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

Paul M. Whalley, David Bakes, Kenneth Grime, Richard J. Weaver*

Division of Metabolism & Pharmacokinetics, Servier Research and Development Ltd., Fulmer Hall, Windmill Road, Slough SL3 6HH, UK

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Abstract

A rapid high-performance liquid chromatography (HPLC) method is described for the quantitation of hydroxytestosterone metabolites. The method combines a Hypersil BDS C_{18} analytical column (10 cm×0.46 cm) and a linear mobile phase (1.25 ml/min) gradient of tetrahydrofuran–acetonitrile–water (10:10:80, v/v) changing to tetrahydrofuran–acetonitrile–water (14:14:72, v/v) over 10 min then remaining isocratic for 3 min. The total run time for the chromatographic separation of eight metabolites of testosterone is 15 min. Detection by UV is linear between 300 ng/ml and 10 μg/ml with a limit of detection on column of 300 ng/ml. A method for the direct HPLC analysis of liver microsomal incubates of [^{14}C]testosterone is also briefly described and when combined with the HPLC method, offers a distinct advantage over previously reported methods for the rapid screening of testosterone hydroxylase activity in rat and human liver microsomes.
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Keywords: Testosterone

1. Introduction

The cytochromes P450 (CYP) are a superfamily of enzymes that span the entire flora and fauna kingdoms [1–3]. Each of these enzymes exhibits unique catalytic activities yet is able to support the biotransformation of large numbers of lipophilic molecules to more water-soluble components. In man and toxicology species, cytochromes P450 are pivotal in determining the potential toxicity and efficacy of pharmaceutical drugs. Evaluating the role of these enzymes in drug metabolism often requires that there are a number of well-characterised cytochrome P450 selective substrates available to probe cytochrome P450 enzyme activities.

The majority of cytochrome P450 probe substrates are used in drug metabolism studies to help characterise structure–activity relationships, assess enzyme induction, predict drug–drug interactions and facilitate drug candidate selection. Among the different cytochrome P450 substrate assays available, the regio- and stereo-selective hydroxylation of testosterone is one of the mostly commonly used in in vitro metabolism assays for the assessment of cytochrome P450 enzyme activity in humans and toxicology species. Since the CYP specific regio- and stereo-selective hydroxylation of testosterone is particularly well characterised in the rat and man, it can

*Corresponding author. Tel.: +44-1753-662-647; fax: +44-1753-664-423.

E-mail address: weaver@netgrs.demon.co.uk (R.J. Weaver).

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be used routinely to assess different cytochrome P450 enzyme activities in a single assay. Testosterone is metabolised by several rat cytochromes P450 including, for example, CYP3A1/2-dependent formation of 6β-hydroxytestosterone CYP2C11-dependent formation of 16α- and 2α-hydroxytestosterone, CYP2B1-dependent formation of 16β-hydroxytestosterone, and CYP2A1-dependent formation of 7α-hydroxytestosterone [4,12,15]. Human CYP3A4-dependent formation of 6β-hydroxytestosterone is widely used as a measure of CYP-dependent hydroxylation of testosterone by human liver microsomes [9,17,18].

A number of thin-layer chromatographic and liquid chromatographic methods currently exist for the measurement of hydroxytestosterone metabolites [4–9]. Unfortunately, many of these methods, owing to the subtle changes in the physico–chemical properties of the hydroxylated products, required typically long complex gradients to achieve resolution of the various hydroxylated metabolites of testosterone. Because a number of the hydroxytestosterone metabolites possesses not only the same parent ion in mass spectrometry but also the same product ion transition, metabolites cannot be identified by mass transition alone and therefore require separation prior to analysis by mass spectrometry. The aim of this study was to develop a rapid, simple and robust high-performance liquid chormatography (HPLC) method suitable for the quantitative analysis of testosterone hydroxylase activity.

2. Materials and methods

2.1. HPLC standards and reagents

Testosterone, hydroxytestosterone metabolites (2α, 2β, 6β, 7α, 11β, 16α, 16β and androstenedione) and NADPH were purchased from Sigma–Aldrich (Poole, UK). The [^{14}C]-labelled testosterone was purchased from Amersham Life Science (Buckinghamshire, UK). The radiochemical purity of [^{14}C]testosterone (specific activity 56 mCi/mmol) by HPLC was >98%. All organic solvents were SpS grade (Romil, Cambridge, UK). Water was purified to 18 MΩ using an Elga UHQ purifier (Elga, Buckinghamshire, UK).

Rat liver microsomes were prepared in the laboratory as previously described [10]. Human liver microsomes pooled from male (n=8) and female (n=7) Caucasians were supplied by In Vitro Technologies (Totam Biologicals, Northampton, UK).

2.2. HPLC apparatus and data analysis

The HPLC system comprised a series 200 pump and an ISS 200 autosampler (Perkin-Elmer, Berkshire, UK), a Hypersil BDS C_{18}, 3 μm (10 cm×0.46 cm) analytical column (Phenomenex, Macclesfield, UK) maintained at a temperature of 40°C. A linear mobile phase (1.25 ml/min) gradient [tetrahydrofuran–acetonitrile–water (10:10:80, v/v; mobile phase A) changing to tetrahydrofuran–acetonitrile–water (14:14:72, v/v; mobile phase B)] was run over 10 min, with a further 3 min run isocratically. Freshly prepared tetrahydrofuran was used to prepare the mobile phase to ensure accuracy of the composition of the mobile phase. The column was re-equilibrated for a further 2 min with mobile phase A before the next sample was injected. Detection of the analytes was by in-line UV detection at 255 nm using an ABI detector (Applied Biosystems, CA, USA) and a Packard 150TR liquid-flow radiochemical detector with 1:3 mixing of HPLC eluent-liquid scintillent (Ultima Flo M, Packard, Meriden, CT, USA). Data was acquired through the use of a PE Nelson 900 series interface and the chromatograms analysed using Turbochrom 6.1.1 software (Perkin-Elmer, San Jose, CA, USA).

2.3. Linearity

All measurements were made in triplicate using testosterone and hydroxylated testosterone standards prepared in methanol, and suitably diluted in mobile phase (tetrahydrofuran–acetonitrile–water, 10:10:80, v/v). Linearity of UV response was determined using non-weighted curve fitting for all analytes between 300 ng/ml and 10 μg/ml.

2.4. Microsomal incubations

Reaction mixtures comprised 250 μg of human or Wistar rat liver microsomal protein, 100 μM [^{14}C]testosterone (56 mCi/mmol), 1 mM NADPH

and Tris buffer (0.1 *M*, pH 7.4), prepared to a total volume of 250 μl. All incubation mixtures were pre-incubated for 5 min at 37°C in a shaking water bath. Reactions were initiated by the addition of NADPH and after a suitable period of incubation, terminated by the addition of 250 μl ice-cold methanol. Microsomal protein was removed from suspension by centrifugation (18 500 *g*, 10 min) and 50 μl of the supernatant injected directly onto the HPLC system.

2.5. Method validation

Stock solutions of testosterone and each of the hydroxylated standards (1 mg/ml) were prepared in methanol and an equal volume of each taken to prepare standard mixtures of 111 μg/ml. Replicate injections ($n=10$) of the standard mixtures were used to determine variability of the retention time and the peak area, reported relative to the testosterone peak. High column concentrations of analyte, used to validate the method, were selected to accommodate potential saturation effects on-column, which may lead to variability in retention time and peak response. For each reference standard the mean relative retention time to testosterone and the maximum and minimum relative retention times were determined. The maximum and minimum values required accuracy to be within 5% of the mean for each standard. In addition, the maximum and minimum UV peak response of each reference standard was required to be within 10% of the mean value for each reference standard.

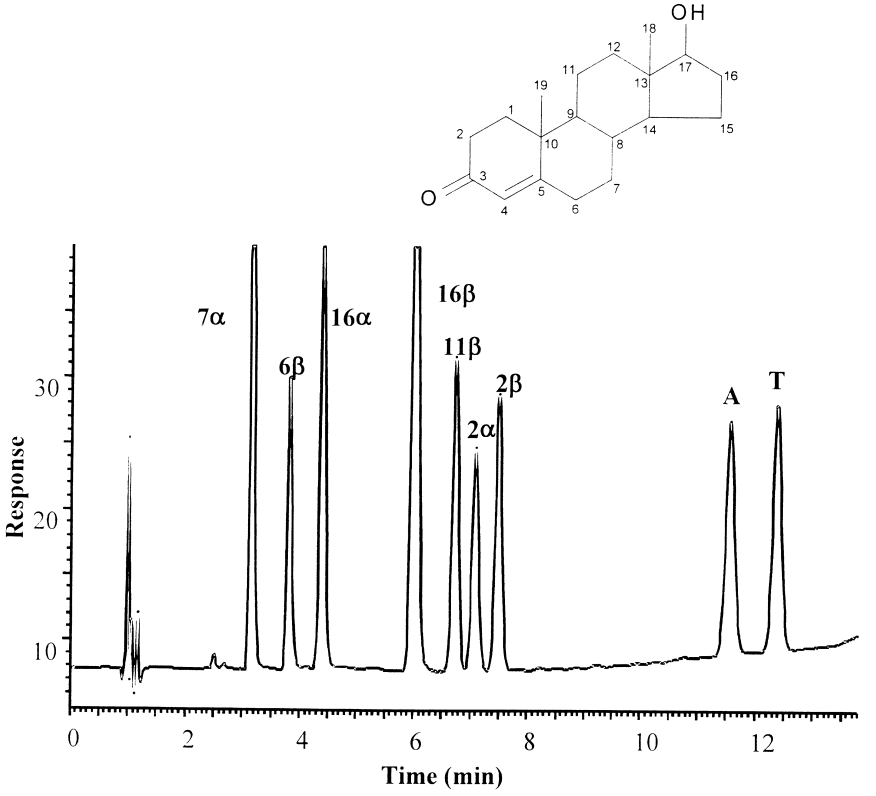
3. Results and discussion

Testosterone is a widely used substrate for the assessment of cytochrome P450 (CYP) enzyme activity. The regio- and stereo- selective hydroxylation of testosterone by different CYP enzymes has been well-characterised [4,5,8] and therefore measurement of these different activities provides a useful tool for the identification of CYP enzymes in metabolism studies [4]. The analysis of testosterone metabolites formed in liver microsomal incubates have required complex gradient systems which can significantly affect the UV-baseline [4,7]. Contemporary assays for complex mixtures of small molecules tend to use tandem mass spectrometry with rapid HPLC allowing the identification of metabolites from the molecular mass and product-ion transitions. The majority of hydroxylated testosterone metabolites, however, possess similar or identical parent-product transitions, making time–resolution an absolute requirement for the use of mass spectrometry to also be appropriate in the analysis and quantitation of mixtures of these hydroxylated metabolites. There was therefore a need to re-develop the HPLC assay to significantly reduce the run time whilst maintaining baseline resolution to achieve greater efficiency in sample analysis during drug screening and drug development programmes.

The original HPLC method employed in our laboratories, based on the method of Sonderfan et al. [5], used an Ultrasphere ODS C_{18}, 5 μm (25 cm× 0.46 cm) analytical column (Phenomenex) maintained at a temperature of 50°C. Mobile phase A comprised 30% methanol in water at pH 4.5 and mobile phase B comprised 90% methanol in acetonitrile. The mobile phase gradient comprised of 10 min at 11% mobile phase B, 30 min to 28%, 20 min at 28% B and 5 min at 0% B. There was an additional 5 min equilibration of 11% mobile phase B thereby comprising a total run time of 65 min. Simply by changing to a Hypersil C_{18} BDS 10 cm column reduced the run time, but with poor resolution. Resolution was improved using an isocratic blend of methanol–acetonitrile–tetrahydrofuran–water (12:7:9:72, v/v), respectively. The basis of the separation is that whilst the total modifier concentration will determine the overall retention, the blend of modifier characteristics; relative proton donor/acceptor and dipole moment, from the reversed-phase Solvent Selectivity Triangle [13] determines the system's selectivity. Whilst it is acknowledged that tetrahydrofuran (THF) is seldom used in modern reversed-phase HPLC, due to certain hardware incompatibilities, it was required in this instance in order to achieve the desired selectivity for these metabolites. No pH control was necessary and indeed could not be used as an alternative to this solvent. This quaternary modifier blend provided the isocratic conditions for metabolite separation on a UV flat baseline, up to 12 min. The methanol concentration in the mobile phase was then increased to accelerate

the elution of androstenedione and testosterone. The assay was run at 1 ml/min at 40°C with the same detection as before. Removing the methanol and introducing a shallow gradient with equal concentrations of tetrahydrofuran and acetonitrile shortened the run time, yet maintained resolution. The final mobile phase comprised a blend of tetrahydrofuran–acetonitrile–water (10:10:80, v/v), changing to tetrahydrofuran–acetonitrile–water (14:14:72, v/v) and then remaining isocratic for 3 min (Fig. 1). These conditions allow for a total cycle time of 15 min, including a 2 min re-equilibration before injection of the next sample. The other distinguishing characteristic of this method is the flat UV baseline throughout the entire chromatographic run. The absence of salts in the mobile phase, coupled with the shallow gradient, enables this flat baseline to be achieved. It is not just the molar absorption of the organic modifiers that can cause a sloping baseline, but also the changing mobile phase density that in turn alters the refractive index and therefore the proportion of incident light transmitted along the flow cell to the photomultiplier tube. Through keeping the change in modifier concentration, notably the THF, to a minimum, both the molar absorption and refractive index changes are minimised.

Separation of the 7α- and 6β-hydroxytestosterone and the 11β- and 2α-hydroxytestosterone pairs were the a priori requirements for routine metabolism studies. Resolution of these pairs necessitated ex-



Key

7α = 7α -hydroxytestosterone 6β = 6β-hydroxytestosterone, 16α = 16α-hydroxytestosterone, 16β = 16β-hydroxytestosterone, 11β = 11β-hydroxytestosterone, 2α = 2α-hydroxytestosterone, 2β = 2β-hydroxytestosterone, A= Androstenedione, T= Testosterone.

Fig. 1. Representative UV chromatogram of testosterone and hydroxytestosterone metabolite standards.

ploiting the subtle differences in the intra-molecular properties, such as hydrogen bonding, present in the aplanar diasteromeric forms, thus altering the analyte's partitioning. A variety of C_{18} columns were investigated using a mobile phase comprising a blend of tetrahydrofuran–acetonitrile–water (10:10:80, v/v) changing to tetrahydrofuran–acetonitrile–water (14:14:72, v/v). The Prodigy ODS 3 (100×4.6 mm, 5 μm) allowed a run time within 12 min. However, resolution of the 7α- and 6β-hydroxytestosterone and the 11β- and 2α-hydroxytestosterone pairs was not achieved. Separation of these base pairs required a significant increase in run time, which was contrary to the objective of this study. Likewise, the Waters SymmetryShield RP_{18} (100×4.6 mm, 3.5 μm) column failed to separate these same base pairs, and also required a much longer run time. Using a Hypersil ODS (100×4.6 mm, 5 μm) column, testosterone and its metabolites were eluted within 13 min, the peak shape was symmetrical, but again baseline resolution between the key base pairs was not achieved. Of the columns evaluated, only Hypersil BDS C 18 (100×4.6 mm) with a 3 μm particle size provided the necessary peak resolution within a short run time of 15 min. This was used with a 10 min linear mobile phase gradient (1.25 ml/min) comprising a blend of tetrahydrofuran–acetonitrile–water (10:10:80, v/v) changing to tetrahydrofuran–acetonitrile–water (14:14:72, v/v).

These columns are all based on the primary interaction of the analyte partitioning between the octadecylsilane and mobile phase, yet exhibit quite significant differences in selectivity. This variation in selectivity is believed to be due to the differences in the silanophilic associations on the stationary phase support. The regio- and stereo-specific hydroxylations of testosterone do not effect any gross changes in the analyte's inherent polarity, yet the localisation of the testosterone carbon skeleton in the brush border domain may appear to confer some degree of orientation of the hydroxyl moieties relative to the aprotonated silanols on the silica surface. All the stationary phases considered are monomeric and classed as end-capped, with the exception of the Symmetry Shield RP_{18} which has a polar embedded group. They are all designed to reduce silanophilic interaction, yet the very basis of this metabolite resolution must be attributable to varying amount of silanophilic interaction. If the separation was based solely upon the differences in polarity between these isomeric forms and their resultant association with the C_{18} ligand, then the differences between these stationary phases may be expected predominantly to be confined to capacity factor and not selectivity, as reported here.

A shorter run time and decrease in peak width

Table 2
Linearity values for eight testosterone metabolites

Analyte	Peak area		Peak height	
	Slope ($\cdot 10^{-6}$)	RSD (%)	Slope ($\cdot 10^{-6}$)	RSD (%)
7α-OHT	8.86	0.13	5.24	0.42
6β-OHT	4.57	0.16	31.5	0.41
16α-OHT	11.5	0.39	8.40	0.86
16β-OHT	5.15	0.23	4.62	0.66
11β-OHT	12.2	0.34	10.7	0.38
2α-OHT	7.09	0.36	6.74	0.42
2β-OHT	13.3	0.28	13.1	0.56
Androstenedione	8.79	0.20	10.9	0.69
Testosterone	7.59	0.46	9.27	0.34

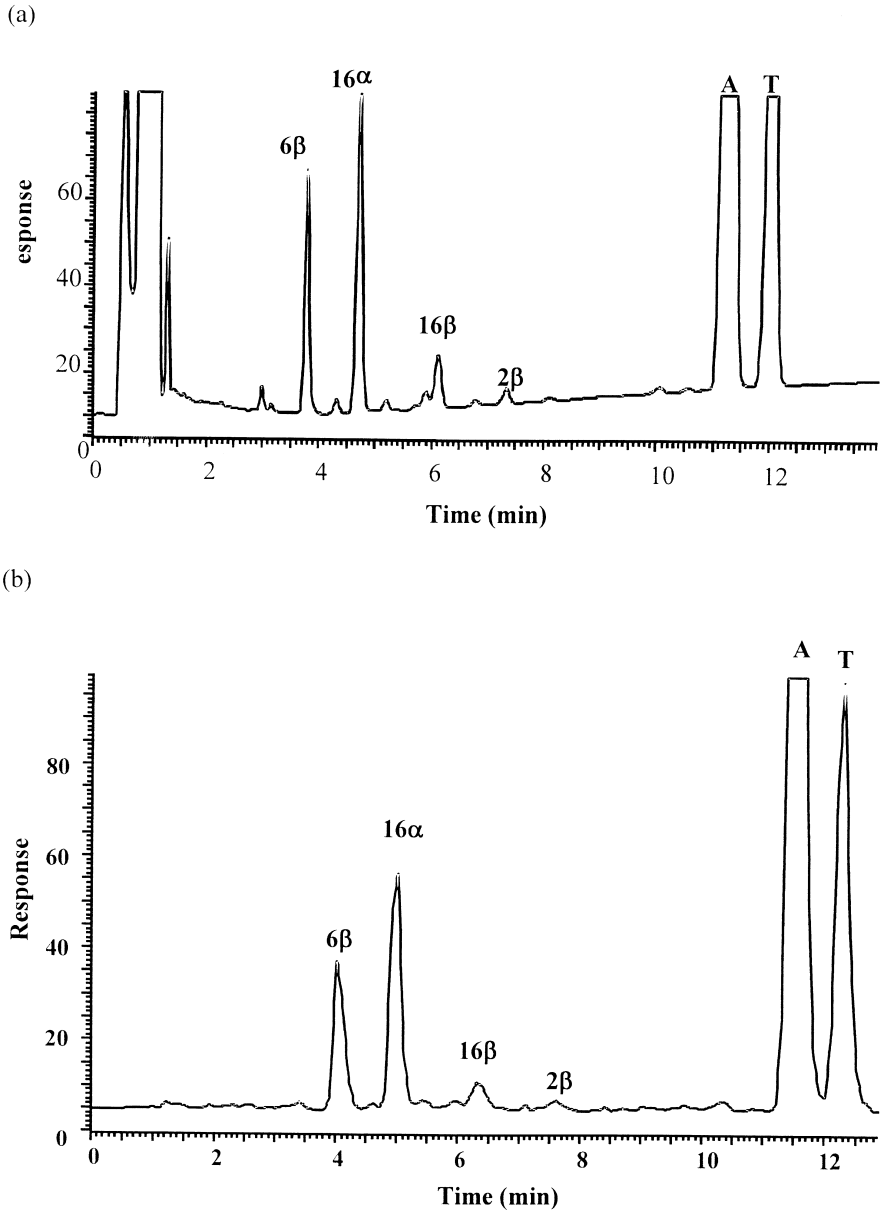
OHT=Hydroxytestosterone. The regression coefficient (r^2) was greater than 0.996 for all metabolites.

Table 1
Retention times and peak areas of eight testosterone metabolites relative to testosterone

	7α-OHT	6β-OHT	16α-OHT	16β-OHT	11β-OHT	2α-OHT	2β-OHT	A
Mean t_R (n=10)	0.23	0.25	0.31	0.42	0.51	0.56	0.61	0.95
RSD t_R (%)	1.26	1.25	1.38	1.25	1.24	1.35	1.34	0.21
Mean area (n=10)	0.73	1.06	2.27	1.50	0.70	1.03	1.45	0.66
RSD area (%)	0.69	0.66	4.28	1.01	0.62	0.88	0.58	0.37

t_R=Retention time, OHT=hydroxytestosterone, A=androstenedione. The means and RSDs of the retention times relative to testosterone were calculated from replicate injections (n=10); each set performed with a fresh batch of mobile phase (n=3). The means and RSDs of the peak areas were calculated from replicate injections (n=10) from solutions prepared from the same stock (1 mg/ml) solutions.

will, for any given quantity of analyte, result in greater peak height and consequently an improvement in assay sensitivity (Table 1). A summary of the retention times (relative to testosterone) and peak area of testosterone and eight testosterone metabolites, presented in Table 1 shows the relative retention time and peak area for each of the eight hydroxylated testosterone metabolites analysed. The



Key

6β = 6β-hydroxytestosterone, 16α = 16α-hydroxytestosterone, 16β = 16β-hydroxytestosterone, 2β = 2β-hydroxytestosterone, A= Androstenedione, T= Testosterone

Fig. 2. UV (a) and radiochemical (b) chromatograms representative of the human microsomal metabolite profile of testosterone.

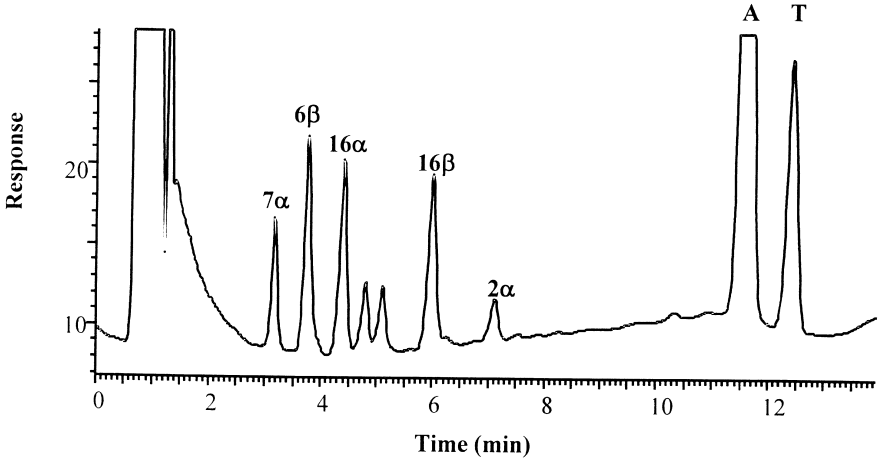
inter-batch relative standard deviations (RSDs) were less than 5% for the peak area response of the metabolites and less than 1.5% for the relative retention time of all metabolites investigated (Table 1). Linearity values for the determination of the eight metabolite standards by HPLC–UV are given in Table 2.

For the rapid screening of enzymatic reactions, drug inhibition studies and enzyme induction studies, it is frequently easier to use radiolabeled substrates for the qualitative and quantitative analysis of metabolite profiles. The suitability of the re-developed testosterone assay for use with in-line radiochemical detection was investigated by the incubation of rat and human liver microsomes with [^{14}C]testosterone. Radiochemical detection typically incorporates a 250 μl flow cell compared to a conventional 8–12 μl UV flow cell. To accommodate for the change in flow cell size, the band spacing between analytes was adjusted accordingly for radiochemical–HPLC analysis by increasing the flow-rate to 1.25 ml/min.

In the human microsomal incubates (Fig. 2) the predominant metabolite was androstenedione and to a lesser extent 6β-, 16α-, 16β- and 2β-hydroxytestosterones. In the rat microsomal incubates (Fig. 3), the predominant metabolite was androstenedione and to a lesser extent 7α-, 6β-, 16α-, 16β- and 2α-hydroxytestosterones. These chromatograms demonstrate that there were no significant interferences associated with these microsomal incubations. The distinct advantage of using radiolabeled testosterone is that it enables the direct and simultaneous determination of the rate of metabolite formation without the requirement of sample extraction and use of internal standards, therefore facilitating the rapid screening of selected cytochrome P450 activities.

4. Conclusions

Several testosterone HPLC methods have been previously reported [4–9,16]. However, these HPLC methods have typically used complex gradient systems [6] that in some instances do not necessarily resolve all the hydroxylated testosterone metabolites of interest [14]. Significant changes in baseline can also accompany gradients, which can affect detection and quantification of later eluting peaks [4,5]. Previously reported chromatographic methods have employed C_{18} stationary phases and a combination of complex step gradient and solvent systems of various combinations of acetonitrile, methanol and tetrahydrofuran to achieve resolution, often with changes to the elution order of the metabolites [11]. More



Key

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Fig. 3. UV chromatogram representative of the rat microsomal metabolite profile of testosterone.

recently, methods have been developed that have minimised baseline complications, by using a simple one step linear gradient [7] or by running under isocratic conditions [16]. However, these methods have run times in excess of 30 min.

In the analysis of hydroxylated testosterone metabolites from P450 metabolism studies, there exist methods of varying degrees of complexity and run times. The selection here of optimal column chemistry balanced with appropriate organic modifiers has enabled the development of a simple and rapid method with adequate sensitivity and no baseline complications. Incubations of rat and human liver microsomes show that the metabolite profiles and catalytic activities determined using this method are similar to those obtained using previously reported methods [4,9,12].

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