methanol–water–tetrahydrofuran (35:55:10, v/v, pH 4.0) and detected by ultraviolet (UV) absorption at 245 nm. UV or on-line radioactivity detection can be used although, due to differences in detector cell volumes, peak resolution is slightly better with UV detection. Selectivity was validated by collecting HPLC peaks and verifying their identity by gas chromatography–mass spectrometry after derivatization by N,O-bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane. A three-day validation was performed to determine the linearity, repeatability, reproducibility and accuracy of the method, using corticosterone as internal standard. The method is applicable to the measurement of cytochrome P-450 isoenzyme activities in rat liver.

1. Introduction

Cytochrome P450 (CYP) represents a family of haemoproteins capable of metabolising a wide range of endogenous and xenobiotic compounds [1]. They have been extensively characterised and shown to exist in multiple forms, or isoenzymes, which show broad substrate specificity and differing catalytic activity [2]. One of the major obstacles in the identification of different CYP isoenzymes involved in the metabolism of a drug is the lack of specific yet simple enzymatic assays to distinguish the individual isoforms [3–9]. The regio- and stereospecific hydroxylation of the steroid nucleus is, however, a sensitive fingerprint for the identification of a specific CYP isoenzyme [4–6]. For example, in rat, CYP2A1 [4,5] metabolizes testosterone mainly to 7α-hydroxytestosterone, whereas CYP2B1 forms 16α- and 16β-hydroxytestosterone and androstenedione [4], and CYP3A3, -3A4 and -3A5 form predominantly 6β-hydroxytestosterone [10,11]. The metabolism of testosterone has thus been used to probe in vitro preparations of rat liver for CYP isoenzyme activities [7–9].

Several HPLC methods have been reported for the separation of testosterone and its hy-

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droxylated metabolites [3–5]. The majority of these methods are, however, characterized by complex gradient systems and, in some cases, require a second elution step to bring about further separation of metabolites [4,7,12]. In this paper we present an isocratic HPLC method, with UV and on-line radioactivity detection, for the separation of testosterone and eight of its hydroxylated metabolites. Previous studies with purified rat liver microsomal P450 isoenzymes have shown that these metabolites are the major oxidative metabolites of testosterone in rat microsomal preparations [3–6].

2. Experimental

2.1. Chemicals

Testosterone, androstenedione, 16α- and 2α-hydoxytestosterone and corticosterone were obtained from Sigma (St. Quentin Fallavier, France); 6α-, 7α-, 6β-, 16β-, 2β- and 19-hydroxytestosterone were obtained from Steraloids (Wilton, NH, USA); 1α-, 15β- and 18-hydroxytestosterone were kindly donated by G.D. Searle (Skokie, IL, USA). 17β-N,N-Diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one (4-MA) was a kind gift from Dr. Rassmusson (Merck, Rahway, NJ, USA). 4-[^{14}C]testosterone was obtained from Amersham (Les Ulis, France). Methanol (HPLC reagent grade) and acetic acid (AnalaR grade) were obtained from Merck (Darmstadt, Germany). Tetrahydrofuran (HPLC grade) was purchased from J.T. Baker (Deventer, Netherlands), and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from Fluka (Buchs, Switzerland). Ultima-flo LSC-cocktail was obtained from Packard (Groningen, Netherlands).

2.2. Preparation of standard solutions

Testosterone, androstenedione and hydroxy metabolites were dissolved in methanol to give 3.5, 3.5 and 3.3 μM stock solutions, respectively, which were stored at 4°C. Working solutions were made by a 1:10 dilution of stock solutions with methanol–water (1:1, v/v). Corticosterone, the internal standard [6], was similarly prepared.

2.3. Preparation of microsomes

Livers of adult male Sprague–Dawley rats weighing 200 to 250 g were homogenized in ice-cold 50 mM (pH 7.4) Tris buffer, 154 mM KCl using a Potter–Elvejehem PTFE glass homogeniser. The homogenate was centrifuged at 10 000 g for 30 min at 4°C and the supernatant was then centrifuged at 105 000 g for 60 min at 4°C. The resulting pellet was resuspended, to the same volume of supernatant, in homogenising buffer and again centrifuged at 105 000 g for 60 min at 4°C. The washed pellets were resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol. The microsomal suspensions were stored in 500-μl aliquots in Eppendorf tubes at −80°C until use.

The protein content of the microsomal preparation was measured by the method of Bradford [13] with bovine serum albumin as standard. The P-450 content was determined spectrophotometrically according to the method of Omura and Sato [14].

2.4. Incubation of testosterone with rat liver microsomes

Testosterone was incubated for 15 min at 37°C in a total incubation mixture volume of 200 μl containing potassium phosphate buffer (80 mM, pH 7.4), $MgCl_2$ (5 mM), NADP (1 mM), glucose-6-phosphate (10 mM), glucose-6-phosphate dehydrogenase (2 U/ml), testosterone (250 μM) and rat liver microsomes (0.5 mg protein/ml). Linearity of the formation of the metabolites as a function of time and protein concentration was previously verified (results not shown). Reactions were started by the addition of the NADPH-generating system and stopped by the addition of 200 μl of ice-cold methanol containing 3 nmol of the internal standard corticosterone. The samples were centrifuged at 1500 g for 5 min, and 200 μl of the supernatant

were analyzed by HPLC. When used, 4-[^{14}C]testosterone was evaporated to dryness to remove carrier toluene and reconstituted in working solutions of cold testosterone to give a specific activity of 4 μCi/μmol.

2.5. High-performance liquid chromatography

The HPLC system was composed of a Waters 510 pump (Waters Millipore, St. Quentin Yvelines, France), a WISP 712 autosampler, a Waters 484 UV detector and a Flo-One A-515 on-line radioactivity detector (Packard Instrument, Rungis, France), which was used for signal acquisition and peak integration. The Ultrasphere IP 5-μm HPLC column (250 × 4.6 mm I.D.) (Beckman, Gagny, France) was used with a Waters μBondapak C_{18} Guard-Pack pre-column (10 μm particle size, 6 × 4 mm I.D.). The column was eluted isocratically with a mobile phase of methanol–water–tetrahydrofuran (35:55:10). Milli-Q water (resistance >10 MΩ cm^{-1}) was used to prepare the mobile phase, and its pH was adjusted to 4.0 by drop-wise addition of glacial acetic acid. The solvent was filtered through a 0.2-μm Zetapor membrane (Cuno, Meriden, CT, USA) before use. A flow-rate of 1.0 ml/min was used and the UV detector set at 245 nm. All chromatography was performed at room temperature (22–24°C).

2.6. Fraction collection

In order to verify the purity of metabolite peaks, individual HPLC peaks obtained from a representative rat liver microsomal incubation were collected manually, and the corresponding fractions were evaporated to dryness. The fractions collected from four injections were combined in order to obtain sufficiently high concentrations of metabolites. Before gas chromatography–mass spectrometry (GC–MS) analysis, the dry fractions were derivatized with 100 μl of BSTFA–TMCS (80:20, v/v) and heated for 2 h at 70°C. The samples were then evaporated to dryness under a stream of nitrogen and reconstituted in 50 μl of *n*-hexane.

2.7. Gas chromatography–mass spectrometry

MS analyses were carried out on a QMD 1000 GC–MS system from Fisons Instruments (Arcueil, France). The chromatographic column was a 30 m × 0.32 mm I.D. 5% phenylmethylsilicone fused-silica capillary column (Supelco, Saint Germain-en-Laye, France). The film thickness was 0.52 μm and the carrier gas was helium at a pressure of 70 kPa at the head of the column. The samples were injected via a splitless injector at a temperature of 280°C. To elute the steroids, the oven was programmed from 80 to 230°C at a rate of 5°C/min and then at 2°C/min to 260 and 5°C/min to 285°C. The interface of the gas chromatograph–mass spectrometer was kept at 295°C. Mass spectra were obtained in the positive-ion chemical ionization (PICI) mode with ammonia as reagent gas at an ion source pressure of 0.02 Pa. In the full-scan mode (100–600 a.m.u.) the scan time was set at 450 ms with a photomultiplier voltage of 550 V. In the selected-ion monitoring mode a dwell time of 150 ms per ion was used with the photomultiplier voltage kept at 750 V.

2.8. Recovery, quantitation and accuracy

The extraction efficiency (recovery) of the sample preparation procedure was tested by comparing the peak-area ratios obtained from chromatography of aqueous standards with those of extracted incubation mixtures. Recovery was determined at concentrations of 0.5 and 5 nmol/ml for the hydroxylated metabolites and at 50 and 375 nmol/ml for testosterone.

Calibration curves were obtained by spiking heat-inactivated microsomal preparations with testosterone, its eight metabolites and corticosterone as internal standard. Concentrations ranging from 0.25 to 10 nmol/ml were used for the calibration curves. Linear regression plots of peak-area ratios against concentration were constructed and concentrations of testosterone and its metabolites determined from the peak-area ratio relative to the calibration graph.

The precision and accuracy of the method

were determined by performing repeated analyses of spiked microsomal preparations containing two different concentrations of testosterone and the eight metabolites. The concentrations were selected to cover the low and high ranges of the calibration curve. Six replicates of each concentration were analysed on three different days. Two calibration graphs were included each day, one at the beginning and one at the end of the run. Within-day and day-to-day precision were expressed as coefficient of variation (C.V., %). Day-to-day accuracy was determined by calculating the difference between the mean observed value and the theoretical value as a function of the theoretical value, and the relative error (R.E.) expressed as a percentage.

3. Results and discussion

3.1. HPLC separation of testosterone and its metabolites

In previously published HPLC methods [3–5] the determination of testosterone and its hydroxylated metabolites was performed by UV detection at wavelengths ranging from 240 nm [15] to 284 nm [16]. As the absorbance maxima

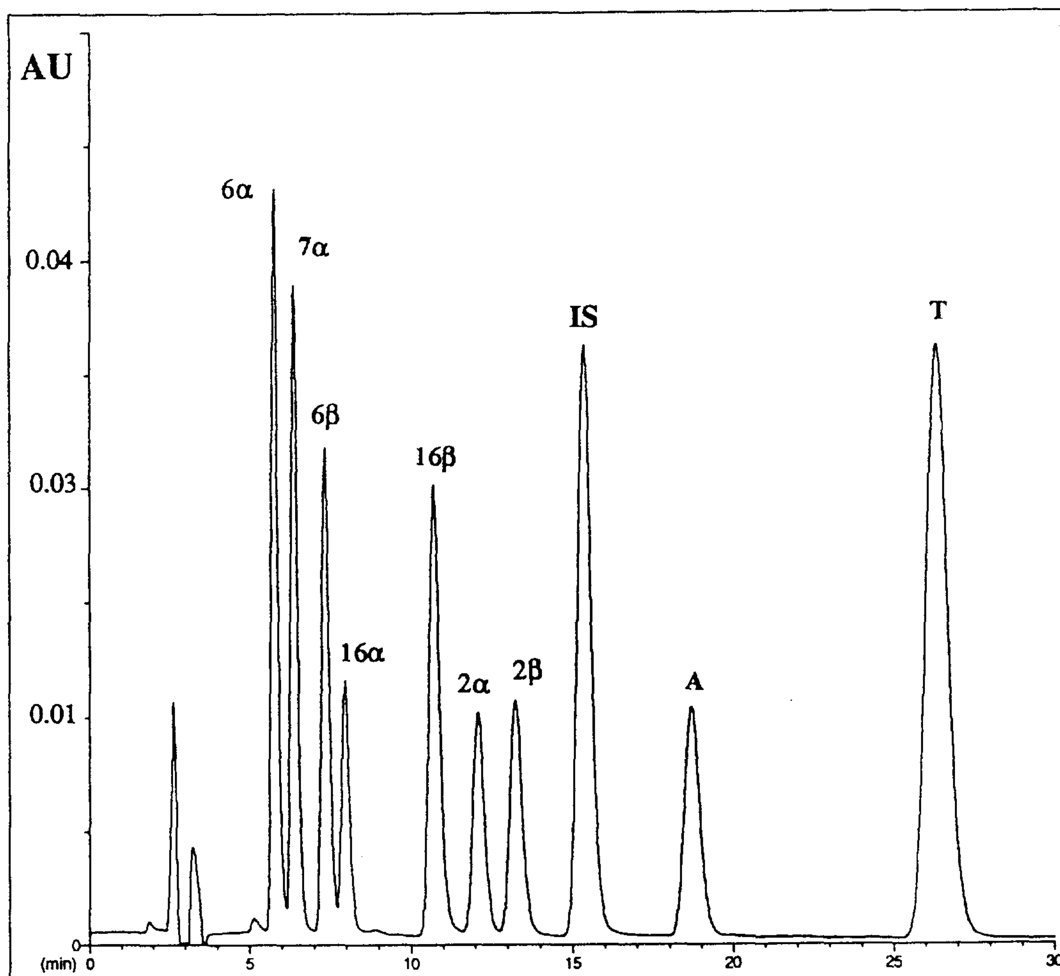

Fig. 1. HPLC separation of testosterone (T, 25 nmol/ml), corticosterone (IS, 6 nmol/ml) and eight authentic standards (5 nmol/ml): 6α-, 7α-, 6β-, 16α-, 16β-, 2α-, 2β-hydroxytestosterone and androstenedione (injection volume 200 μl).

Table 1
HPLC and GC retention times of steroid standards and major ions formed by GC–MS

Compound	HPLC retention time (min)	GC retention time (of di-TMS derivative) (min)	Major ions
6α-Hydroxytestosterone	5.8	43.9	$[M+H]^+$ 449
7α-Hydroxytestosterone	6.4	40.6	$[M+H]^+$ 449
6β-Hydroxytestosterone	7.3	40.7	$[M+H]^+$ 449/$[MH-90]^+$ 359
16α-Hydroxytestosterone	7.9	45.2	$[M+H]^+$ 449
16β-Hydroxytestosterone	10.6	45.8	$[M+H]^+$ 449
2α-Hydroxytestosterone	12.0	43.9	$[M+H]^+$ 449
2β-Hydroxytestosterone	13.1	42.2	$[M+H]^+$ 449/$[MH-16]^+$ 433
Androstenedione	18.5	37.5[a]	$[M+H]^+$ 287
Testosterone	26.1	38.8[b]	$[M+H]^+$ 361
Corticosterone	15.1	N.D.[c]	N.D.[c]

[a] Not derivatized.
[b] Mono-TMS derivative.
[c] N.D. = not determined.

of testosterone and its metabolites was observed to be closer to 245 nm, in the present study this wavelength was selected. The majority of the above-reported methods also used complex gradient elution systems and, in some cases, required a second elution step [3–5], whereas separation of the metabolites of testosterone in the present method was achieved by isocratic elution on a 25-cm reversed-phase column.

A representative chromatogram of a mixture of eight authentic standards, reported as being the major testosterone metabolites formed by rat liver microsomes [3], is shown in Fig. 1. Retention times of testosterone and its metabolites are presented in Table 1. The compounds were well resolved and eluted in the same order as previously described [15]. In the present method, corticosterone was used as the internal standard instead of 11β-hydroxytestosterone [4,17] because 11β-hydroxytestosterone was not baseline-separated from 2β-hydroxytestosterone using the present HPLC conditions. The overall run-time for the chromatographic separation was 30 min.

3.2. Specificity of the method

GC–MS analysis of testosterone and available steroid standards is shown in Fig. 2. Most of the compounds present a major ion corresponding to the $[M+H]^+$ ion and some fragments corresponding to the loss of a methyl group $[MH-16]^+$ or a silanol group $[MH-90]^+$ (Table 1). 7α-Hydroxytestosterone presented a major ion at m/z 449 and 6β-hydroxytestosterone a characteristic ion at m/z 359; although these compounds were not well separated by GC–MS, the specificity of these ions allowed their separation and identification. Similarly, 2β- and 15β-hydroxytestosterone, which had characteristic ions at m/z 433 and 359, respectively, could also be separated. Unfortunately, 2α- and 6α-hydroxytestosterone could not be separated by their GC retention time or by the presence of specific ions. In all cases the two compounds were well separated by HPLC.

HPLC peaks that showed both UV and radioactivity responses were collected, after injecting a representative rat liver microsomal incubation, in order to verify that no endogenous compounds or unidentified metabolites coeluted. Fractions were analyzed by GC–MS after derivatization.

A chromatogram of rat liver microsomes incubated with 4-[^{14}C]testosterone is shown in Fig. 3. The top trace is that of the on-line radioactivity detector and the bottom trace of the UV detector. Peaks that were collected are shown

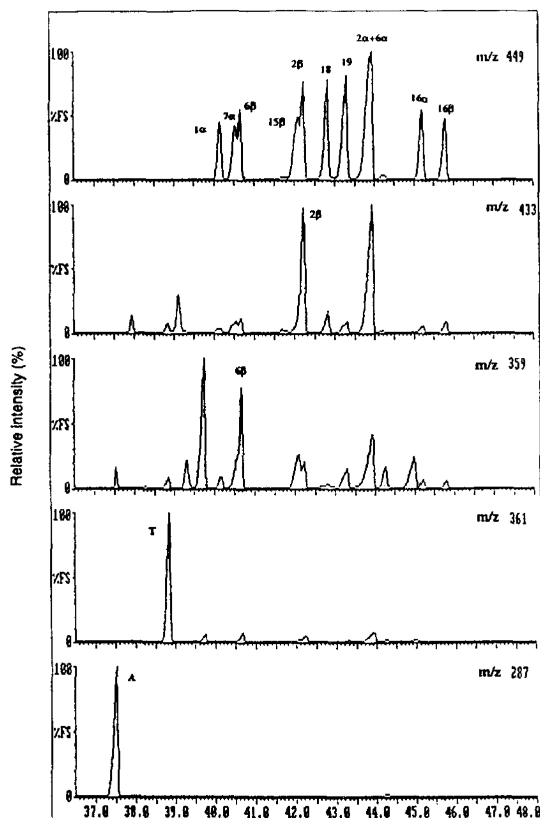

Fig. 2. GC–MS reconstructed ion chromatograms of androstenedione (A) and the TMS derivatives of testosterone (T), 7α-, 6β-, 2β-, 18-, 19-, 6α-, 2α-, 16α- and 16β-hydroxytestosterone (0.7 nmol) and 1α- and 15β-hydroxytestosterone (2 nmol).

numbered on the bottom UV trace. It can be seen that all UV peaks characterized as testosterone metabolites, when their retention times were compared to those of authentic standards, gave a corresponding ^{14}C trace. The identity of the collected fractions was assigned after GC–MS analysis, when spectra were compared to standards. A summary of the results is shown in Table 2.

All fractions were found to be pure except peak 5, which was a minor HPLC peak. Although its HPLC retention time was consistent with that of 2β-hydroxytestosterone, GC–MS analysis revealed the presence of two compounds (Fig. 4). The first compound, with a GC–MS retention time of 42.2 min and quasimolecular ion $[M+H]^+$ at m/z 449, was similar to 2β-hydroxytestosterone. The second compound, with a retention time of 42.8 min, demonstrated a quasimolecular ion $[M+H]^+$ at m/z 451 with a characteristic fragment ion $[MH-90]^+$ at m/z 361 corresponding to the loss of a silanol group. A molecular mass of 306 was assigned to the non-derivatized compound which, according to MS data, had two hydroxyl groups and was reduced at position 4. This testosterone metabolite may correspond to a hydroxydihydrotestosterone analogue but, as no reference compound was available, the position of hydroxylation could not be confirmed. It has been shown previously that hydroxytestosterone metabolites are substrates for steroid 5α-reductase and that this enzyme is completely inhibited by 4-MA [18]. The co-eluting peak disappeared when testosterone was incubated with rat liver microsomes in the presence of 1 μM 4-MA, thus confirming the hypothesis that this metabolite corresponded to a hydroxydihydrotestosterone derivative.

An unknown HPLC peak with a retention time of 20.2 min (peak 7) was detected in all incubations. After derivatization with BSTFA–TMCS, GC–MS analysis showed one peak that represented a major characteristic quasimolecular ion $[M+H]^+$ at m/z 359. A molecular mass of 286 was assigned to the non-derivatized compound, which contained one hydroxyl function,

Table 2
Identification by GC–MS of the collected fractions

Fraction No.	Structure identified by GC–MS
1	7α-Hydroxytestosterone
2	6β-Hydroxytestosterone
3	16α-Hydroxytestosterone
4	2α-Hydroxytestosterone
5	2β-Hydroxytestosterone + hydroxydihydrotestosterone
6	Androstenedione
7	Dehydrotestosterone
8	Testosterone

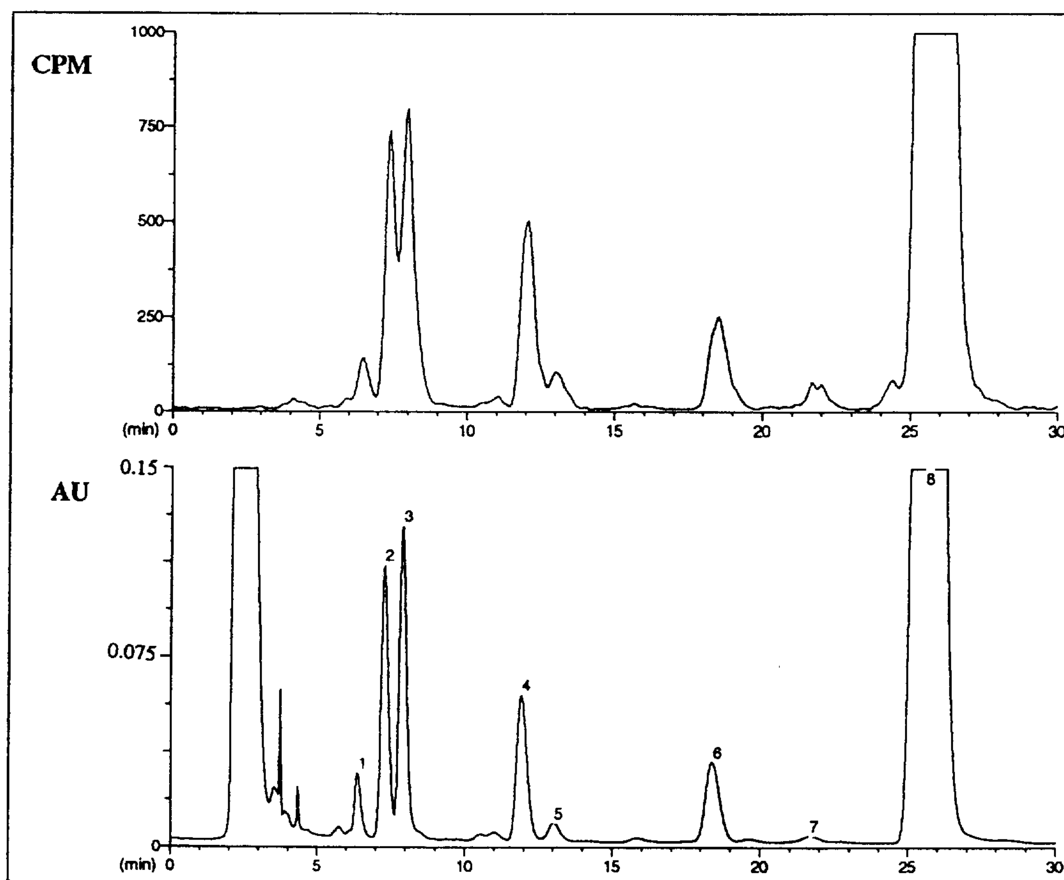

Fig. 3. Chromatogram of rat liver microsomes (0.5 mg protein/ml incubation mixture) incubated with 4-[^{14}C]testosterone (250 μM). The top trace is from the on-line radioactivity detector and the bottom trace from the UV detector. Numbered peaks on the bottom UV trace correspond to the collected fractions.

confirmed by the presence of one trimethylsilyl (TMS) group, and two double bonds. As the unknown metabolite gave the same mass spectrum as 1-dehydrotestosterone but with a different retention time, it was thought to be 6-dehydrotestosterone, which has been reported to be a testosterone metabolite [3].

Thus, under the isocratic HPLC conditions used, complete separation of the testosterone metabolites produced by rat liver microsomes was achieved and GC–MS analysis showed no major interfering compound. Although 6α- and 16β-hydroxytestosterone were available as standards, they were not formed by rat liver microsomes.

3.3. Recovery, linearity and accuracy

All recoveries of testosterone metabolites at concentrations of 0.5 and 5 nmol/ml were greater than 94% (Table 3).

Linearity of the method was established over the concentration range 50–375 nmol/ml for testosterone and 0.25–10 nmol/ml for metabolites (with the exception of 16β-hydroxytestosterone, which was found to be linear within the range 0.25–5 nmol/ml). Typical correlation coefficients were greater than 0.99 for all compounds.

The within-day precision (repeatability) for the analysis of testosterone and its metabolites is

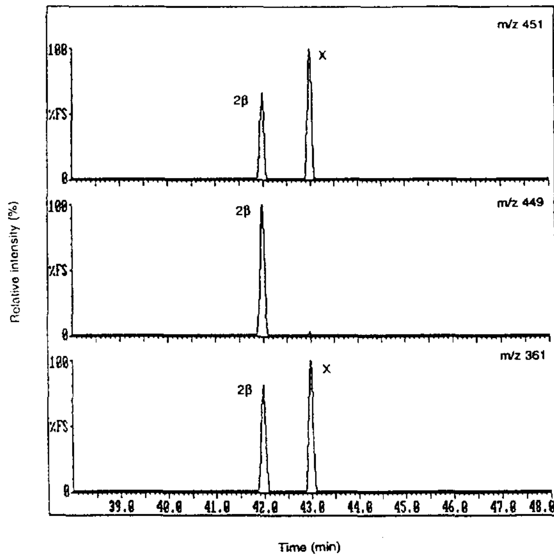

Fig. 4. GC–MS reconstructed ion chromatograms of the BSTFA–TMCS-derivatized fraction corresponding to HPLC peak 5. Peaks: 2β = 2β-hydroxytestosterone; X = hydroxydihydrotestosterone.

shown in Table 4. The values were less than 8% for the lower concentration studied, except for 2α-hydroxytestosterone where they were 12.4%. At the higher concentration studied, all values were less than 6%. The day-to-day C.V. (reproducibility) ranged from 3 to 14% for all the compounds at the concentrations studied (Table 5).

Day-to-day accuracy, expressed as the relative error (R.E.), is shown in Table 5. R.E.s for testosterone and its metabolites were less than ± 8% for both concentrations.

Table 3
Recovery of testosterone and its metabolites

Steroid	Recovery (mean ± S.D.) (%)	
	0.5 nmol/ml ($n = 5$)	5 nmol/ml ($n = 6$)
6α-Hydroxytestosterone	99.5 ± 6.3	93.5 ± 5.3
7α-Hydroxytestosterone	100.3 ± 9.4	93.9 ± 5.0
6β-Hydroxytestosterone	102.4 ± 9.0	94.4 ± 5.4
16α-Hydroxytestosterone	108.7 ± 9.5	95.4 ± 5.4
16β-Hydroxytestosterone	103.4 ± 8.7	93.9 ± 4.5
2α-Hydroxytestosterone	101 ± 11.1	94.1 ± 4.1
2β-Hydroxytestosterone	103.8 ± 11.0	94.8 ± 4.5
Corticosterone	102.3 ± 1.5	102.4 ± 3.8
Androstenedione	105.7 ± 11.0	94.1 ± 5.2
Testosterone	100.7 ± 1.5[a]	98.9 ± 2.7[b]

[a] Added concentration 50 nmol/ml.
[b] Added concentration 375 nmol/ml.

Table 4
Within-day precision ($n = 6$) for the analysis of testosterone and its metabolites

Steroid	C.V. (%)	
	0.5 nmol/ml	5 nmol/ml
6α-Hydroxytestosterone	3.9	5.7
7α-Hydroxytestosterone	4.1	5.6
6β-Hydroxytestosterone	3.1	5.5
16α-Hydroxytestosterone	3.9	6.1
16β-Hydroxytestosterone	4.6	5.4
2α-Hydroxytestosterone	12.4	5.8
2β-Hydroxytestosterone	7.8	5.9
Androstenedione	3.0	5.4
Testosterone	2.4[a]	0.9[b]

[a] Added concentration 50 nmol/ml.
[b] Added concentration 375 nmol/ml.

4. Conclusions

An isocratic reversed-phase HPLC method has been developed and validated for the resolution of testosterone metabolites produced by rat liver microsomes. The inclusion of 4-MA in the incubation mixture inhibits the production of a reduced hydroxytestosterone metabolite, which co-eluted with 2β-hydroxytestosterone in the described HPLC conditions. The present work demonstrates the complementarity of the two separation techniques of HPLC and GC for verifying the purity of the identified peaks. The developed method can be used to measure CYP isoenzyme activities and has been used to monitor changes in the pattern of testosterone metabolism in rat liver microsomes after treatment with inhibitors or inducing agents, as well as new candidate drugs. In the case of inducing agents (β-naphthoflavone, phenobarbital, 3-methylcholanthrene and dexamethasone), the purity of HPLC peaks was also verified by GC–

Table 5
Day-to-day accuracy (R.E.) and precision (C.V.) ($n = 18$) for the analysis of testosterone and its metabolites

Steroid	0.5 nmol/ml		5 nmol/ml	
	C.V. (%)	R.E. (%)	C.V. (%)	R.E. (%)
6α-Hydroxytestosterone	5.4	3.6	5.0	−6.6
7α-Hydroxytestosterone	8.9	−3.1	8.9	−6.3
6β-Hydroxytestosterone	4.9	4.3	5.1	−6.5
16α-Hydroxytestosterone	5.7	7.8	6.8	−6.1
16β-Hydroxytestosterone	6.4	−0.9	5.3	−3.6
2α-Hydroxytestosterone	8.9	−3.6	4.6	−5.9
2β-Hydroxytestosterone	7.9	5.9	5.0	−5.7
Androstenedione	14.3	0.3	5.2	−0.7
Testosterone	2.9[a]	−4.4[a]	2.7[b]	−1.5[b]

[a] Added concentration 50 nmol/ml.
[b] Added concentration 375 nmol/ml.

MS. This method reflects actual enzyme activity and therefore represents an advantage over other methods, such as spectral measurement of total CYP or Western blot analysis, which only determine the amount of protein. The method has also been applied to monitor CYP isoenzyme activities in rat hepatocytes; in this case ^{14}C-labelled testosterone was used to avoid interfering peaks from the cell culture medium.

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