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Ethyl acetate extraction procedure and isocratic high-performance liquid chromatographic assay for testosterone metabolites in cell microsomes

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Abstract

An isocratic reversed-phase high-performance liquid chromatography (HPLC) method was developed and validated for separation of testosterone and its main metabolites over the nominal range 20 to 40 μg/ml and 280 to 4600 ng/ml, respectively. Mobile phase composition (phosphate buffer–methanol–acetonitrile, 50:38.5:11.5) was optimised by studying the influence of numerous chromatographic parameters. The most critical one was the ratio CH_3CN/CH_3OH. Good recoveries (around 90% for all compounds) and an improved specificity were assessed by a double ethyl acetate extraction of biological samples. According to the performance criteria tested, the method could be applied to enzymatic inhibition and induction in vitro studies. © 1998 Elsevier Science B.V.

Keywords: Testosterone; Ethyl acetate

1. Introduction

Among the numerous enzymes involved in xenobiotic metabolism, the cytochrome (CYP) P450-dependent monooxygenases are the most frequently reported. They are expressed in several organs, including liver, intestine, kidney, skin, lung and placenta [1,2]. The CYP450, superfamily of hemoproteins is present in multiple forms, or isoenzymes. They catalyse a wide range of endogenous and xenobiotics compounds [3]. The regio- and stereospecific hydroxylation of steroids, often used to measure cytochrome isoenzymes activities, are considered as a fingerprint for the identification of the specific isoform of CYP450 [4–6]. The oxidative pathways of testosterone, leading to numerous hydroxylated metabolites are considered to be specific for defined isoenzymes.

Therefore testosterone is a frequently used probe for in vitro inhibition and induction studies involving cytochrome isoenzyme activities.

Several high-performance liquid chromatography (HPLC) methods have been reported for the separation of testosterone and its hydroxylated metabolites. Most of these HPLC methods use a gradient elution and a complex mobile phase [5,7,8]. Run times are generally excessive and between-run reequilibration is required. Up to now, only one isocratic HPLC method with UV absorbance detection has been reported [9]. The mobile phase

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contained tetrahydrofuran (THF) which is known to form peroxides. The present work was undertaken in order to overcome the stability problem caused by THF use and to improve the sensitivity of the assay. The development and the analytical validation results of the isocratic HPLC method and extraction procedure are presented. An in vitro inhibition study is also investigated.

2. Experimental

2.1. Chemicals

Testosterone, 6β-, 16α-, 11β- and 2α-hydroxytestosterone, corticosterone, quercetin, magnesium chloride, ethyl acetate (HPLC-grade) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 7α-, 16β-Hydroxytestosterone, androstenedione were a kind gift from UCB (Braine L'Alleud, Belgium).

Methanol (HPLC, analytical-grade), potassium mono- and dihydrogenphosphate, sodium mono- and dihydrogenphosphate were obtained from Merck (Darmstadt, Germany).

Acetonitrile (HPLC, analytical-grade) was purchased from Lab-Scan (Dublin, Ireland).

Cell microsomes expressing human CYP450 3A4 isoenzyme and P450 reductase were obtained from Gentest (Woburn, MA, USA).

2.2. Preparation of working standard solution

Stock solutions of testosterone (10.7 m*M*), corticosterone, the internal standard (I.S.) (1.5 m*M*) and 6β-, 16α- and 2α-hydroxytestosterone (10 m*M*) were prepared in methanol and stored at 4°C until use. Testosterone and metabolite working solutions were prepared by serial dilution in a methanol–potassium phosphate buffer (1:1) (100 μ*M*, pH 7.4) mixture in order to achieve a final concentration of 1.7 m*M* and 0.25 m*M*, respectively.

Corticosterone (I.S.) working solution was prepared by dilution in a potassium phosphate buffer (100 μ*M*, pH 7.4) to achieve a final concentration of 0.15 m*M*.

Quercetin was dissolved in ethanol to give a stock solution of 10.9 m*M*. Working solutions were prepared by serial dilution in a methanol–potassium phosphate buffer (100 μ*M*, pH 7.4) mixture.

2.3. Incubation of testosterone with cell microsomes

Testosterone (520 μ*M*, 50 μl) was incubated for 15 min at 37°C in a total incubation mixture volume of 250 μl containing potassium phosphate buffer (100 μ*M*, pH 7.4), $MgCl_2$ (25 m*M*, 25 μl), NADP (6.5 m*M*, 25 μl), glucose-6-phosphate (16 m*M*, 25 μl), glucose-6-phosphate dehydrogenase (10 U/ml, 25 μl) and cell microsomes (1 mg/ml, 100 μl). The production of testosterone metabolites was linear over time and protein microsomal concentration (results not shown). The reaction was initiated by the addition of cell microsomes at 4°C (the addition of ice cold microsomes to prewarmed incubation mixture was recommended by Gentest). After 15 min incubation, the reaction was stopped by addition of 500 μl of ethyl acetate containing 7.3 nmol of the I.S., corticosterone. The sample was Vortex-mixed for 1 min and centrifuged at 1100 *g* for 5 min. After removal of 350 μl of the ethyl acetate extract, the extraction was repeated with 500 μl of ethyl acetate. After Vortex-mixing and centrifugation, 500 μl of the organic phase was removed. The pooled extract was evaporated under a stream of nitrogen. The residue was reconstituted with 150 μl of mobile phase. An aliquot fraction was analysed by HPLC.

2.4. Preincubation with quercetin

Action of quercetin at $1.09 \cdot 10^{-7}$ *M* and $1.09 \cdot 10^{-6}$ *M*, on testosterone metabolism in vitro was investigated. Quercetin was preincubated for 5 min at 37°C in a total mixture of 225 μl containing $MgCl_2$ (25 m*M*, 25 μl), NADP (6.5 m*M*, 25 μl), glucose-6-phosphate (16 m*M*, 25 μl), glucose-6-phosphate dehydrogenase (10 U/ml, 25 μl) and cell microsomes (1 mg/ml, 100 μl). After 5 min, 25 μl of testosterone (1.04 m*M*) was added and the experiment was then conducted as described in Section 2.3.

2.5. High-performance liquid chromatography

The HPLC system included a Gilson 307 pump (Gilson, Villiers Le Bel, France), a HP UV series

1050 detector (Hewlett-Parckard, Wilmington, DE, USA) operating at 242 nm. Signal acquisition and peak integration were achieved by a Borwin chromatography software (JMBS, Le Fontanil, France). A Waters Symmetry RP 18 HPLC column (5 μm, 250×4.6 mm I.D.) protected by a Sentry Symmetry C_{18} guard column (20×3.9 mm I.D.) were used (Waters, Milford, MA, USA). The mobile phase was a mixture of sodium phosphate buffer (100 μ*M*, pH 6.0)–methanol–acetonitrile (50:38.5:11.5, v/v/v) and the flow was set to 1.2 ml/min. Milli-Q Water (resistance>15 MΩ cm^{-1}) was used to prepare buffer solution (Millipore, Bedford, MA, USA) and the mobile phase was filtered through a 0.22-μm membrane (Sartorius, Brussels, Belgium). All separations were performed at 40°C.

2.6. Performance criteria

All the performance criteria, except extraction efficiency, were studied by the analysis of the ratio (analyte/I.S.) determined from extracted samples.

Concentrations studied for standard calibration curves covered the range 280 to 4600 ng/ml for the 6β-, 16α- and 2α-hydroxylated metabolites and 20 to 40 μg/ml for testosterone. Linear regression plots for hydroxylated metabolites and for testosterone were constructed [10,11]. The extraction efficiency was estimated by comparing peak-area of spiked extracted samples to those of non-extracted standards [12]. The within-day, between-day and total precision were expressed by the relative standard deviation (R.S.D.) [13]. Accuracy was estimated by calculating the difference between mean observed value and the theoretical value. It was expressed as a percentage by the relative error (R.E.) [14].

3. Results and discussion

3.1. HPLC separation for testosterone and its metabolites

The isocratic HPLC method precedently reported for the separation of testosterone and eight of its metabolites has the drawback of THF use [9]. Indeed, THF, an unstable solvent, generates peroxides [15,16]. These peroxides are able to oxidise some components of the biological system and so, in some instance disturb metabolism results. Moreover, THF often requires precautionary measures before use (such as distillation) to discard impurities [15,17].

In short, in order to reduce the risk of peroxide formation and for easiness and precautionary reasons, THF has to be avoided. Our alternative isocratic HPLC method improves the isocratic mobile phase by using a more stable and pure organic modifier, namely acetonitrile. The composition of the new mobile phase was adjusted by optimisation of several chromatographic parameters namely buffer concentration, buffer pH, temperature, flow, proportions of CH_3CN and CH_3OH. The relative proportion of CH_3OH and CH_3CN had a drastic incidence on the separation of the 6β/7α-, 6β/16α-, 7α/16α-testosterone metabolites (results not shown). In the optimised conditions allowing adequate separations of 6β/7α-, 6β/16α-, 7α/16α-testosterone metabolites, corticosterone appeared to be a better alternative than 11β-hydroxytestosterone as internal standard. A good resolution and selectivity were obtained for a ratio CH_3CN/CH_3OH less than 0.500 and with a buffer percentage of 55% (results not shown). Using these conditions, the run time was too long. By decreasing the CH_3CN/CH_3OH ratio to 0.3, buffer percentage to 50% and by increasing the temperature to 40°C, the run time was reduced to 35 min without any significant loss of resolution and selectivity. A representative chromatogram of a mixture of testosterone, six of its main metabolites and the internal standard, corticosterone is shown in Fig. 1.

3.2. Extraction

Sample purification improves selectivity and sensibility of chromatographic assay and enhances the column and guard-column life time. Ethyl acetate extraction appeared to be a simple, rapid and efficient way of improving the sensitivity of the assay. Ethyl acetate (HPLC-grade) was chosen on the basis of a study of the extraction recovery of several solvents (results not shown). It appeared that ethyl acetate was essential to combine high extraction recoveries and no interferences from biological matrices. Extraction has also the advantage of con-

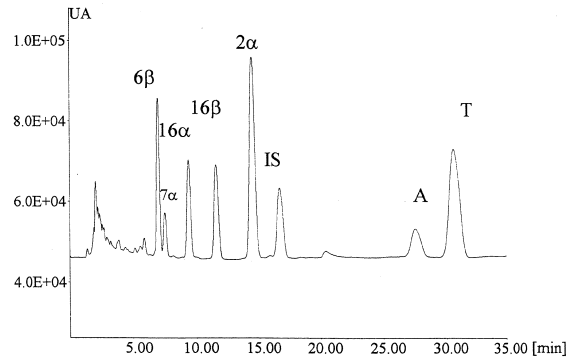

Fig. 1. Chromatogram of a mixture of testosterone, its main metabolites and the internal standard. HPLC separation of testosterone (T, $1.0 \cdot 10^{-8}$ g), corticosterone (I.S., $3.1 \cdot 10^{-7}$ g) and six testosterone metabolites ($1.9 \cdot 10^{-8}$ g): 6β-, 7α-, 16α-, 16β-, 2α-hydroxytestosterone and androstenedione (A).

centrating the sample. No influence of partial miscibility between ethyl acetate and aqueous phase on extraction precision and accuracy has been statistically proved ($p<0.05$). The efficiency of the extraction procedure was measured for 6β-, 16α-, 2α-hydroxytestosterone and testosterone and the recovery ranged between 84 to 102%. The results of the extraction efficiency are summarised in Table 1. We could expect a good extraction efficiency for 7α- and 16β-hydroxytestosterone and androstenedione. These metabolites have lyophilic and chromatographic behaviour intermediate to 6β-hydroxytestosterone and testosterone.

6β-, 16α- and 2α-Hydroxytestosterone were selected for additional validation parameters as the assay was intended to be used for future inhibition and induction investigations of cytochrome P450 3A4 (6β), 2C11 (16α, 2α) isoenzymes.

3.3. Specificity

The specificity of the analytical procedure was assessed by studying the possible interference of the incubation system including NADPH generating system, $MgCl_2$, microsomes. Representative microsomal system incubation without NADPH and representative microsomal system incubation without substrate were also investigated. Moreover 7α-, 16β-hydroxylated metabolites and androstenedione did not interfere with the retention time of 6β-, 16α- and 2α-hydroxytestosterone. In short, no peak interfered with the retention time of testosterone and its main hydroxylated metabolites (Fig. 2a,b).

3.4. Linearity

Relationships between peak area ratios (analyte/I.S.) and concentrations were linear over the range 280 to 4600 ng/ml for the metabolites and 20 to 40 μg/ml for testosterone. Over this range, there was evidence for heteroscedasticity, assessed following Bartlett's test, for metabolite peak area ratios. Therefore data were log pretransformed prior to the calculation of the calibration curve parameters (least squares regression analysis). However, for testosterone, no pretransformation was required.

Slope, intercept and correlation coefficient values of three calibration lines, are summarised in Table 2.

Table 1
Extraction efficiency of testosterone and metabolites

Concentration (M)	Hydroxylated testosterone metabolites (% recovery)			Concentration (M)	Substrate (% recovery)
	6β OH-testo	16α OH-testo	2α OH-testo		Testosterone
$9.12 \cdot 10^{-7}$	89	85	96	$6.96 \cdot 10^{-5}$	95
$2.43 \cdot 10^{-6}$	93	92	95	$7.97 \cdot 10^{-5}$	98
$5.47 \cdot 10^{-6}$	93	90	97	$8.96 \cdot 10^{-5}$	98
$9.12 \cdot 10^{-6}$	96	86	88	$1.07 \cdot 10^{-4}$	102
$1.22 \cdot 10^{-5}$	94	84	86	$1.18 \cdot 10^{-4}$	87
$1.46 \cdot 10^{-5}$	90	86	86	$1.28 \cdot 10^{-4}$	94

Six independent sets of samples were prepared at six different concentrations. The extraction efficiency was determined by comparing peak area ratio of spiked extracted samples to those of non-extracted standards.

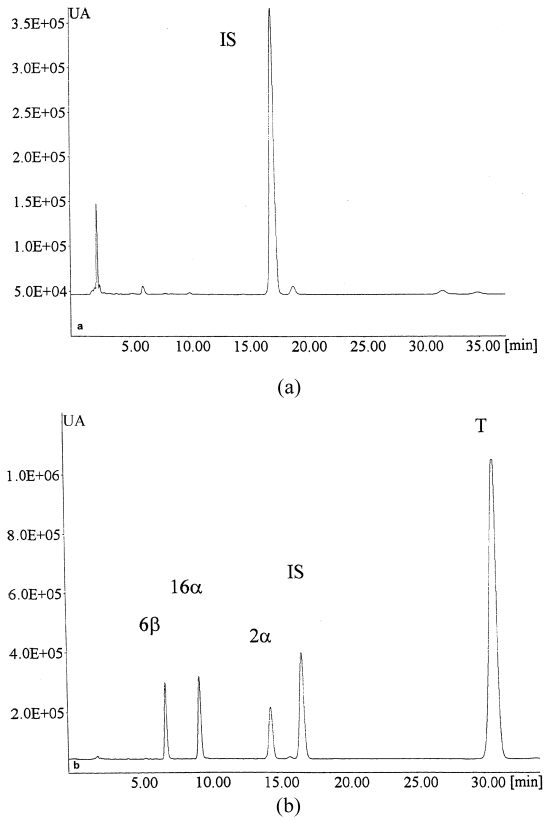

Fig. 2. Specificity of the HPLC method. (a) Chromatogram of a representative microsomal system incubated without testosterone. (b) HPLC separation of testosterone (T, $1.2 \cdot 10^{-6}$ g), corticosterone (I.S., $1.7 \cdot 10^{-6}$ g) and 6β-, 16α-, 2α-hydroxytestosterone ($1.5 \cdot 10^{-7}$ g) after extraction of a spiked sample.

The correlation coefficient was always >0.997 for the metabolites. As the slopes and the intercepts on three days were not significatively different ($p>0.01$), it was not necessary to prepare each day a new calibration curve (results not shown). Concentrations adjusted to peak area ratios were not significatively different (variance analysis, $p>0.05$). The correlation coefficient for testosterone was >0.97. Sensitivity, represented by the calibration curve slope was fairly constant for each compound. The sensitivity for the hydroxylated metabolites was expressed by the log detransformed intercept value. For testosterone, it was the slope. The unit of sensitivity was l/mol.

3.5. Precision

The precision was assessed from peak area ratio measurements at four concentrations included in the range of the calibration standards. For each level of concentration, six microsomal independent replicates were prepared on three different occasions. The pooled within-day, between-day and total precision were estimated following the method of Krouwer and Rabinowitz [13]. Results are summarised in Table 3. The pooled within-run component of imprecision (PWR) (the precision), expressed by the R.S.D., was <9%. The imprecision was greater for the lower concentrations. The between-run component of imprecision (BR), expressed by the R.S.D.,

Table 2
Assay linearity for testosterone and its main metabolites

Compound	Transformation	Correlation coefficient (r) (mean±S.D.) R.S.D. (%)	Slope (mean±S.D.) R.S.D. (%)	Intercept (mean±S.D.) R.S.D. (%)	Sensitivity (mean±S.D.) R.S.D. (%)
6β OH-testo	log–log	$0.99877 \pm 0.6 \cdot 10^{-4}$ 0.006	1.01±0.01 1.1	4.41±0.06 1.3	26 000±3000 12.6
16α OH-testo	log–log	$0.99877 \pm 0.6 \cdot 10^{-4}$ 0.006	0.97±0.01 1.0	4.32±0.05 1.2	21 000±2000 11.6
2α OH-testo	log–log	$0.9975 \pm 2.0 \cdot 10^{-4}$ 0.02	0.936±0.003 0.3	4.13±0.01 0.3	13 300±400 3
Testosterone	No	0.97±0.06 0.65	42 000±1000 3.4	−0.7±0.1 14	42 000±1000 3.4

Three calibration curves were studied on one day. The six concentrations covered the range from 280 to 4600 ng/ml for the metabolites and 20 to 40 μg/ml for testosterone. The results were obtained after extraction of spiked samples.

Table 3
Assay precision for testosterone and 6β-, 16α- and 2α-hydroxylated metabolites

Products	Concentration (*M*)	R.S.D. (%) (PWR)	R.S.D. (%) (BR)	R.S.D. (%) (T)
6β-Hydroxytestosterone	$9.00\cdot10^{-7}$	5.0	2.4	5.6
	$5.40\cdot10^{-6}$	2.2	0.3	2.2
	$1.22\cdot10^{-5}$	2.4	1.7	2.9
	$1.46\cdot10^{-5}$	4.6	10.7	11.7
16α-Hydroxytestosterone	$9.25\cdot10^{-7}$	8.8	3.6	9.5
	$5.55\cdot10^{-6}$	2.8	0	2.8
	$1.22\cdot10^{-5}$	3.1	1.6	3.4
	$1.50\cdot10^{-5}$	1.3	7.4	7.5
2α-Hydroxytestosterone	$9.12\cdot10^{-7}$	6.0	10.9	12.4
	$5.40\cdot10^{-6}$	1.8	1.0	2.0
	$1.22\cdot10^{-5}$	3.1	0	3.1
	$1.46\cdot10^{-5}$	3.5	6.2	7.1
Testosterone	$6.96\cdot10^{-5}$	3.4	3.7	5.0
	$7.97\cdot10^{-5}$	4.4	5.6	7.1
	$1.07\cdot10^{-4}$	4.3	0	4.3
	$1.28\cdot10^{-4}$	1.5	2.2	2.6

Precision was determined for four concentrations, each concentration replicated six times. PWR: The pooled within-run component of imprecision; BR: between-run component of imprecision; T: total imprecision.

was <11%. The total imprecision (T) (the reproducibility), expressed by the R.S.D., was <13%.

3.6. Accuracy

The accuracy was assessed at three different concentrations. Four spiked microsomal preparations at each level and a calibration curve were prepared. Concentrations were determined from back-calculated concentrations relative to the calibration curve. Actual concentrations were compared to the expected concentrations, and the relative error (R.E.) was calculated. The results of accuracy, presented in Table 4, ranged from −13% to 4%.

3.7. Stability

Three sets of extracted samples were used for stability assessment. The stability of the solutions was studied over three days at room temperature. Since there was no evidence of any consistent degradation of the compounds, the stability results are considered satisfactory.

3.8. In vitro inhibition test

An illustration is presented in Fig. 3a,b. The percentage of inhibition was determined by comparing a mixture without inhibitor (100% control activi-

Table 4
Assay accuracy for testosterone and its main metabolites

Concentration (*M*)	Hydroxylated testosterone metabolites [% relative error (R.E.)]			Concentration (*M*)	Substrate (% R.E.)
	6β OH-testo	16α OH-testo	2α OH-testo		Testosterone
$1.70\cdot10^{-6}$	−11.5	−12.9	−10.9	$8.85\cdot10^{-5}$	0.04
$7.29\cdot10^{-6}$	−2.6	−3.5	1.5	$9.60\cdot10^{-5}$	0.05
$1.10\cdot10^{-5}$	3.9	0.6	2.4	$1.22\cdot10^{-4}$	3.5

Accuracy was determined for three concentrations, each concentration replicated four times.

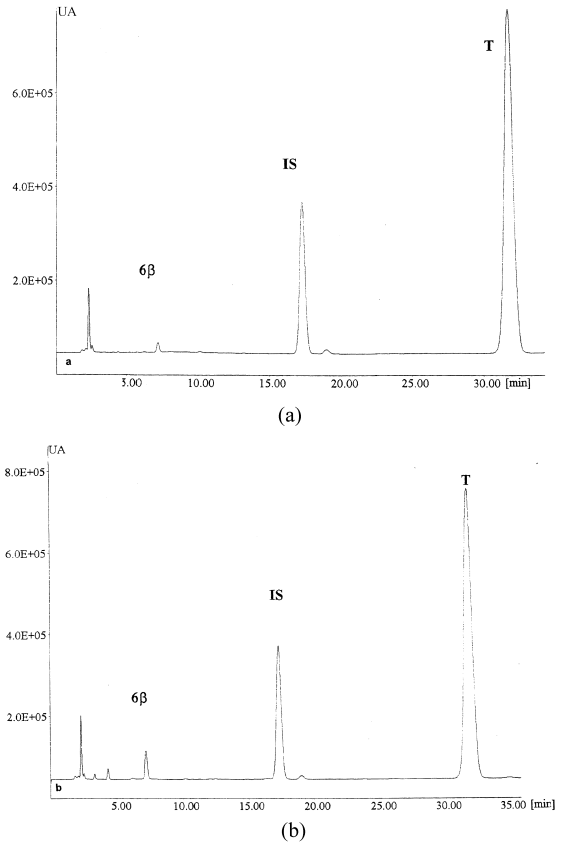

Fig. 3. (a) Action of quercetin ($1.09 \cdot 10^{-6}$ *M*) on testosterone metabolism. (b) Action of quercetin ($1.09 \cdot 10^{-7}$ *M*) on testosterone metabolism.

ty) with a mixture containing the inhibitor. Inhibitions reached 76±4% and 66±5% for the $1.09 \cdot 10^{-6}$ *M* and $1.09 \cdot 10^{-7}$ *M*, respectively. Quercetin is thus, a potent inhibitor, in vitro, of the cytochrome P450 3A4, by inhibiting 6β-hydroxytestosterone formation.

4. Conclusions

The isocratic HPLC method permits the separation of testosterone and six of its metabolites with good resolution and good selectivity. The performance criteria, such as linearity, specificity, precision, accuracy, extraction efficiency and stability, indicate that the proposed HPLC method is suitable for the determination of testosterone and its main metabolites over the nominal range 20 to 40 μg/ml and 280 to 4600 ng/ml, respectively. The simple, rapid extraction procedure enables one to purify and to concentrate the samples. This extraction procedure associated with the HPLC method is well adapted to future induction and inhibition studies.

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