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Liquid chromatographic–mass spectrometric method to assess cytochrome P450-mediated metabolism of testosterone by rat everted gut sacs

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Abstract

A rapid, sensitive and specific method was developed for the simultaneous assay of testosterone, androstenedione and 6β-hydroxytestosterone (6β-OHT) in the TC199 tissue culture medium used in intestinal drug metabolism studies with the rat everted gut sac model. An electrospray LC–MS method was validated in the concentration range of 0.025–9.5 μM (7.2 ng–2.7 μg/mL) for testosterone and androstenedione and 0.01–4 μM (3 ng–1.2 μg/mL) for 6β-hydroxytestosterone. The limits of quantification (LOQ) with an injection volume of 10 μL were 0.0005 μM (4.9 fmol, 1.4 pg injected), 0.004 μM (0.04 pmol, 11.4 pg injected) and 0.03 μM (0.3 pmol, 91 pg injected), respectively. The method also detected the other testosterone metabolites, the 16α-, 16β-, 2β- and 2α-hydroxytestosterones and was then used to study the metabolism of testosterone during its absorption by rat intestine in vitro, using everted gut sacs.

Keywords: Testosterone; Cytochrome P450

1. Introduction

The metabolism of drugs when passing through the intestinal enterocytes has been a focus of attention in recent years with the realisation that the intestine can play an important role in the biotransformation of xenobiotics which undergo first-pass metabolism in the gastro-intestinal tract. The major enzymes involved in drug metabolism in man are members of the cytochrome P450 (CYP) super family of enzymes of which the isoforms CYP1A, CYP2C9, CYP2D6 and CYP3A4 collectively participate in the oxidation of about 95% of drugs [1]. In man the CYP3A is the most important predominent sub-family [2] and this enzyme also exhibits a high potential for drug-drug interactions. Testosterone 6β-hydroxylation (6β-hydroxytestosterone (6β-OHT) formation) is widely used as probe to measure the activity of the CYP3A enzymes and is also a marker of the 1A group. The rat small intestine is reported to express several of the CYP isoforms, including 1A1, 2B1, 2C6, 2C11, and 3A1, with the 1A1 being the most predominant [3]. However, there are considerable discrepancies in the literature, with some of the enzymes, e.g. CYP2C11, reported by certain authors and not detected at all by others [3–7]. This may be as a consequence of the different methods employed which often detect the presence of the appropriate messenger RNA (Northern blotting) or enzyme protein using antibodies (Western blotting). An additional isoform, 2J4, has also recently been described [8].

The problem with these methods is that they give no information on the actual *activities* of the enzymes in the intestinal epithelial cells and the effect this may have on the first-pass metabolism of a drug during its absorption by the intestine. With this latter objective in mind, we have turned to the use of our improved everted rat gut sac system which has proved very valuable for studying various aspects of drug absorption in vitro [9–12]. Caco 2 and other intestinal cell cultures [1,13,14] have also been used to study intestinal metabolism and inhibition or induction related drug interactions. While in vitro systems offer many advantages, only small quantities of metabolites are formed, and so very sensitive assays are required. We report herein the development of a sensitive LC–MS assay for 6β-OHT, the major CYP

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3A/1A metabolite of testosterone, so that the metabolism of testosterone could be accurately quantified in the rat everted gut sac model. A requirement of the assay was that the metabolic products could also be satisfactorily recovered from the complex TC 199 culture medium.

Other metabolism studies using hepatic microsomes or hepatocytes have relied primarily on HPLC [1,14–17] or radiochemical techniques [18,19], but these are not sufficiently sensitive to quantify 6β-hydroxytestosterone formation in the everted gut sac, or other in vitro systems used to study metabolism, such as cell cultures. More sensitive analytical techniques like GC–MS [20] and LC–MS [21,22] have also been used for the quantification of testosterone but usually 6β-OHT was not quantified at the same time, or no details of the limits of quantification (LOQ) were reported [23,24]. While GC–MS can be very sensitive, the derivatisation of the analytes is required [25].

Therefore we present the details of the LC–MS system which has the sensitivity to detect the small amounts of 6β-hydroxytestosterone, and other metabolites produced during testosterone transport by everted gut sacs or other in vitro systems. The method was fully validated for 6β-hydroxytestosterone and also for the non-hydroxylated metabolite, androstenedione. In addition, the method also has the versatility to separate and detect other potential products of testosterone metabolism such as the 16α-, 16β-, 2β- and 2α-hydroxytestosterones.

2. Experimental

2.1. Chemicals

Testosterone, androstenedione, 6β-hydroxytestosterone (6β-OHT), 16α-hydroxytestosterone (16α-OHT), 2α-hydroxytestosterone (2α-OHT) progesterone, tissue culture medium TC 199 (10× concentrated with Earle's salts), glutamine and ammonium formate were purchased from Sigma–Aldrich Chimie (St Quentin Fallavier, France). 2β-Hydroxytestosterone (2β-OHT) and 16β-hydroxytestosterone were purchased from Ultrafine (Manchester, UK). Ultrapure water was obtained using a Millipore Simplicity 185 apparatus. Dichloromethane, hexane and methanol (SDS, France) were of HPLC grade and used without further purification.

2.2. Gut sac preparation and incubation

Male Sprague–Dawley rats (220–240 g weight, DEPRE, Saint Doulchard, France) were used in our experiments. The medium was TC 199 (with Earle's salts), pH 7.3, and gassed by bubbling at 37 °C with 95% O_2/5% CO_2. Adult rats were starved for 24 h, killed by cervical dislocation and the entire small intestine quickly excised and flushed through several times with NaCl solution (0.9%, w/v) at room temperature. The intestine was immediately placed in warm (37 °C), oxygenated TC 199 and then gently everted over a glass rod (2.5 mm diameter). One end of the intestine was clamped and the whole length of the intestine was filled with fresh oxygenated medium and sealed with a second clamp and the resulting large gut sac divided into sacs of approximately 2.5 cm in length using braided silk sutures. For each experiment, 12–15 sacs were prepared, starting from the end of the duodenum, to ensure that sacs were from the upper/mid jejunum where metabolic activity is maximal. Each experiment was carried out using the intestine from one rat with each sac being placed in an Erlenmeyer flask (50 mL) containing a solution of testosterone (100 μM) in TC 199 pregassed with 95% O_2/5% CO_2 at 37 °C. Flasks were stoppered with gas-tight silicon bungs and incubated at 37 °C in a shaking water bath (60 cycles/min). At the appropriate time points, sacs were removed, washed three times in saline and blotted dry. The sacs were cut open and the serosal fluid drained into small tubes. Each sac was weighed before and after serosal fluid collection to calculate accurately the volume inside the sac. The sacs were then digested individually in 25 mL of 1 M NaOH at 37 °C for 2 h. Samples of the medium and serosal fluid were kept for extraction for the LC–MS analysis. The protein content of the digest was determined spectrophotometrically using the method described by Peterson [26] with bovine serum albumin as standard.

2.3. Sample preparations for LC–MS analysis

For the analysis of 6β-OHT in the serosal fluid, 0.2 mL were added to 0.295 mL of TC 199 and 5 μL of progesterone used as internal standard (IS) (stock solution, 16 μM), and the mixture then extracted with 0.5 mL of a mixture of hexane/dichloromethane (1:1, v/v). After agitation, the organic layer was removed and evaporated to dryness under nitrogen and the residue was dissolved in a 0.1 mL of a methanol/water (1/1, v/v) mixture for the quantification of the 6β-OHT by LC–MS. Thus the final IS concentration was 0.8 μM. For the quantification of testosterone in the serosal fluid, 50 μL of IS was added to 0.2 mL of serosal fluid and diluted with 0.250 mL of TC 199 medium. After extraction and the evaporation of the organic layer, the residue was dissolved this time in 1 mL of methanol/water (1/1) mixture, and consequently the final IS concentration was also 0.8 μM. For the mucosal fluid, 50 μL was removed, diluted with 0.4 mL of TC 199 and added to 50 μL of IS (16 μM). Samples were then extracted as described for serosal fluid and the residue, and after evaporation dissolved in 1 mL of a methanol/water mixture (1/1, v/v), giving a final concentration of 0.8 μM for the IS.

2.4. LC–MS conditions

The LC–MS system consisted of an Alliance 2690 separation module interfaced to a ZQ mass spectrometer equipped with an electrospray ionisation source (Waters, St. Quentin, France). A Waters Sentry C_{18} (2.1 mm × 10 mm,

Table 1
Gradient program for the separation of testosterone and metabolites

Time (min)	Water (%)	Methanol (%)	Ammonium formate (100 mM)
0	35	60	5
5	15	80	5
6	5	90	5
9	5	90	5
10	35	60	5
20	35	60	5

3.5 μM) guard column and a Waters Symmetry C_{18} column (2.1 mm × 150 mm, 5 μM) were used for the chromatographic separations. Analyses were run in positive mode with the capillary and cone voltages set to 3 kV and 25 V, the temperature of the heated capillary at 280 °C and the nitrogen nebulizing gas flow set at 350 L/h. The mobile phase consisted of a mixture of water (A), methanol (B), and ammonium formate 100 mM (C) (Table 1) with a flow rate of 0.2 mL/min for a run time of 20 min. The concentration of progesterone used as internal standard was 0.8 μM for all the quantifications. The apparatus was managed with a Masslynx software (Micromass, version 3.5).

2.5. Recovery of testosterone and metabolites in TC 199

Recovery studies were performed by extracting the target compound from TC 199 medium (1 mL) in three replicates. One milliliter of TC 199 was spiked with the appropriate quantity of testosterone or metabolites and then extracted with a mixture of hexane and dichloromethane (1:1, v/v), and the organic layer was evaporated to dryness under nitrogen and redissolved in methanol (1 mL) before analysis. The recovery was determined by comparing the chromatogram area obtained after extraction with the area obtained with the target compound prepared in methanol but without extraction. Recoveries were carried out in triplicate for different concentrations within the calibration range. The results are shown in Table 2.

2.6. Method validation

TC 199 medium was tested after extraction for any matrix effects and no significant matrix variation was observed with six replicates of TC 199 blanks without analytes but spiked with the IS. The specificity of the method was also tested to ensure that 6β-OHT was correctly resolved from the other hydroxylated metabolites that might have been formed during intestinal testosterone metabolism. The chromatographic resolution of testosterone, 6β-OHT, 16β-OHT, 16α-OHT, 2β-OHT, 2α-OHT and androstenedione was verified by analysing a mixture of them all. A stock solution of each standard (in methanol) was diluted in water to produce the mixture of standard compounds at a concentration of 0.4 μM for each one. The retention time of each analyte was pre-determined after injection of each compound individually (see Table 3).

Within-day precision and accuracy were determined by preparing five replicates on the same day at three concentrations (0.06; 0.6; 2.5 μM) for 6β-OHT or four concentrations (0.06; 0.6; 2.5; 7.5 μM) for testosterone and androstenedione as the linearity range was greater. This operation was repeated by preparing and analysing fresh independent replicates for 3 days to assess between-day precision and accuracy.

Table 2
Extraction recoveries from TC 199 tissue culture medium

Concentration (ng/μL)	Recoveries (%)		
	Testosterone	6β-OHT	Androstenedione
0.05	99.6 ± 0.4[a]	78.7 ± 1.1	101.4 ± 9.4
0.5	99.5 ± 3.5	80.7 ± 6.2	84.2 ± 8.7
2.5	105.2 ± 5.5	n.d.	90.3 ± 13.7

n.d.: not determined.
[a] Values are means of three replicates ± S.D.

Table 3
Retention times of testosterone, progesterone (IS) and testosterone metabolites

Compound	Retention time (min)
6β-OHT	4.28
16α-OHT	5.01
16β-OHT	5.99
2α-OHT	6.71
2β-OHT	7.12
Androstenedione	8.50
Testosterone	9.63
Progesterone	11.9

3. Results

3.1. Chromatographic separation and detection

Analyses were run in the selected ion recording (SIR) mode by selecting the protonated molecular ion (MH^+) for each compound. The SIR chromatogram following the analysis of standard compounds spiked in TC 199 and extracted as described in Section 2 is presented in Fig. 1A. As can be seen, a satisfactory separation of testosterone and each metabolite was obtained with the chromatographic conditions used; retention times are reported in Table 3. The mass spectra of testosterone, 6β-OHT, androstenedione and the IS are shown in Fig. 2. The MH^+ ions were selected for subsequent analysis at m/z = 289.14; 287.14; 305.14 and 315.13, respectively for testosterone, androstenedione, 6β-OHT and the IS. For 6β-OHT, the formation of an adduct with methanol (m/z: 337.15 MH^+ (MeOH)) and sodium (m/z: 327.12 MNa^+, m/z: 359.14 MNa^+ (MeOH)) was observed, which may explain the lower sensitivity of the

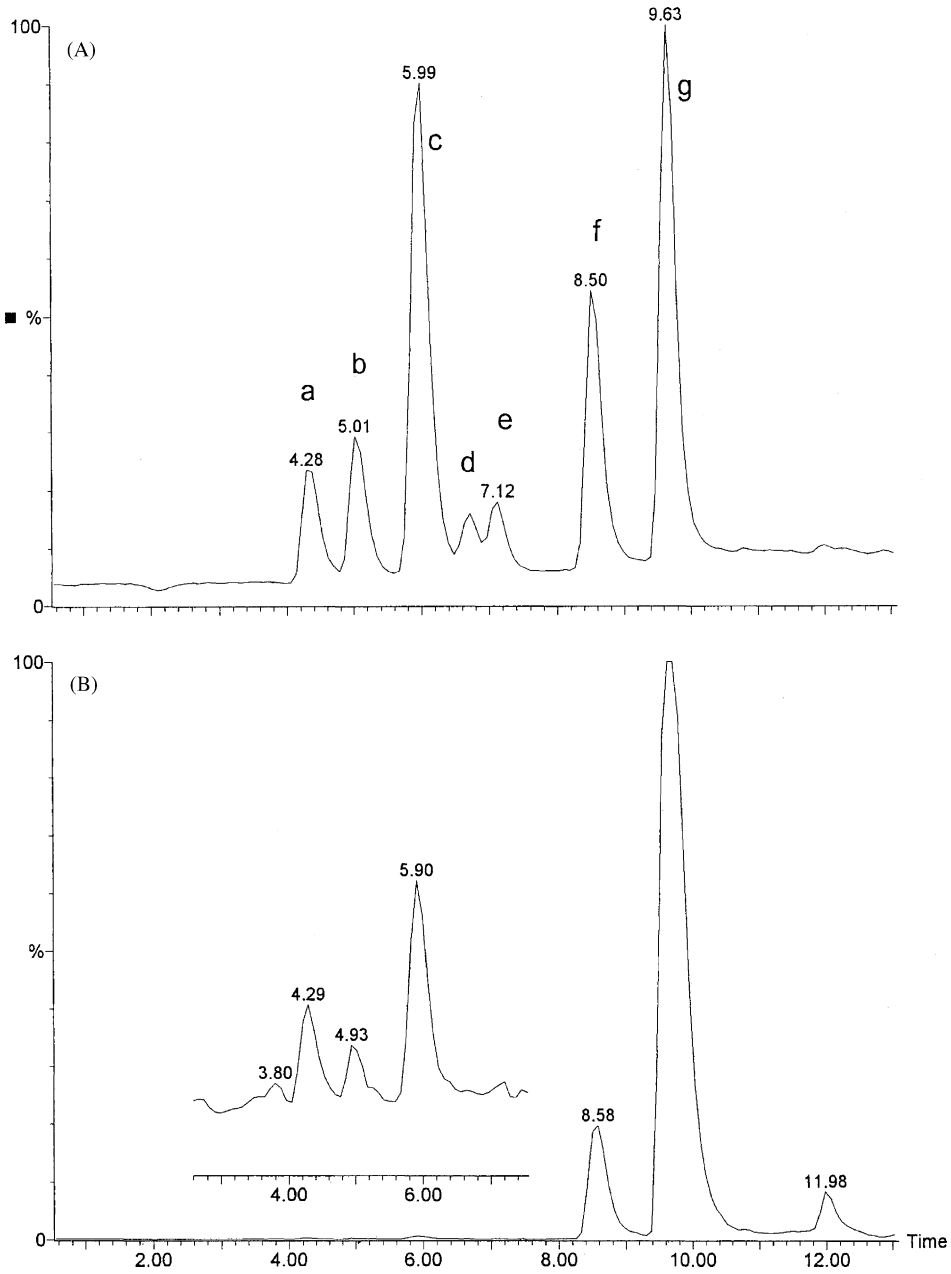

Fig. 1. (A) Selected ion recording (SIR) chromatogram of testosterone and metabolites (0.4 μM) added to TC 199 culture medium and then extracted as described in Section 2: (a) 6β-OHT, (b) 16α-OHT, (c) 16β-OHT, (d) 2α-OHT, (e) 2β-OHT, (f) androstenedione, (g) testosterone. (B) Selected ion recording (SIR) chromatogram of the serosal content (inside) of an everted gut sac after incubation for 60 min in TC 199 with testosterone (100 μM) on the mucosal side (outside). The inset shows an enlargement of the region between 4 and 8 min. The peak at retention time 11.98 min was the internal standard, progesterone.

method for this metabolite. Attempts to increase the yield of *m/z* 305 (MH^+) for 6β-OHT using different additives in the LC eluent such as formic acid, acetic acid (0.1 or 0.3%) or ammonium acetate, did not provide efficient ionisation of the sample. Lou et al. [18] reported recently that they failed to quantify hydroxytestosterone by ESI^+ LC–MS and that APCI LC–MS was not very successful because of the very low abundance of expected ions for androstene-3-α, 17-β-diol.

Calibration graphs were constructed by plotting peak area ratios versus analyte concentrations using a least-square linear regression model. The linearity was very good for all analytes in the concentration range tested: 0.025–9.5 μM for testosterone and androstenedione and 0.01–4 μM for 6β-OHT as indicated by the regression data: $y = 0.821x + 0.059$ ($r^2 = 0.999$) for testosterone; $y = 0.138x - 0.011$ ($r^2 = 0.999$) for 6β-OHT; $y = 0.535x + 0.011$ ($r^2 = 0.997$) for androstenedione.

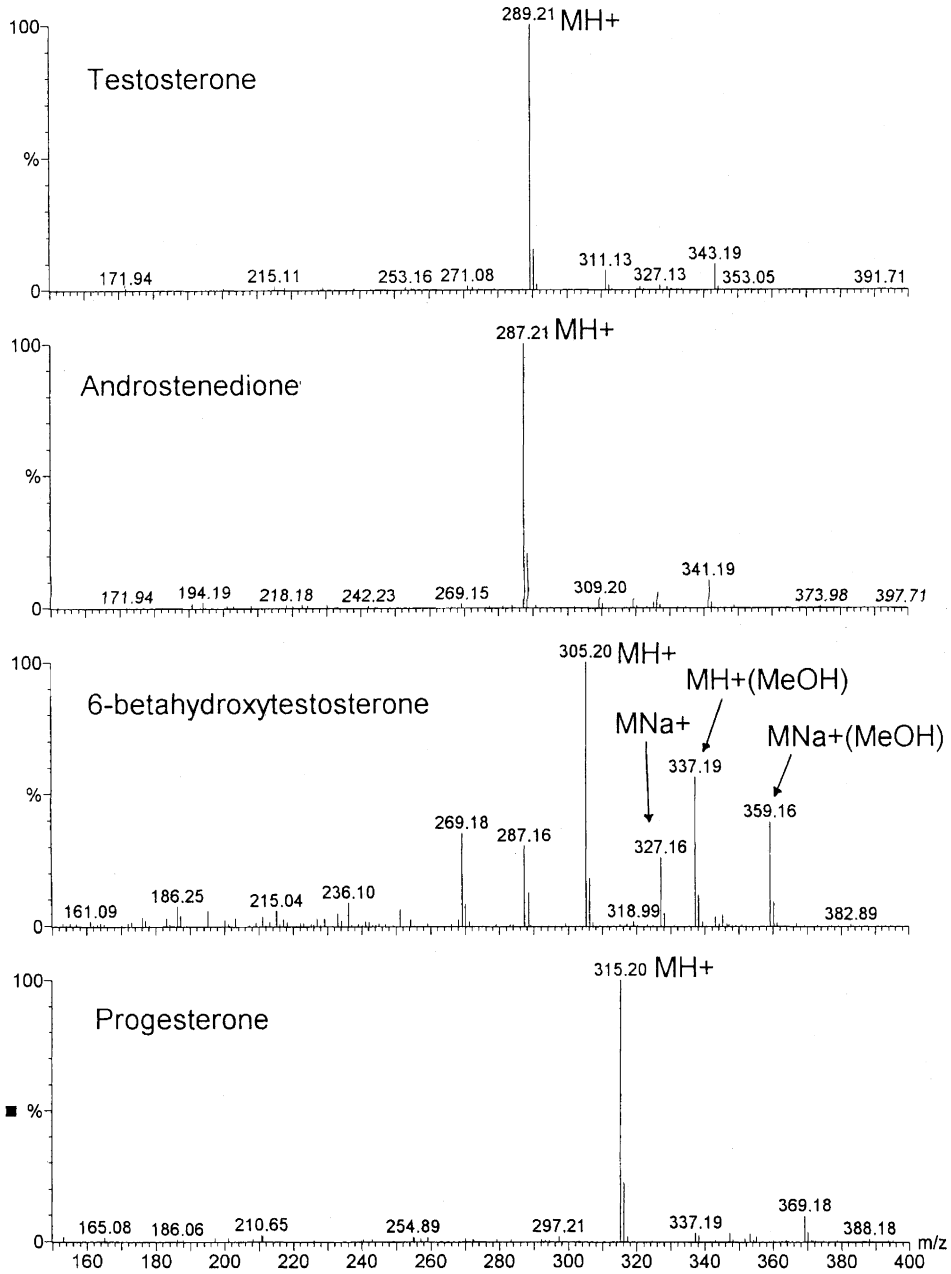

Fig. 2. Mass spectra of testosterone, androstenedione, 6β-OHT and progesterone (IS).

3.2. Precision and accuracy

Relative standard deviation (R.S.D. %) was calculated as an estimation of precision. Accuracy (relative error) was expressed as a percentage and calculated by the agreement between the measured and the nominal concentration of the spiked standard samples. As can be seen in Table 4, for within-day analyses the overall precision ranged from 2.3 to 16.8%, and accuracy (% bias) ranged from −4.13 to 5.76%. The overall precision for between-day assays ranged from 3.9 to 15.9% while the between-day accuracy ranged from −1.56 to 12.78%. These values were considered satisfactory, given the complexity of the matrix, and these results demonstrated that the method has good precision and accuracy.

3.3. Sensitivity

The limits of quantification were determined with the Signal to Noise ratio greater than 10 (S/N > 10). With an injection volume of 10 μL they were: 0.0005 μM (4.9 fmol, 1.4 pg injected) for testosterone; 0.004 μM (0.04 pmol, 11.4 pg injected) for androstenedione; and 0.03 μM (0.3 pmol, 91 pg injected) for 6β-OHT. Thus the sensitivity was better for testosterone and androstenedione than for the hydroxylated metabolite. Furthermore, the sensitivity of the method was

Table 4
Precision and accuracy of the LC–MS determination of testosterone, 6β-hydroxytestosterone and androstenedione

Concentration (μM)	Within-day ($n = 5$)			Between-day ($n = 11$)		
	Testosterone	6β-OHT	Androstenedione	Testosterone	6β-OHT	Androstenedione
0.06						
Mean	0.061 ± 0.01	0.063 ± 0.01	0.059 ± 0.003	0.066 ± 0.01	0.064 ± 0.006	0.067± 0.010
Precision CV (%)	16.8	6.6	4.2	15.6	9.9	14.8
Accuracy (% bias)	2.50	0.43	−0.42	10.17	0.60	12.78
0.6						
Mean	0.61 ± 0.016	0.59 ± 0.082	0.62 ± 0.018	0.596 ± 0.024	0.59 ± 0.055	0.59 ± 0.035
Precision CV (%)	2.6	9.2	2.9	3.9	9.2	5.9
Accuracy (% bias)	1.67	1.66	3.67	0.55	1.55	0.61
2.5						
Mean	2.62 ± 0.082	2.52	2.64 ± 0.089	2.62 ± 0.155	2.49 ± 0.13	2.59± 0.133
CV (%)	3.1	2.3	3.3	5.9	5.4	5.15
Accuracy (% bias)	4.88	1.12	5.76	4.87	0.36	3.53
7.5						
Mean	7.19 ± 0.277	n.d.	7.50 ± 0.265	7.38 ± 0.601	n.d.	7.50 ± 0.74
CV (%)	3.8		3.5	8.1		9.9
Accuracy (% bias)	4.13		0.11	1.56		0.01

n.d.: not determined.

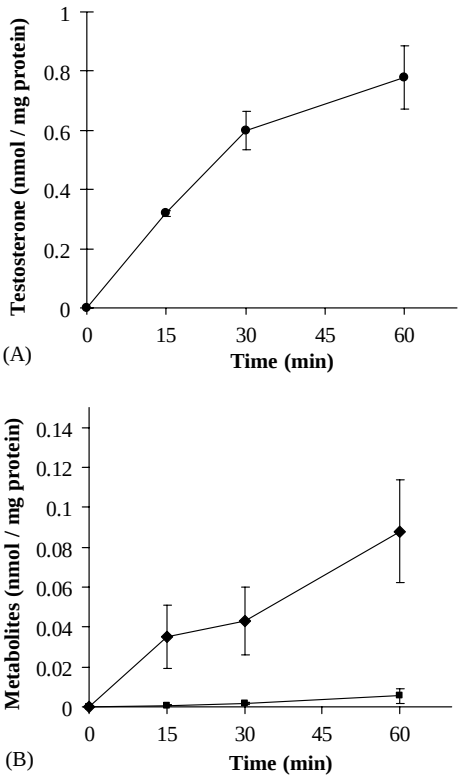

Fig. 3. (A) Accumulation of testosterone (●) inside (serosal content) an everted gut sac incubated at 37 °C in TC 199 medium containing 100 μM testosterone. (B) Accumulation of 6β-OHT (■) and androstenedione (◆) inside (serosal content) an everted gut sac incubated at 37 °C in TC 199 medium containing 100 μM testosterone. Data is expressed as the nmol accumulated per mg of tissue protein. Each point is the mean ± S.D. of three sacs.

respectively fifteen to twenty fold better than the LC–UV [17] and APCI LC–MS [21] techniques used for the quantification of 6β-hydroxytestosterone as published by other researchers. For testosterone, the LOQ was established to be 1.4 pg (4.9 fmol) injected, which is a good result and in the same range as that reported with a GC–MS method [20].

3.4. Testosterone metabolism in the rat everted gut sac

Fig. 1B shows the HPLC–MS SIR trace of an everted gut sac contents, removed after incubation of the sac in a 100 μM testosterone solution in TC 199 for 60 min and extracted as described in Section 2. It can be seen that in addition to the formation of major metabolite, 6β-OHT, the presence of 16α-, 16β-, 2β-hydroxytestosterone and androstenedione in the serosal medium was also detected as can be seen by comparing the retention times with those of standards of the metabolites (Fig. 1A). Testosterone, 6β-OHT and androstenedione were quantified in the gut sac medium from the serosal side. As can be seen in Fig. 3, testosterone, 6β-OHT and androstenedione all appeared in serosal medium during incubation of the sacs, and the amount of testosterone and androstenedione found was much greater than the amount of 6β-OHT.

4. Discussion

The objective of this study was to develop an analytical method that would enable direct quantitative and qualitative studies to be made of drug metabolism during intestinal absorption using an in vitro system. Testosterone was chosen as the test molecule because it is a substrate of a number of the principle cytochrome P450 drug metabolising

enzymes. Indeed, the position of the hydroxylation of the molecule can give valuable insight into which isoforms of the enzymes are active in the tissue. The small quantities of material available from in vitro methods meant that the analytical system needed to be very sensitive, accurate, and discriminatory as well as capable of analysing the metabolites in the presence of tissue culture medium, which contains a wide variety of chemical components. The LC–MS method described fulfills the requisite criteria. For the metabolites, 6β-hydroxytestosterone and androstenedione, subjected to a standard validation procedure, the LOQ were 0.3 and 0.04 pmol, respectively. At the same time it was possible to measure testosterone over a wide concentration range (0.025–9.5 μM) in the presence of its metabolites. The validation criteria, as shown in Table 4, were very satisfactory.

To illustrate the value of the technique for studying drug metabolism in vitro an example of some preliminary data obtained with the rat intestine everted gut sac technique is shown. In this method, the tissue is rapidly removed from the animal and with gentle handling, and is then placed immediately in oxygenated tissue culture medium, which ensures maximum viability and metabolic activity. The everted sacs are incubated in tissue culture medium with the test drug on the mucosal side. As the drug is absorbed across the intestinal mucosa it will appear in the serosal medium inside the sac along with any metabolites that have been formed by cytochrome P450 activity during the passage of the molecule across the epithelial cell layer. Thus the method gives a direct indication of the extent of first pass metabolism in the intestinal mucosa. Fig. 3A shows the appearance of testosterone inside the sac. The rate of transport is expressed as nmol/mg of sac protein and is similar to the rate obtained from other studies in our laboratory using radiolabelled testosterone. Fig. 3B shows the appearance of the two metabolites, 6β-hydroxytestosterone and androstenedione in the serosal medium, and it is clear that there is a much greater production of androstenedione. As discussed earlier in the introduction, there are considerable differences in the literature concerning which isoforms of the P450 enzymes are present in the rat intestine. While there is a concensus that isoforms of the family 3A and 1A are present, the precise isoforms and their abundance and/or activity are less clear [3–7,15,27,28]. A number of authors also report the presence of isoforms of the 2B group [3–5,15,29]. The situation regarding enzymes of the 2C group is more confused with some researchers citing their presence [3,4] and others unable to detect them [5,6,15]. As can be seen in Fig. 2, in addition to the formation of the 3A/1A products, 6β-OH-testosterone and androstenedione, the 16α-, 16β- and 2β-hydroxy metabolites were also detected (though not quantified in these preliminary studies), indicating that isoforms of the 2B and 2C families of the P 450 enzymes are active in the mucosal cells. This has recently been corroborated by studies in our laboratory using microsomes prepared from rat intestine, where as well as 3A-type activity, 2B, 2C and 2D have been found using specific substrates. This work is currently in submission to the pharmaceutical literature.

In conclusion, we present a sensitive and versatile LC–MS method for analysing the various hydroxylated metabolites of testosterone which are indicative of the drug metabolising enzymes that are active in the intestinal mucosa and which bring about first-pass metabolism during drug absorption. No derivatisation is required. The sensitivity of the method enables it to be used for in vitro studies with small amounts of tissue, as demonstrated in the initial studies with rat everted gut sacs. Given that the rat is a widely used species for the pre-clinical assessment of drug absorption and metabolism, such studies are very valuable in evaluating the role of mucosal drug metabolism in oral drug bioavailability.

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