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Journal of Chromatography B, 784 (2003) 49–61
JOURNAL OF CHROMATOGRAPHY B
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Determination of testosterone metabolites in human hepatocytes I. Development of an on-line sample preparation liquid chromatography technique and mass spectroscopic detection of 6β-hydroxytestosterone

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Received 28 February 2002; received in revised form 23 September 2002; accepted 23 September 2002

Abstract

A rapid and sensitive RP–HPLC assay for determination of 6β-hydroxytestosterone in human hepatocytes with corticosterone as the internal standard is described. The procedure employs on-line sample enrichment using a BioTrap 500 MS™ (20×4 mm I.D.) extraction pre-column and subsequent gradient separation on a Prontosil 60-5 C_{18}-H (250×2 mm I.D., 5 μm particle size) analytical column in the back-flush mode using a ternary eluent system composed of methanol, tetrahydrofuran and water. Signal monitoring was done by measurement of the responses from liquid chromatography coupled to mass spectroscopy (LC–MS/MS) using an atmospheric pressure chemical ionization (APCI) source conducted in the selected reaction monitoring (SRM) mode. Mean recoveries of 6β-hydroxytestosterone from an estimate of the biological matrix, i.e., Dulbecco's modified Eagle medium ''High Glucose'', ranged from 101.8–104.4% for samples containing the target analyte at the 250, 500 and 1000 ng/ml level. The limit of quantitation (LOQ) was 20 ng/ml at an injection volume of 100 μl determined in the same matrix. Linearity of signal responses versus concentration for all three analytes was accomplished in the range of 100–4000 ng/ml. Mean values of the coefficients of variation (C.V.) for the target analyte obtained for the concentrations 250, 500 and 1000 ng/ml at 5 different days in quintuplicate ranged from 1.5–7.7% (within-day) and 4.8–7.3% (between-day). The corresponding values for the accuracy ranged from 87.7–106.1% for the within-day and from 98.8–102.5% for the between-day measurements. The target analyte was sufficiently stable at both storage and sample preparation conditions because no substantial deviations between analyte concentrations measured before and after subsequently performed freeze and thaw cycles were observed.

Keywords: Testosterone; 6β-Hydroxytestosterone

1. Introduction

Oxidative enzymes of the cytochrome P450 type, which all are members of the hemoprotein family, play an important role in biotransformation of either

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00754-7

drugs or endogenous substrates, as, e.g., steroids [1–6] and about 25% of all xenobiotic compounds are metabolized by these liver enzymes. In particular cytochrome oxidase P450 3A4 (CYP 3A4) is involved in regio- and stereospecific hydroxylation of a wide variety of xenobiotics and endogenous compounds exerting biological activity [7–13]. The family of cytochrome P450 isozymes is induced by a lot of compounds widely differing in chemical structure. In particular, phenobarbital, 3-methylcholanthrene and Aroclor 1254, which is composed of a mixture of chlorinated biphenyls [7,8] as well as rifampicin [9], the latter being a typical activator of CYP 3A4 activity, are widely used as the standard inducers of CYP 3A4 activity. Testosterone, as a typical example of such endogenous targets for CYP 3A4 [7–13], undergoes oxidization to 6β-hydroxytestosterone (Fig. 1a) and it is easily comprehensible that extensive hydroxylation will be associated with

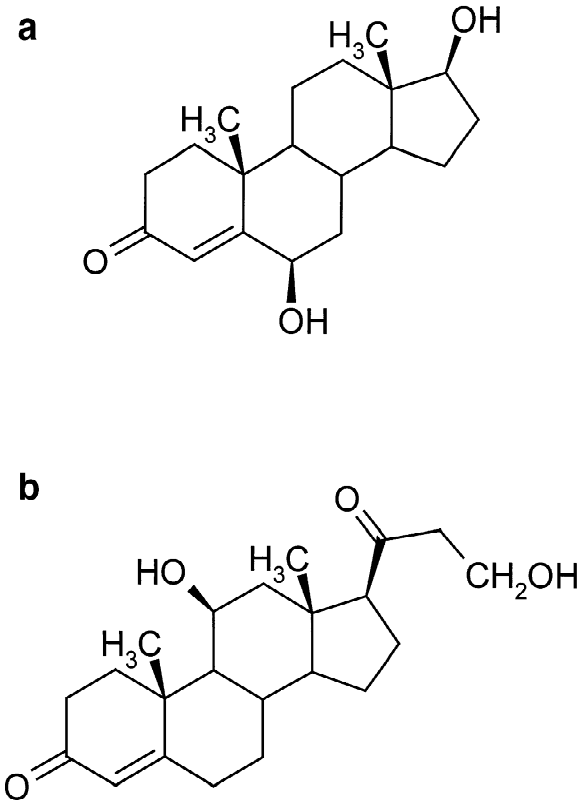

Fig. 1. (a, b) structural formula of 6β-hydroxytestosterone (a) and corticosterone (b).

fertility disorders of male subjects. 6β-Hydroxytestosterone represents the main testosterone metabolite in hepatic microsomes of untreated rats accounting for about two-thirds of total metabolites [8]. However, despite the observation of a marked increase in cytochrome P450 oxidase activity after treatment of rats with phenobarbital, 3-methylcholanthrene and Aroclor 1254, a decrease in formation of 6β-hydroxytestosterone occurs, but nevertheless it still remains the principal testosterone metabolite [8]. A similar influence of phenobarbital, 3-methylcholanthrene and other kinds of inducers, such as pregnenolone-16α-carbonitrile (PCN), dexamethasone, erythromycin estolate, troleandromycin and spironolactone was reported by Sonderfan et al. [10], while in contrast, rifampicin fails to induce liver microsome 2β-, 6β- and 15β-hydroxylase activity [10]. These findings were at least partially supported by Kern et al. [9] who found a substantial effect of rifampicin on 6β-hydroxylase activity in cultured human hepatocytes, whereas no effect was observable in rat hepatocytes. As a consequence, testosterone is a frequently used probe for in vitro inhibition and induction studies with respect to cytochrome isoenzyme activities. So for this reason, in order to get a better insight into the extent of CYP 3A4 activation after prior enzyme induction, it will be advisable to determine the concentration of 6β-hydroxytestosterone in hepatocyte-containing culture media when subjected to different activators of CYP 3A4 activity.

Plenty of papers dealing with analytical investigations of sex steroid hormones and anabolic steroids in biological media is available in the scientific literature. The action as well as regulation of hydroxylation exerted by hepatic cytochrome P450 isozymes was investigated by Wood et al. [8], Kern et al. [9], Sonderfan et al. [10], van der Hoeven [7,14], Woertelboer et al. [15], Arlotto et al. [12] and Williams et al. [16] using high-performance liquid chromatography (HPLC) for separation and identification of testosterone metabolites. HPLC as well as gas chromatography (GC) coupled to mass spectroscopy (GC–MS) was applied by Khalil and Lawson [17] to determine steroid concentrations in porcine follicular fluid. GC–MS after prior pre-fractionation by HPLC was applied by Andersson and Sjövall [18] for determination of unconjugated ster-

oids in rat testicular tissue and by Daeseleire et al. [19] for anabolic agents in tissues and urines. Conventional HPLC was used by Hayata et al. [20] for analysis of sex steroid hormones in serum and by Laganà and Marino [21] for determination of anabolic steroids in tissues. In both cases electrochemical detection was additionally applied for monitoring of easily oxidizable compounds, such as estriol. An HPLC assay for determination of testosterone and epitestosterone in urine was described by Navajas et al. [22] and both HPLC and GC–MS was reported by Smith et al. [23] for separation and identification of testosterone and its metabolites in virus-infected hepatic cells, Sanwald et al. [24] in rat liver microsomes and Stolker et al. in bovine urine [26]. Baltes et al. [25] measured testosterone metabolites in cell microsomes and Ekins et al. [27] found quantitative differences in phase I and II metabolism between rat precision-cut liver slices and isolated hepatocytes using HPLC and UV-detection. Shou et al. [28] reported on the use of inhibitory monoclonal antibodies raised against different subtypes of cytochrome oxidase P450 in order to assess the contribution of the individual P450s in their potency to metabolize xenobiotics and endogenous compounds including testosterone β-hydroxylation by means of HPLC and GC–MS. Williams et al. [16] and Draisci et al. [29,30] used the highly specific HPLC–MS technique yielding precise structural assignment of either target compounds or metabolites for in vivo biotransformation studies of anabolic steroids in canine microsomal preparations as well as quantitation of anabolic hormones and their metabolites in bovine serum and urine, respectively. Most recently, Tachibana et al. [31] determined testosterone metabolites, among them 6β-hydroxytestosterone in liver microsomes, using on-line column-switching HPLC. Last but not least, capillary electrophoresis was also applied for separation of testosterone metabolites in microsomal incubates as reported by Fernandez et al. [32].

In the first part of this contribution we report on development of a highly specific gradient reversed-phase HPLC assay for determination of 6β-hydroxytestosterone (Fig. 1a) in human hepatocytes using ''on-line'' extraction column-switching for sample enrichment and pre-purification with corticosterone (Fig. 1b) as the internal standard. Structural assignment was done by mass-spectroscopy in the selected reaction monitoring (SRM) mode. Using the same chromatographic system and partially different SRM conditions, the following further metabolites of testosterone can also be detected and quantified: 2α-, 2β-, 6α, 7α-, 11β-, 15α-, 16α- and 19-hydroxytestosterone. Results will be shown in incubation media of human, rat and pig hepatocytes in part II.

2. Experimental

2.1. Reagents and materials

The testosterone metabolites 6β-hydroxytestosterone (6β-OHT), 6α-hydroxytestosterone (6α-OHT), 16α-hydroxytestosterone (16α-OHT), 2α-hydroxytestosterone (2α-OHT), 2β-hydroxytestosterone (2β-OHT) and corticosterone as the internal standard were obtained from Sigma (St Louis, MO, USA), whereas 7α-hydroxytestosterone (7α-OHT) was a gift kindly donated by Professor J. V. Castell (University of Valencia, Valencia, Spain). Methanol and 2-propanol both gradient grade and formic acid (analytical grade) were purchased from Merck (Darmstadt, Germany) and triethylamine (puriss., p.a.) from Fluka (Buchs, Switzerland). Dulbecco's modified Eagle's medium (DMEM) ''High Glucose'' was obtained from Cell Concepts (Umkirch, Germany). High purity water for the use in HPLC was prepared with a Milli-Q water system™ from Millipore–Waters (Milford, MA, USA). A BioTrap 500 MS™ pre-column (20×4 mm I.D.) from Chromtech (Hägersten, Sweden) and Prontosil 60-5 C_{18}-H analytical column with either 250×2 mm or 150×2 mm I.D. and 5 μm particle size from Bischoff (Leonberg, Germany), were used for sample extraction and separation, respectively.

2.2. Preparation of calibration samples, internal standard and quality controls

A stock solution of 1 mg/ml of the internal standard corticosterone was prepared in methanol–water 1:1 (v/v), which was diluted to a working concentration of 20 μg/ml with DMEM ''High Glucose''. A stock solution of 1 mg/ml of 6β-OHT was prepared in methanol–water 1:1 (v/v), which

after sequential dilution with DMEM ''High Glucose'' yielded the final concentrations of the individual calibration solutions, i.e., 100, 250, 500, 1000, 2000, 4000 ng/ml. In the same way ''artificial'' samples, i.e., samples mimicking the situation in a true biological environment, were prepared in methanol–water 1:1 (v/v) yielding final concentrations of 250, 500 and 1000 ng/ml after sequential dilution with DMEM ''High Glucose''. The solutions of calibration standards, samples and internal standard prepared in this manner were stored at −20 °C until use. Prior to sample preparation 50 μl of internal standard (20 μg/ml) was added to 500 μl of either calibration solutions or ''artificial'' samples from which 100 μl were subjected to the sample extraction procedure.

2.3. Sample extraction and chromatographic separation

For sample enrichment using the BioTrap 500 MS™ pre-column an identical on-line column-switching procedure as that one previously described for lonazolac was applied [33]. Extraction was performed with 10 m*M* triethylammonium formate (TEAF) buffer (pH 6) at 3.2 ml/min. Back-flush of analyte onto the analytical C_{18} column and subsequent chromatographic separation was accomplished in the gradient mode (gradient program see Table 1) with mobile phase A composed of water–methanol–tetrahydrofuran (73:20:7, v/v/v) and mobile phase B composed of methanol and THF (93:7, v/v) at a flow-rate of 0.2 ml/min. Signal monitoring was effected by MS detection (conditions see below) as well as measurement of UV responses at 254 nm. The time events and experimental conditions of sample extraction on the pre-column, back-flush onto the analytical column and chromatographic separation including the steps of individual re-equilibration of either pre-columns and analytical columns is depicted in Table 2, whereas the column-switching scheme is shown in Fig. 2.

Table 1
Gradient program for separation on the analytical column

Time (min)	Mobile phase A	Mobile phase B
0	90	10
5	90	10
39	52	48

2.4. LC–MS conditions

The LC–MS/MS analysis was carried out on a type LCQ™ ion-trap mass spectrometer purchased from ThermoQuest Finnigan (Bremen, Germany) equipped with an atmospheric pressure chemical ionization (APCI) source operated in the selected reaction monitoring (SRM) mode. Mass spectra were obtained in the positive ion mode applying a source voltage of 5.0 kV and a discharge current of +5 μA. The vaporizer temperature was set at 450 °C and the heated capillary temperature at 200 °C, while the settings for sheath and auxiliary gas both being high purity nitrogen (99.999%) were adjusted to 70 and 10 units, respectively. Data acquisition was done by either Full Scan MS/MS between 2.5 and 39 min in the mass range 90–400 a.m.u. or by the selected reaction monitoring (SRM) technique by means of the transitions m/z 305.3→[267.7–270.7], 29% collision energy, 3 a.m.u. collision width, for 6β-OHT and m/z 347.1→[327.7–330.7], 29% collision energy, 3 a.m.u. collision width, for corticosterone.

3. Results

3.1. Sample extraction, chromatographic separation and detection

As revealed in Fig. 3 showing the HPLC–MS/MS SRM traces of a mixture of 6β-OHT, 6α-OHT, 7α-OHT, 16α-OHT, 2α-OHT, 2β-OHT (transition m/z 305.3→[267.7–270.7]) (Fig. 3 upper trace) and corticosterone (transition m/z 347.1→[327.7–330.7]) as the internal standard (Fig. 3 lower trace) in medium, i.e., DMEM ''High Glucose'' as well as in stimulation experiments in human hepatocytes[1)] containing testosterone (2 m*M*) as the substrate of CYP 3A4 and clofibric acid (1 m*M*) as the inducer of CYP 3A4 activity (Fig. 4, upper trace), satisfac-

[1]A more detailed treatment with respect to either structure or quantitative determination of additional metabolites obtained from incubation of testosterone in media containing human, pig and rat hepatocytes will be reported in part II of this contribution.

tory and sometimes almost baseline separation of the compounds of interest was achieved using on-line sample extraction by means of the BioTrap 500 MS™ pre-column and subsequent back-flush onto the analytical Prontosil 60-5 C_{18}-H column. Slightly deviating from the experimental conditions of method development performed with a 250×2 mm support, a 150×2 mm column was used now for these experiments. However, either gradient program or time events remained the same as applied with the longer stationary phase. Furthermore, a control experiment carried out in the absence of inducer is shown in Fig. 5. As recently seen with lonazolac applying an identical experimental design [33], no measurable peak broadening occurred compared with direct injection of the analyte mixture without preconcentration (results not shown), which in a corresponding way, underlines the efficiency of the back-flush column-switching technique. For quantitative determination of 6β-OHT, measurement of both the UV signal responses at 254 nm as well as those from MS/MS detection in the SRM mode was applied. However, due to the fact that MS detection provides a substantial gain in sensitivity over UV detection, only data from LC–MS experiments were presented.

Considering the limit of quantification (LOQ) as that concentration with a C.V. of 20%, a value of 20 ng/ml was found for an injection volume of 100 μl in the case of the LC–MS/MS technique conducted in the SRM mode. In most cases this value satisfies the requirements of expected concentration ranges of the target analyte in biological media, although the LOQ further decreases to 2.0 ng/ml when using a sample volume of 1000 μl. For comparative purpose, the LOQ at an injection volume of 1000 μl increased to 5 ng/ml when UV detection at 254 nm was applied.

3.2. Recovery

Recovery values for 6β-OHT have been determined in quintuplicate at the 250, 500 and 1000

Table 2
Different time-dependent analytical events used in the column-switching procedure

Time (min)	Analytical events on PC-1/AC-1	Analytical events on PC-1/AC-1	MS/MS events
0.0–2.5	Sample injection-sample extraction on PC-1 with 10 m*M* TEAF (pH 6.0) by means of pump C at 3.2 ml/min.	Start cleaning of AC-2 with methanol–tetrahydrofuran 93:7 (v/v) by means of pump B at 0.2 ml/min	Cleaning ion-source with methanol–water 1:1 at a flow-rate of 0.2 ml/min by means of pump E
2.5–6.0	Back-flush PC-1/AC-1 and begin of analytical separation with pump A at 0.2 ml/min (conditions see Experimental)	Cleaning loop and PC-2 by means of pump D with methanol–water 1:1 at 0.2 ml/min Cleaning of AC-2 with methanol–water 1:1 by means of pump B continued	Cleaning ion-source continued
6.5–21.5	Analytical separation on AC-1 continued	Cleaning loop and PC-2 continued End of cleaning AC-2 (20.5 min) and start equilibration of AC-2 with 90% methanol/water/tetrahydrofuran 20:73:7 (v/v/v) and 10% methanol/tetrahydrofuran 93:7 (v/v) at 0.2 ml/min by means of pump B	Segment *m/z* 305→*m/z* 269
21.5–32.0	Analytical separation on AC-1 continued	Cleaning loop and PC-2 continued Equilibration of AC-2 continued	Segment *m/z* 347→*m/z* 329
32.0–38.0	Analytical separation on AC-1 continued	Equilibration of PC-2 with 10 m*M* TEAF (pH 6.0) by means of pump C at 3.2 ml/min Equilibration of AC-2 continued	
38.0–39.0	End of analytical separation	End equilibration of PC-2 End equilibration of AC-2	Cleaning of ion-source by means of pump E

Pump A=elution pump; pump B=cleaning and equilibrating pump; pump C=extraction pump; pump D=cleaning pump; pump E=ion source cleaning pump.

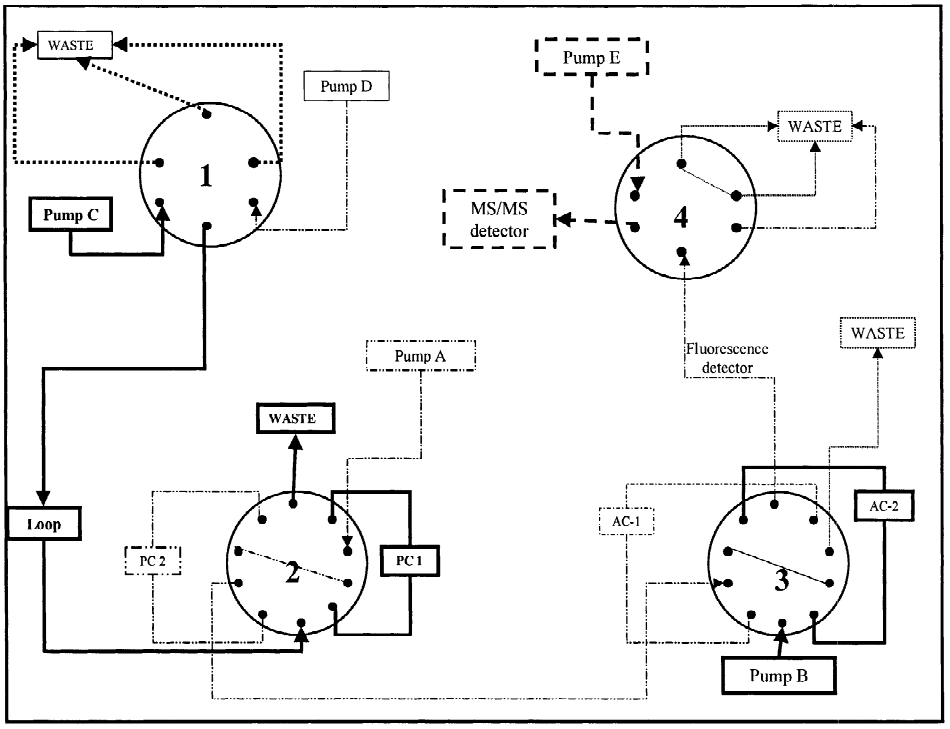

Fig. 2. Insert column-switching scheme PC1/AC1—PC2/AC-2 used within the course of the study (from Ref. [33] with permission from Elsevier Publishers).

Table 3
Determination of within-day and between-day data for precision and accuracy

	Day 1	Day 2	Day 3	Day 4	Day 5	Between-day data
	Nominal concentration (ng/ml)					
	250					
Within-day data	Mean (n=5): 87.74	Mean (n=5): 103.24	Mean (n=5): 101.64	Mean (n=5): 100.50	Mean (n=5): 100.74	n=25
	SD:±7.31	SD:±9.94	SD:±18.95	SD:±8.69	SD:±16.58	Mean: 246.34
	% C.V.: 3.3	% C.V.: 3.9	% C.V.: 7.5	% C.V.: 3.5	% C.V.: 6.6	SD:±17.93
	% Accuracy: 87.7%	% Accuracy: 103.2	% Accuracy: 101.6	% Accuracy: 100.5	% Accuracy: 100.7	% C.V.: 7.3
						% Accuracy: 98.8
	500					
Within-day data	Mean (n=5): 482.76	Mean (n=5): 504.83	Mean (n=5): 518.82	Mean (n=4): 530.74	Mean (n=5): 520.66	n=24
	SD:±37.16	SD:±8.50	SD:±17.79	SD:±17.0	SD:±18.71	Mean: 512.34
	% C.V.: 7.7	% C.V.: 1.7	% C.V.: 3.4	% C.V.: 3.0	% C.V.: 3.6	SD:±24.38
	% Accuracy: 96.6	% Accuracy: 101.0	% Accuracy: 103.8	% Accuracy: 106.1	% Accuracy: 104.1	% C.V.: 4.8
						% Accuracy: 102.5
	1000					
Within-day data	Mean (n=5): 919.35	Mean (n=5): 1026.6	Mean (n=5): 1010.0	Mean (n=5): 1047.53	Mean (n=5): 975.99	n=25
	SD:±28.06	SD:±32.38	SD:±23.81	SD:±23.90	SD:±14.23	Mean: 995.32
	% C.V.: 3.1	% C.V.: 3.2	% C.V.: 2.4	% C.V.: 2.3	% C.V.: 1.5	SD:±53.73
	% Accuracy: 91.9	% Accuracy: 102.7	% Accuracy: 101.0	% Accuracy: 97.6	% Accuracy: 97.6	% C.V.=5.4
						% Accuracy: 99.5

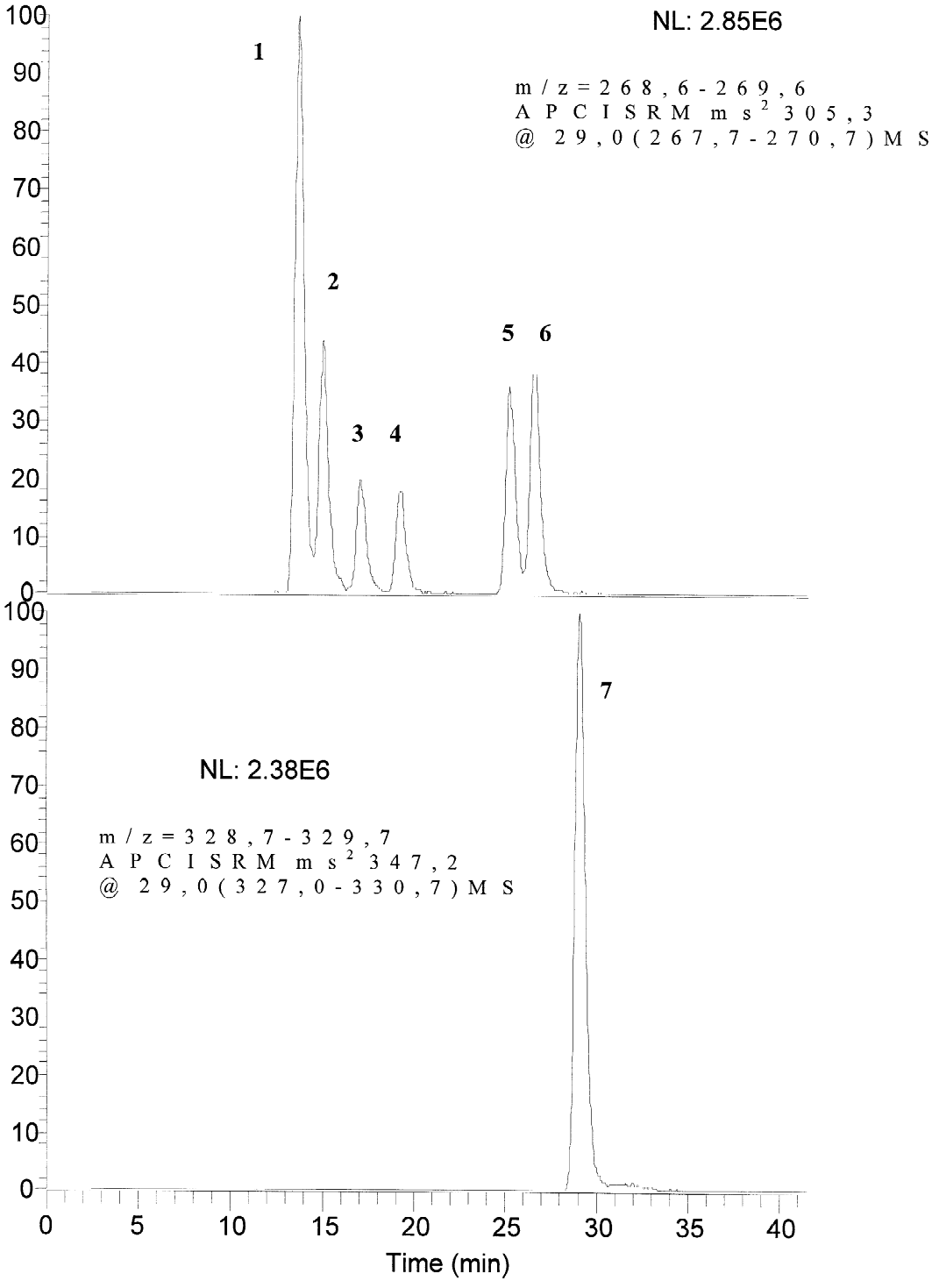

Fig. 3. Upper trace: HPLC–MS/MS SRM chromatogram of a mixture of testosterone metabolites, i.e., 6α-OHT (1), 7α-OHT (2), 6β-OHT (3), 16α-OHT (4), 2α-OHT (5), 2β-OHT (6), in DMEM ''High Glucose'' (transition m/z 305.3→[267.7–270.7]). Lower trace: HPLC–MS/MS SRM chromatogram of corticosterone (7) as the internal standard (transition m/z 347.1→[327.7–330.7]) in DMEM ''High Glucose'' (for details, see Experimental).

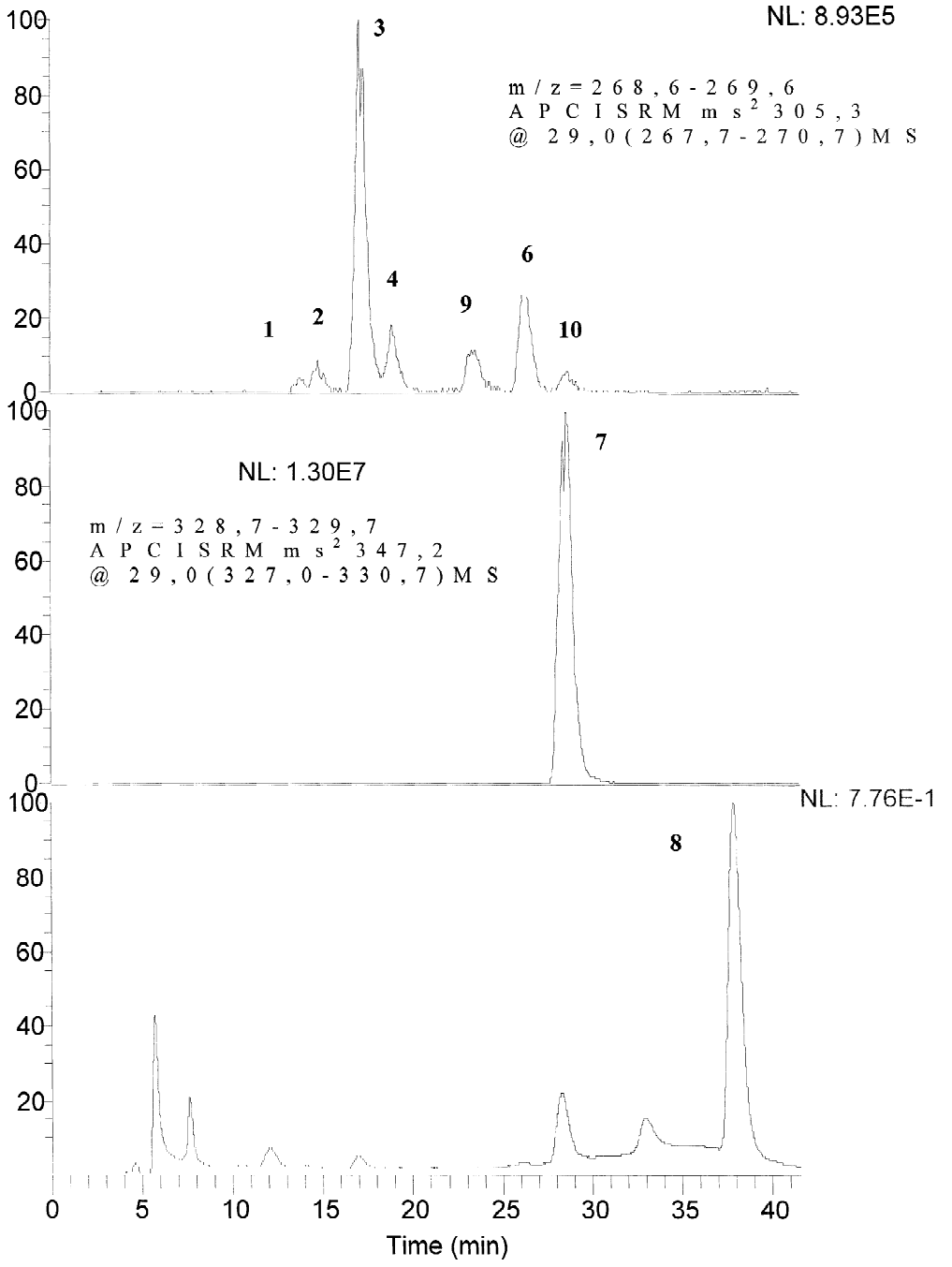

Fig. 4. Upper trace: HPLC–MS/MS SRM chromatogram (transition m/z 305.3→[267.7–270.7]) of the mono-hydroxylation products formed after stimulation of CYP 3A4 activity in human hepatocytes with clofibric acid (1 m*M*) to which testosterone (2 m*M*) was added as the substrate of CYP 3A4; numbering of compounds as in Fig. 3 (for details, see Experimental) Middle trace: HPLC–MS/MS SRM chromatogram of corticosterone (7) as the internal standard (transition m/z 347.1→[327.7–330.7]). Lower trace: HPLC–UV chromatogram recorded at 254 nm showing the peak of testosterone (8) used as the substrate for CYP 3A4 activity. Peaks 9 and 10 represent metabolites of hitherto unknown structure.

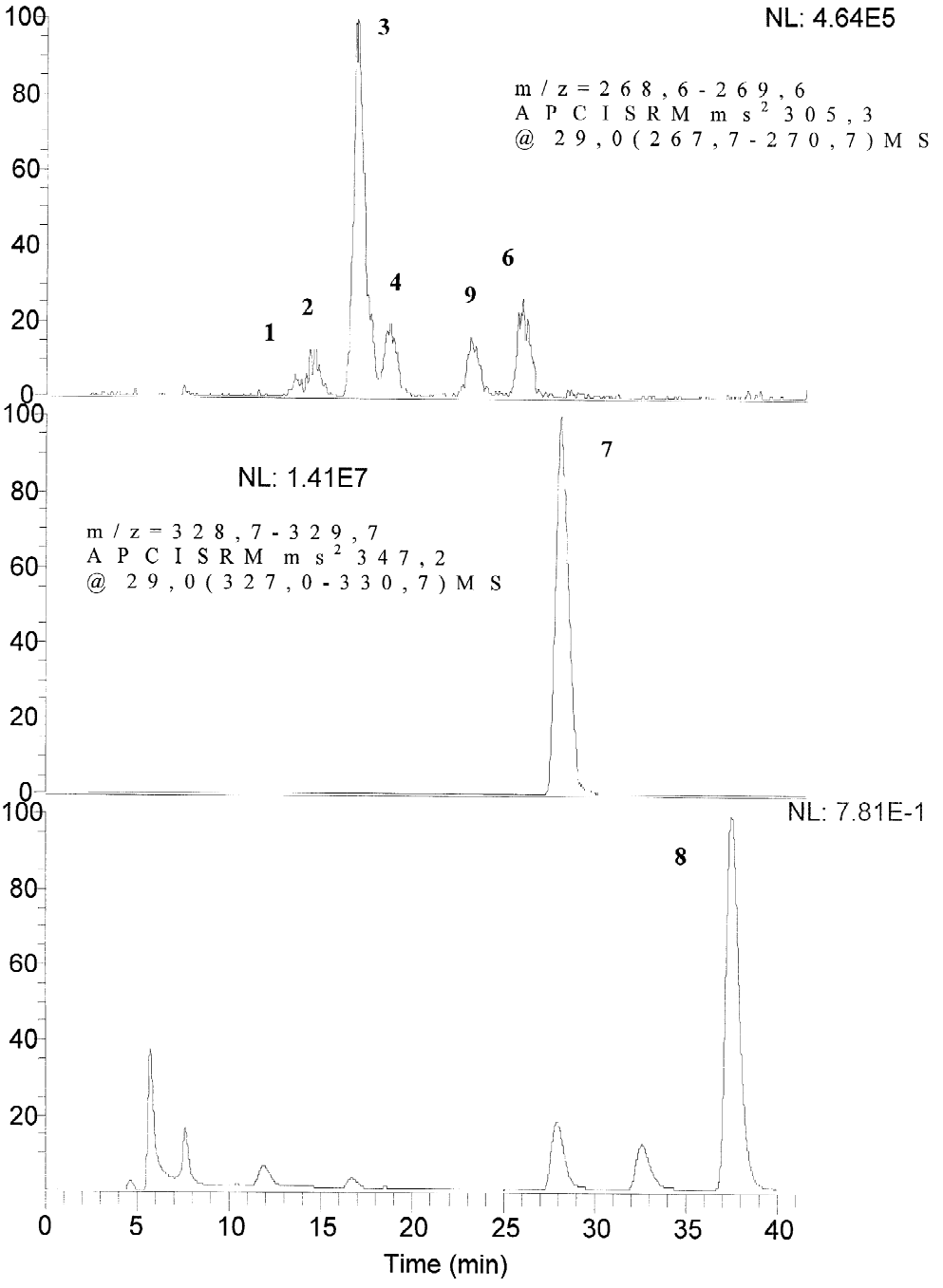

Fig. 5. Upper trace: HPLC–MS/MS SRM chromatogram (transition m/z 305.3→[267.7–270.7]) of the mono-hydroxylation products formed in human hepatocytes from testosterone (2 m*M*) in the absence of clofibric acid as the inducer of CYP 3A4 activity; numbering of compounds as in Figs. 3 and 4 (for details, see Experimental). Middle trace: HPLC–MS/MS SRM chromatogram of corticosterone (7) as the internal standard (transition m/z 347.1→[327.7–330.7]). Lower trace: HPLC–UV chromatogram recorded at 254 nm showing the peak of testosterone (8) used as the substrate for CYP 3A4 activity.

ng/ml level, using the peak area ratios obtained from target compound and internal standard in the biological matrix to those of them dissolved in buffer only. The recoveries obtained for the three concentrations were 104.4%, 103.0% and 101.8%, respectively, for 6β-OHT, whereas recovery of the internal standard corticosterone used at the concentration added to the analyte dissolved in DMEM ''High Glucose'' was 92.1%.

3.3. Linearity

Linearity was achieved in the concentration range of the standard curve, i.e., from 100–4000 ng/ml including six external standards (for individual concentrations see Experimental). In all cases the mean values for the correlation factor R^2 of 6β-OHT were >0.99 (mean=0.9985).

3.4. Specificity

As shown in Fig. 4, depicting the HPLC–MS/MS SRM trace (transition m/z 305.3→[267.7–270.7) obtained from a stimulation experiment of CYP 3A4 activity in human hepatocytes using testosterone (2 m*M*) as the substrate and clofibric acid (1 m*M*) as the inducer, 6β-OHT formed as the main product during the action of CYP 3A4, was clearly separated from either 7αOHT or 16α-OHT, which are the metabolites most closely eluting to the target analyte. Therefore, the complete absence of matrix effects was verified and thus interfering compounds from the used medium as well as from human hepatocytes can extensively be ruled out.

3.5. Precision and accuracy

Precision and accuracy data of 6β-OHT have been determined in quintuplicate at the 250, 500 and 1000 ng/ml level at 5 different days over a period of about 3 weeks. Sample concentrations have been measured with a total of six external standards ranging from 100–4000 ng/ml using internal standardization whereby linear regression was used to fit lines to the data. Either calibration standards or samples used for this procedure have been dissolved in DMEM ''High Glucose''. This medium is representative for all media used in hepatocyte cultures, which only slightly differ from each other. During on-line sample preparation hydrophilic constituents of the media are quantitatively removed and thus do not interfere with the assay.

As can be seen from Table 3, the values for the within-day coefficients of variation (C.V.) and accuracy, respectively, measured in quintuplicate at 5 different days ranged from 3.3–7.5% and 87.7%–103.2% for 250 ng/ml, 1.7–7.7% and 96.6%–106.1% for 500 ng/ml and 1.5–3.2% and 91.9–102.7% for 1000 ng/ml, respectively. The corresponding between-day C.V. and accuracy values measured at 5 different days, as also shown in Table 3, were 7.3% and 98.8% for 250 ng/ml ($n=25$), 4.8% and 102.5% for 500 ng/ml ($n=24$) and 5.4% and 99.5% for 1000 ng/ml ($n=25$).

3.6. Freeze and thaw stability

No detrimental effect of three freeze–thaw cycles, i.e. thawing from −20 °C to room temperature, performed in duplicate at three different days took place. Recoveries at the 250 ng/ml and 1000 ng/ml level were 101.7±4.6% and 101.7±4.7%, respectively, and thus underline the stability of the drug under real-life conditions.

4. Discussion

4.1. Sample preparation, chromatographic separation and detection

Due to the multitude of testosterone metabolites, all exhibiting similar structural properties, separation of them represents a challenge to the analyst and for reliable quantification almost baseline resolution from neighboring components is advisable. In this respect, at first sight, gradient HPLC should be the method of choice and indeed, this technique [7–10,12,14–17,19,21–23,27,31] proves to be the preponderantly applied one, although isocratic separation [18,20,24–26,29,30] is still in use. Extraction of testosterone and its metabolites from the biological matrix was done ''off-line'' by solid-phase extraction (SPE) [17–19,29,30], supercritical fluid extraction (SFE) [26], liquid–liquid extraction (LLE) [7,8,10,12,14–16,18,20,22,31] and also ''on-

line'' by column-switching HPLC using a pre-column for sample enrichment and complete removal of unwanted by-products [31]. Chromatography of the markedly hydrophobic target analytes was almost exclusively effected on C_{18} materials. Different kinds of mobile phase systems were used for separation including mixtures of hydro-organic solvents without addition of additives, such as acids and buffers [7,8,10,12,14–17,19,21–24,26–28,31]. In addition, the use of acidic and buffered hydro-organic mobile phases is reported [9,20,22,25,28,29]. Owing to the fact that the 3-keto-4-ene group of testosterone and its metabolites is a strong chromophore showing an absorption range of 240–280 nm [16], they can be measured by UV-detection with sufficient sensitivity. For this reason, testosterone and its metabolites have been detected at 254 nm [8–10,12,15,17,19,26, 27,31], 245 nm [24], 242 nm [16,20,25], 240 nm [7,14,21,22] and at both 242 and 282 nm [23].

As reasonably expected, GC–MS provides the best sensitivity and values for the limit of detection (LOD) of 0.6 pg [18], 0.3 ppb [19] and 5 ng/ml [26] have been reported for steroids and their derivatives, but time-consuming derivatization procedures are required, which on the other hand give rise to more or less severe side-reactions and thus calls for more convenient and less time-consuming analytical procedures. Due to the excellent sensitivity being achievable even by use of spectrophotometric detection, HPLC proves to be much more suited for analysis of steroids and related components and LOD values of about 0.1 μM [31], 50 ng/ml [26] and in the lower ng/ml range [18,20,21] were observed, whereas a limit of quantitation (LOQ) of 20 ng/ml was reported in Ref. [22]. Nevertheless, when using HPLC–MS, a further gain in sensitivity is achieved and LOD and LOQ values of 6–7 pg [29] and 0.1 ng/ml [30], respectively, have been determined for anabolic steroid hormones in biological fluids.

Successful chromatography of a wide variety of metabolites is reported by Wood et al. [8], Sonderfan et al. [10], Kern et al. [9] as well as Woertelboer et al. [15]. As can be concluded from the investigations of a lot of authors [7,8,12,14,24,31], separation of the pair 6β-OHT/7α-OHT marks the most critical event and in addition, complete separation of the pair 6β-OHT/16α-OHT may also give rise to problems in some cases [14,24,25,31].

Due to the fact that 6β-OHT does not contain any ionizable functional groups, in general no buffer additives and/or acidic mobile phase modifiers are necessary, although in a lot of cases admixture of small amounts of acid or volatile buffers in the eluent system facilitates ionization of the target analyte in LC–MS applications.

Most recently Tachibana and Tanaka [31] provided evidence that admixture of THF to the mobile phase both reduced elution time and increased peak resolution, advantageous properties already reported in 1991 by Laganà and Marino [21]. Therefore, we decided to apply a similar mobile phase system as, in addition, was also successfully used previously by other authors [14,23,24]. As impressively demonstrated in Fig. 3, satisfactory and sometimes almost baseline separation of testosterone metabolites, which according to common experience elute most closely together, including the critical pair 6β-OHT/7α-OHT was accomplished. Furthermore, no interference between 6β-OHT and 7α-OHT as well as additional metabolites formed during stimulation of CYP 3A4 activity in human hepatocytes with clofibric acid (1 mM) in the presence of testosterone (2 mM) as the substrate (see Fig. 4) was observed. The same holds true for the control experiment performed under identical conditions but lacking the inducer (Fig. 5). In accordance with van der Hoeven [14], corticosterone was used as the internal standard, because it elutes after the hydroxylated products and therefore, does not interfere with quantitative determination of the more polar metabolites.

Due to the use of the native analytes, liquid chromatography coupled to mass-selective detection bears considerable advantages over the GC alternative and often provides sensitivity in a comparable order of magnitude. In this respect, LC-ion spray-tandem MS in the SRM mode was applied by Draisci et al. for both structural confirmation of anabolic hormones in bovine blood [29] and quantitation of anabolic hormones in bovine serum and urine [30], whereas Williams et al. [16] have chosen LC–ESI/MS for their investigations of in vitro biotransformation of anabolic steroids in canines applying an up-front collision-induced dissociation (UF–CID) technique, in which ion fragmentation was generated in the first rather than the second mass analyzer. Compared with UV detection at 254 nm, marked

improvement of sensitivity is observed in our investigations by the switch to MS detection in the SRM mode and a quantification limit of about 2.0 ng/ml is achieved using an injection volume of 1000 μl. Although these data substantially lie above the values reported in Refs. [16,29] and [30], but still are satisfactory for the expected concentration range of 6β-OHT within the course of our investigations, it may be expected that LOQs will perhaps further decrease by use of a mobile phase containing small amounts of organic acid and/or buffer or alternatively, by admixture of acid and/or buffer to the column efflux in the post-column addition mode before entering the LC–MS interface.

4.2. Recovery, linearity, precision and accuracy

As impressively recognizable from the data given in the Results, i.e., Table 3, evidence for reliable and reproducible determination of 6β-OHT in biological media is provided. Values obtained for both recovery of 6β-OHT from the biological matrix at three concentrations in the lower, medium and higher concentration range as well as linearity of the signal responses over the whole concentration range of 100–4000 ng/ml are satisfactory as also holds true for the data obtained from measurements of the between-day and within-day coefficients of variation as well as values for accuracy of 6β-OHT levels in quintuplicate at three different concentrations and 5 different days over a period of about 3 weeks.

5. Conclusions

A sensitive on-line sample enrichment procedure based on column-switching gradient high-performance liquid chromatography for determination of 6β-OHT levels in cell culture media is reported. This technique, the principle of which was first described in 1981 [34], employs sample concentration on a biocompatible pre-column allowing interfering compounds to be diverted to waste, while the components of interest are quantitatively retained and therefore assures the non-perturbed, rapid and reproducible analysis of large sample numbers. After back-flushing the target analyte onto an the analytical column, separation is performed by gradient elution with a ternary methanol–water–THF mobile phase system and measurement of signal responses by mass spectroscopic detection in the selected reaction monitoring (SRM) mode yielding a limit of quantification of 20 ng/ml with an injection volume of 100 μl and 2.0 ng/ml with 1000 μl. As previously reported for lonazolac, hydroxy lonazolac and lonazolac sulfate [33], the extensively automated procedure allows processing of large sample numbers making any time-consuming ‘‘off-line’’ extraction and back-extraction steps unnecessary, as, e.g., are required in liquid–liquid or solid-phase analyte enrichment procedures. At first sight the method seems to be rather complicated but once established, it permits rapid processing and quantification of large sample numbers and therefore may be recommended as a powerful alternative to the more conventional time-consuming procedures. Furthermore, the data obtained for linearity, precision and accuracy are satisfactory and therefore, the method is expected to provide reliable results. As previously mentioned in the Introduction, the present chromatographic methodology can be further used for the quantitation of additional metabolites of testosterone in media of human rat and pig hepatocytes, which will be reported in part II of this work.

In conclusion, the new experimental design may also be exploited for quantitative determination of a wide range of biologically active compounds including xenobiotics in plasma, urine and tissue after appropriate modification of the conditions of either pre-column sample enrichment or subsequent chromatographic separation without change of the basic design.

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