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Simultaneous determination of urinary free cortisol and 6β-hydroxycortisol by liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry and its application for estimating hepatic CYP3A induction

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Abstract

A reversed-phase high-performance liquid chromatography coupled to atmospheric pressure chemical ionization tandem mass spectrometry (HPLC–APCI-MS–MS) assay was developed to simultaneously determine monkey urinary free cortisol (C) and 6β-hydroxycortisol (6β-OHC) in 8 min. Urine sample (0.5 ml) containing fludrocortisone acetate (F-C) as the internal standard was extracted with ethyl acetate for 5 min with an extraction efficiency of 90% and 75% for C and 6β-OHC, respectively. A Perkin-Elmer Sciex API 3000 triple quadruple instrument was used for mass spectrometric detection and the column eluent was directed to a heated nebulizer probe. The assay was linear over the range 0.25–10 μ*M* for each analyte. The intra- and inter-day relative standard deviation (RSD) over the entire concentration range for both analytes was less than 10%. Accuracy determined at three concentrations (0.8, 2.0 and 8.0 μ*M*) ranged between 95.5 and 108%. The method described herein is suitable for the rapid and efficient measurement of 6β-OHC/C ratio in Rhesus monkey urine following administration of known hepatic CYP3A inducers and can be used to estimate potential CYP3A induction by drug candidates in the process of early drug development.

Keywords: Cortisol; 6β-Hydroxycortisol

1. Introduction

The cytochrome P450 (CYP) superfamily plays an important role in oxidative biotransformation of many xenobiotics in all species [1–3]. Its 3A family (CYP3A) comprises the most abundant CYP forms found in human liver and catalyzes the metabolism of a host of medications and steroids [4,5]. Therefore, changes in the activity of CYP3A forms may contribute to inter- and intra-individual variation in drug efficacy and potential toxicity associated with drug–drug interactions [6]. Increased CYP3A activity resulting from induction by some drugs, such as rifampin [7], phenobarbital [8] and troglitazone [9], has drawn considerable attention to the timely detection of the effect in the early stage of the drug development process [10]. It has been reported that urinary excretion of 6β-hydroxycortisol (6β-OHC), the major unconjugated metabolite of endogenous

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cortisol, constitutes a non-invasive indicator of CYP3A induction in man [11,12]. However, since urinary 6β-OHC concentrations are variable, its urinary excretion should be expressed as the ratio of 6β-OHC to free cortisol (C) in order to correct for daily and inter-individual variations in adrenal cortisol production [13]. This index has been widely used to assess hepatic CYP3A induction [14], and enzyme activity in human neonates [15] and subjects of different ethnicity [16].

Various methods for the simultaneous detection of 6β-OHC and C have been described, such as high-performance liquid chromatography (HPLC) with UV [17,18] and fluorescence detection [19,20], and gas chromatography [21]. Separate detection of 6β-OHC and C has been performed by radioimmunoassay (RIA) [22–24], enzyme-linked immunosorbent assay (ELISA) [25] and HPLC with UV detection [26,27]. However, RIA suffers from relatively poor specificity due to cross-reactivity of the antibodies [28]. HPLC with UV and fluorescence detection has provided the desired selectivity and sensitivity, but the extensive sample clean-up, multi-step derivatization procedure, as well as time-consuming chromatography, limit its efficiency and capacity. With the increased demand for screening of investigational drugs as inducers of CYP3A activity, the ratio of urinary 6β-OHC to C is an attractive non-invasive index for use in the early-phase of drug development [10]. Liquid chromatography–mass spectrometry (LC–MS) techniques can provide increased specificity not available with conventional HPLC and, therefore, can minimize the extent of sample preparation and reduce the need for sample derivatization. Recently, this technique has been explored for measurement of steroids present in various biological matrixes [29–31], but there is no report describing the simultaneous determination of 6β-OHC and C in urine.

In the present study we describe a rapid, simple and reproducible HPLC–atmospheric pressure chemical ionization (APCI)-MS–MS method for the simultaneous determination of urinary 6β-OHC and C. Because CYP3A is present in the Rhesus monkey [32,33], and has been shown to catalyze the 6β-hydroxylation of cortisol (Tang et al., unpublished results), the urinary 6β-OHC and C ratio in Rhesus monkeys was determined in an attempt to develop an animal model and to validate the reliability of using the ratio as an indicator of CYP3A induction.

2. Materials and methods

2.1. Chemicals and reagents

Cortisol (C, >98%), 6β-hydroxycortisol (6β-OHC,<98%) and fludrocortisone acetate (F-C, >98%) were obtained from Sigma (St. Louis, MO, USA). Stock solutions (10 m*M*) were made by dissolving the respective compounds in a mixture of water–methanol–acetonitrile (25:25:50, v/v/v) and stored at −20°C. Working solutions were freshly prepared by dilution with an aqueous solution of acetonitrile (30%, v/v) to concentrations of 10, 100 and 1000 μ*M* (for C and 6β-OHC) or 2.5 μ*M* (for fludrocortisone acetate). All other chemicals and solvents were of analytical-reagent grade.

2.2. Sample preparation

Both liquid extraction and solid-phase extraction (SPE) procedures were compared.

2.2.1. Method 1A: ethyl acetate extraction

Urine specimens were centrifuged at 1800 *g* for 3 min to remove insoluble matter. To the resulting urine sample (0.5 ml), 25 μl of working F-C solution (internal standard, 2.5 μ*M*) and 3 ml of ethyl acetate were added. The mixture was vortex-mixed on a multi-tube vortexer (Fisher Scientific, Pittsburgh, PA, USA) for 5 min. Following centrifugation at 1800 *g* for ca. 3 min, the ethyl acetate extract was transferred to a clean tube and evaporated to dryness under a flow of nitrogen at 37°C.

2.2.2. Method 1B: ethyl acetate extraction followed by washing

Another set of the same urine samples were treated as in method 1A, except that the organic extract was washed with 1 ml of 1 *M* sodium hydroxide and subsequently with 2 ml of ultra pure water, as reported by Inoue et al. [20].

2.2.3. Method 2A: SPE with methanol elution

Bakerbond C_{18} cartridges of 3 ml with 200 mg of solid-phase mass per column (J.T. Baker, Phillipsburg, NJ, USA) were used. The urine sample (0.5 ml) containing the internal standard was allowed to pass through a preconditioned cartridge (2.5 ml methanol and 2.5 ml water), using a vacuum line to maintain a flow-rate of approximately 1 drop per second at all times. The cartridge was then washed with 2.5 ml of water and the analytes were eluted with 2.5 ml of methanol. The eluted methanol fraction was evaporated to dryness under a stream of nitrogen at 37°C.

2.2.4. Method 2B: SPE with ethyl acetate elution

The urine samples were treated as described in method 2A, except that the cartridges loaded with analytes were eluted with 2.5 ml of ethyl acetate. The ethyl acetate extract was either directly evaporated to dryness under same conditions stated above (method 2B-1), or washed with 1 ml of 1.0 *M* NaOH saturated with Na_2SO_4 followed by 1 ml of 1.0% acetic acid saturated with Na_2SO_4 and finally concentrated to dryness (method 2B-2), according to the method described by Lykkesfeldt et al. [18].

The residues from all treatments were reconstituted in 50 μl of an aqueous solution of acetonitrile (15%, v/v). A 5-μl aliquot was injected into the HPLC–APCI-MS–MS system.

2.3. Chromatography

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) 200 series binary pump and a Perkin-Elmer 200 autosampler. Chromatographic separations were performed on a 5 μm Inertsil ODS-3 (MetaChem, Torrance, CA, USA) column (50×2.0 mm I.D.), operated at ambient temperature. The mobile phase, consisting of 0.02% acetic acid (solvent A, pH adjusted to 4.5 with ammonium hydroxide) and acetonitrile (solvent B), was delivered at a flow-rate of 0.5 ml/min with a linear increase of solvent B from 15% to 65% over 5 min. Equilibration was allowed for additional 3 min, giving a total chromatographic run time of 8 min.

2.4. Mass spectrometry

APCI was used in the present study as an atmospheric pressure ionization technique, in order to minimize the potential matrix effect observed mostly with electrospray ionization technique [34–36].

The tandem mass experiment was performed on a Sciex (Concord, Canada) Model API 3000 triple quadrupole mass spectrometer interfaced to the column eluent via a Sciex heated nebulizer probe operating at 450°C. Operating conditions were optimized by flow injection of a mixture of all analytes (10 μ*M* each) at a flow-rate 5 μl/min, along with the LC flow [500 μl/min, solvent A–B (50:50)], and were determined as follows: nebulizing gas pressure, 80 p.s.i.; auxiliary gas flow, 1.2 l/min; curtain gas, 60 p.s.i.; nebulizing current, 4 μA; orifice voltage, 30 V; ring voltage, 110 V; collision gas (nitrogen) flow, 4. Multiple reaction monitoring (MRM) experiments in the positive ionization mode were performed using a dwell time of 200 ms per transition to detect ion pairs at *m*/*z* 379/343 (6β-OHC), 363/327 (C) and 423/343 (F-C). These product ions were chosen based on their significance in the MS–MS spectra (Fig. 1). The collision voltage was optimized for each analyte, using the autotune feature of the Sciex software (−30 V for 6β-OHC, −33 V for C and −45 V for F-C).

2.5. Quantification

To establish calibration curves, various amounts of standard working solutions were added to pooled monkey urine ($n=3$) to yield eight final concentrations at 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5 and 10 μ*M* for each analyte. Quality control (QC) samples were prepared by adding separately prepared standard working solutions to the same pooled monkey urine to yield three concentrations at 0.8, 2.0 and 8.0 μ*M*, representing low, medium and high QC concentrations. Ratios of peak area of each analyte to that of F-C were computed using Sciex's MacQuan (version 1.6) software. Calibration curves were reconstructed using weighted ($1/x$) linear regression of the standard urine concentrations to measured peak area ratios. Urine concentrations of unknowns were

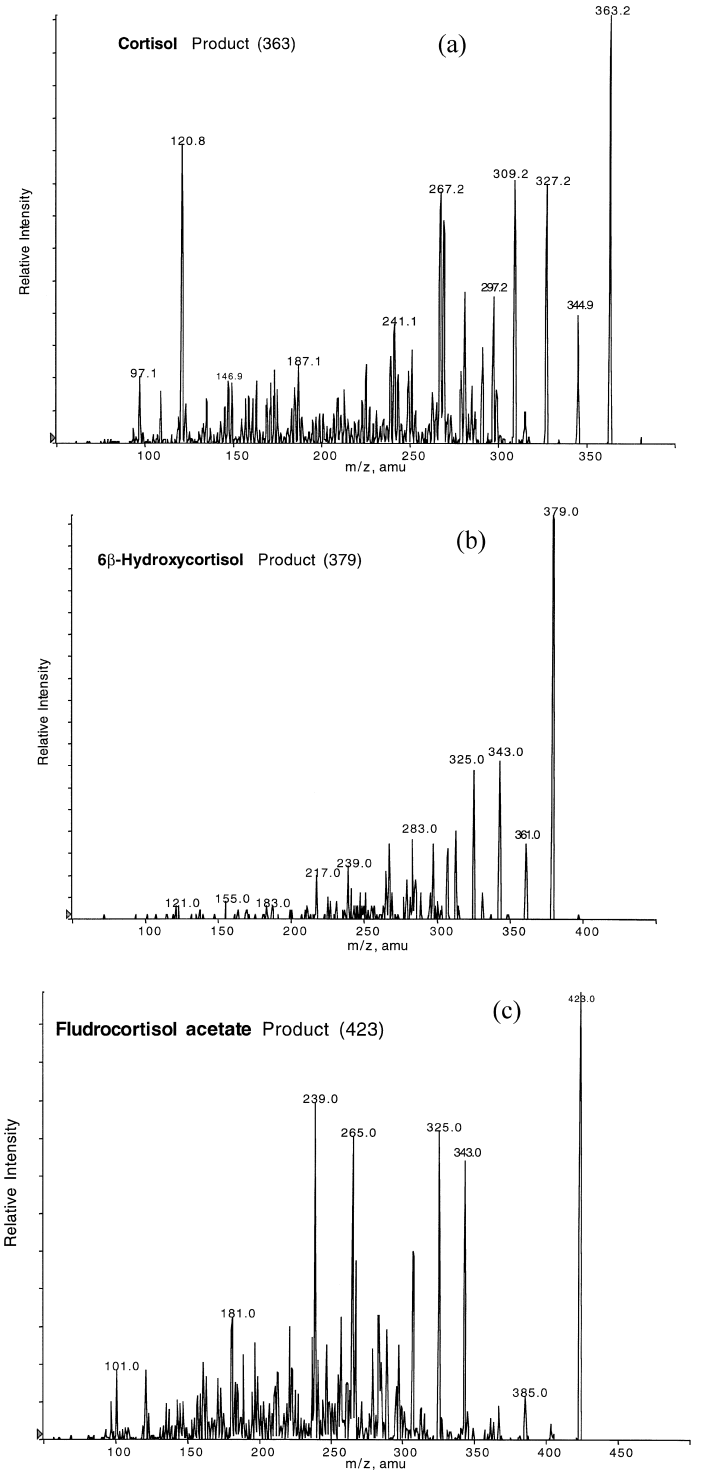


Fig. 1. Product spectra of MH^+ of C (A), 6β-OHC (B) and F-C (C).

determined by extrapolation from calibration curve slopes.

2.6. Treatment of Rhesus monkeys with phenobarbital and rifampin

Based on the procedures reported by other investigators [20,37] for the induction of CYP3A by phenobarbital and rifampin, in the present study sodium phenobarbital and rifampin were dissolved in saline (6 mg/ml) and 2.5% methyl cellulose (4.35 mg/ml), respectively, for oral dosing. Five male Rhesus monkeys were administered phenobarbital (30 mg/kg per day) orally for a 14-day period. Twenty-four-hour cumulative urine samples were collected just before dosing and on days 2, 5, 7, 9, 11 and 14 after administration. After a 2-month washing period, the same monkeys were dosed with rifampin (13 mg/kg per day) orally for 7 days. Similarly, 24-h cumulative urine samples were collected just before dosing and on days 1, 2, 3, 4, 5, 6 and 7 after dosing. Triplicate determinations were performed on each urine sample.

3. Results

3.1. Chromatography and sample clean-up

Separation of 6β-OHC, C and F-C was achieved on a reversed-phase column by gradient elution with acetonitrile and 0.02% acetic acid (pH 4.5) over 8 min. A typical chromatogram obtained with a standard mixture of the three analytes is shown in Fig. 2. The retention times for 6β-OHC, C and F-C were 1.35, 3.4 and 4.3 min, respectively.

An untreated monkey urine sample was directly injected into the LC–MS system and the resulting

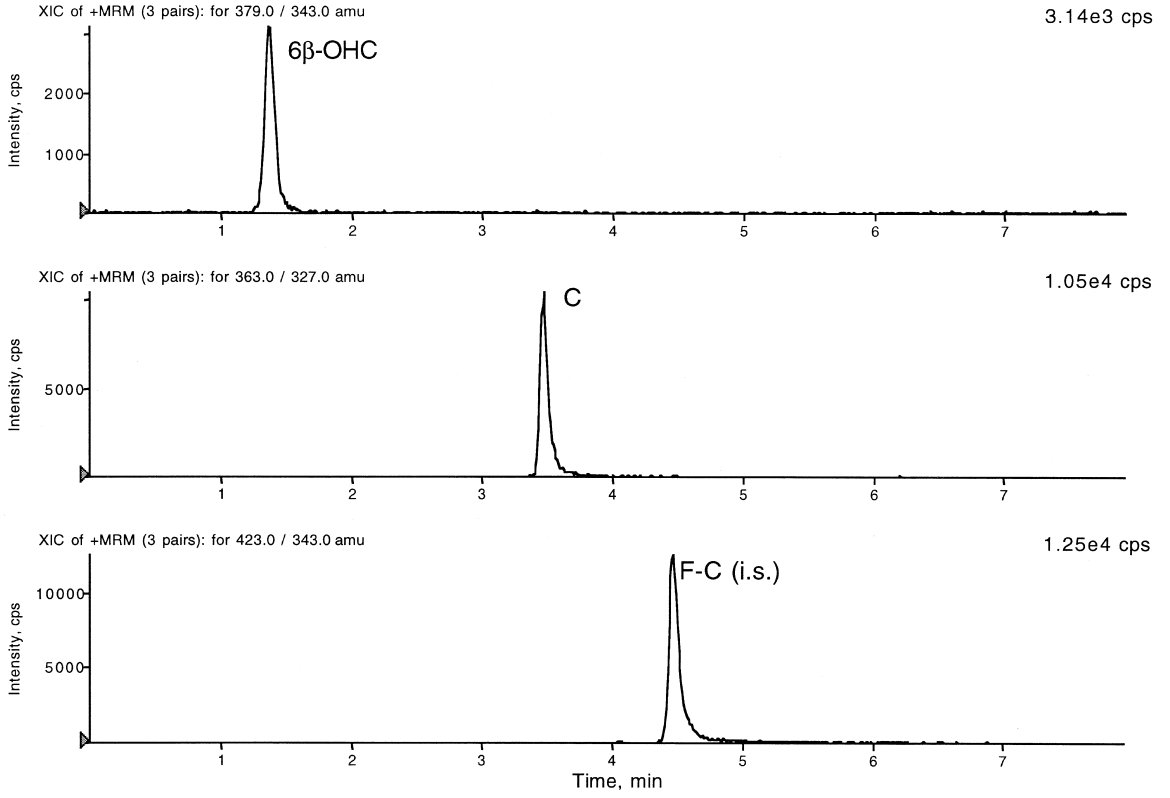


Fig. 2. Multiple reaction monitoring (MRM) chromatograms of standard 6β-OHC, C and F-C. A 5-μl volume of a mixture (0.8 μ*M* for 6β-OHC and C, 10 μ*M* for F-C) was injected.

chromatogram (Fig. 3A) showed very low S/N ratio for 6β-OHC and interference from the urine matrix, indicating that sample clean-up and concentration were needed. As a derivative of fludrocortisone, F-C proved to be a better internal standard than fludrocortisone itself due to its higher mass, allowing for less interference from endogenous steroids. As shown in Fig. 3A, no interfering peaks were detected in the channel (m/z 423/343) for this compound.

Sample preparation by liquid extraction (method 1) and SPE (method 2) were compared. Ten-fold concentration was achieved by both methods, but ethyl acetate extraction yielded much cleaner ion chromatograms (Fig. 3C) than SPE with methanol elution (Fig. 3B). The total ion chromatogram was not improved with further washing of the ethyl acetate extract with sodium hydroxide solution and water (method 1B, data not shown). In addition, SPE with ethyl acetate elution (method 2B-1) and liquid extraction (method 1A) yielded similar results (data not shown). Similarly, further washing with basic and acidic solution caused the loss of all analytes, especially of 6β-OHC (see below).

3.2. Extraction efficiency

The efficiency of extraction methods was examined by comparing the peak area of each analyte determined in samples from two treatments. The results are listed in Table 1. In treatment A, analytes of known amount in 50 μl of an aqueous solution of acetonitrile (15%, v/v) were added to the extracts from 0.5 ml of blank urine. In treatment B, the same amount of analytes was spiked to 0.5 ml of blank urine followed by extraction and the residue was reconstituted in 50 μl of an aqueous solution of acetonitrile (15%, v/v).

In this study, liquid extraction and SPE (methods 1 and 2, as described in Section 3.1), were compared with respect to their extraction efficiency. It was found that liquid extraction using ethyl acetate (method 1A) offered a satisfactory efficiency for all analytes, especially for C and F-C (>90%). The extraction efficiency of SPE with methanol elution (method 2A) is also acceptable (>65%). Elution with ethyl acetate (method 2B-1) showed satisfactory extraction efficiency for all analytes (>75%), but larger variation was observed when compared with liquid extraction with ethyl acetate. Further washing of the ethyl acetate eluent (method 2B-2) resulted in a significant loss of 6β-OHC (Table 1).

Based on the flexibility and simplicity of sample handling, and the results of recovery and extraction efficiency, liquid ethyl acetate extraction was chosen for further validation.

3.3. Calibration curve

Linear calibration curves for 6β-OHC and C were obtained over the range of 0.25–10 μM by plotting the peak area ratio of the respective analyte to F-C against the amount of the steroids added to a pooled blank monkey urine, giving rise to calibration equations of $y=0.196+0.762x$ and $y=0.081+0.154x$ for C and 6β-OHC, respectively. The correlation coefficients of the calibration curves were ≥0.995 for both analytes. The intercept of the curves reflected the amount of 6β-OHC and C present in this typical urine sample and, therefore, the slope was used to calculate the amount in unknown samples. The lower limit of detection was 50 nM and 10 nM for 6β-OHC and C, respectively, at a S/N ratio of 3, when Hank's balanced salt solution (HBSS) was used to construct the calibration curves. However, since the concentration of the analytes present in the blank monkey urine ranged around 0.5 and 0.25 μM in the present study, such a low limit of detection was not required.

3.4. Precision and accuracy

The intra-day precision and accuracy of the assay were determined by analyzing triplicates of standards at all the calibration concentrations. The intra-day precision for all eight concentrations, expressed as RSD, ranged from 1.6 to 7.2% and 2.7 to 9.9% for 6β-OHC and C, respectively. The intra-day accuracy, expressed as a percentage of nominal value, ranged from 96.4 to 104% and 91.8 to 106% for the respective analytes (Table 2).

The inter-day precision and accuracy were determined by analyzing five replicates of QC samples at low, medium and high concentrations through three assay runs. As shown in Table 3, the inter-day precision ranged from 6.8 to 8.8% for 6β-OHC and 2.1 to 6.1% for C. The inter-day accuracy for the

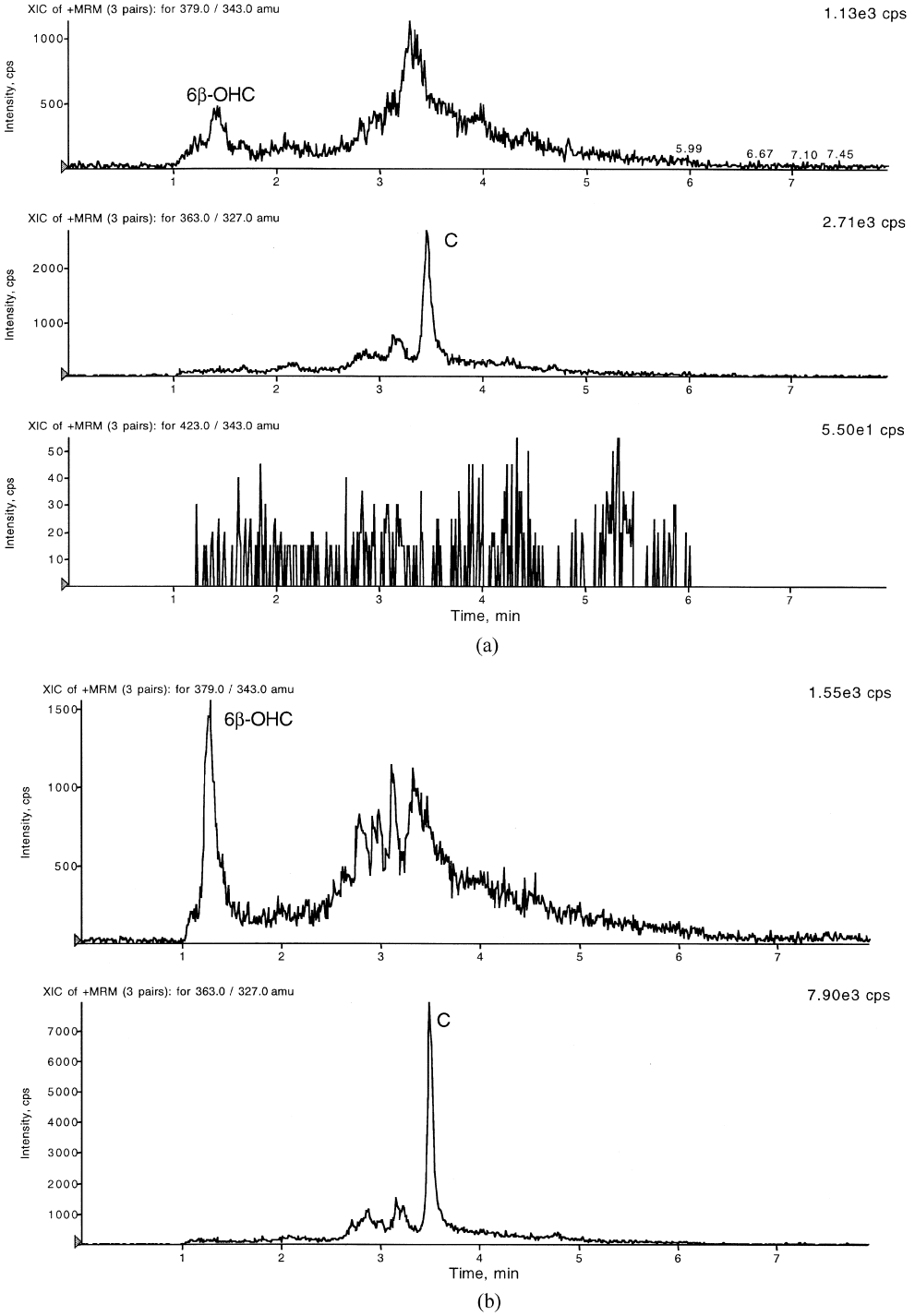
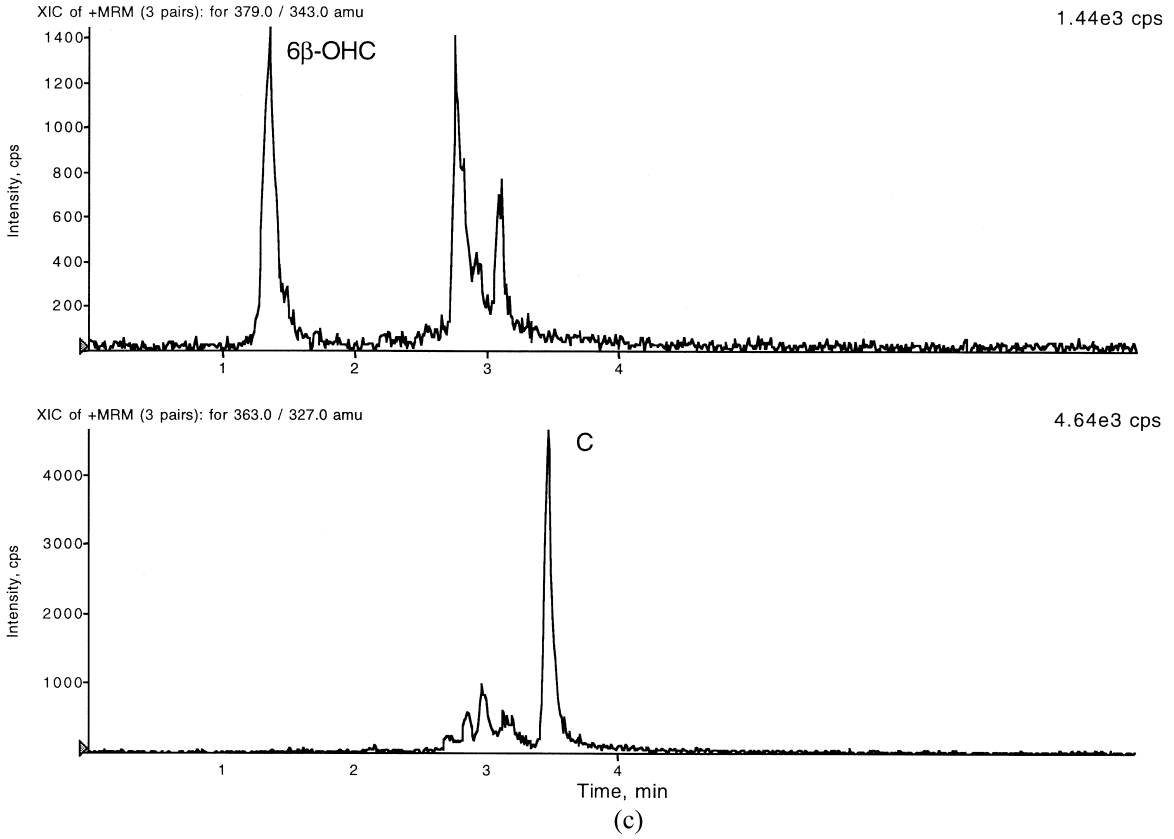


Fig. 3. Multiple reaction monitoring (MRM) chromatograms of an untreated blank monkey urine (A), an extract of 0.5 ml blank monkey urine, which was concentrated with a C_{18} cartridge followed by methanol elution (B), and an extract of 0.5 ml blank monkey urine subjected to ethyl acetate extraction (C).



(c)

Fig. 3. (*continued*)

three QC concentrations ranged from 96.0 to 111% and 95.6 to 111% for the respective analytes.

3.5. Effects of phenobarbital and rifampin on the ratio of urinary 6β-OHC/C in Rhesus monkeys

To investigate the usefulness of the present method for evaluation of CYP3A induction, phenobarbital and rifampin, two well known CYP3A inducers, were administered orally to Rhesus monkeys. Their 24-h cumulative urine samples were collected over a 14-day (phenobarbital) or 7-day (rifampin) period and urinary levels of 6β-OHC and C were measured by the presently described method.

There were considerable inter- and intra-animal variations in urinary excretion of 6β-OHC and C before and during the treatment of the drugs (data not shown). Therefore, no significant changes in 6β-OHC excretion were found during the treatment, although the excretion of C appeared to decrease with time. However, the 6β-OHC/C ratio yielded a much less variation, as also described by others [19,20]. Before the treatment with phenobarbital, the urinary 6β-OHC/C ratio ranged from 0.96 to 2.42 (mean 1.96 ± 0.61, $n=5$). The value changed upon the treatment with phenobarbital, and showed an approximately six-fold increase on day 5 and lasted until day 14 of dosing (Fig. 4). A similar result has been reported by Inoue et al. [20]. The baseline urinary 6β-OHC/C ratio prior to rifampin treatment ranged from 2.79 to 3.80 (mean 3.36 ± 0.48, $n=4$). The treatment with rifampin resulted in a significant increase of the value on day 2 (~2.5-fold) and reached three-fold on day 7 of dosing (Fig. 4). These results demonstrated that the 6β-OHC/C ratio in urine can be used as a non-invasive indicator for monitoring the time-course of CYP3A induction in Rhesus monkeys.

Table 1
Extraction efficiency of different procedures (the data are expressed as mean±SD of triplicates)

Extraction method	Treatment[a]	6β-OHC		C		F-C	
		Area (mean±SD)	RSD (%)	Area (mean±SD)	RSD (%)	Area (mean±SD)	RSD (%)
Neat sample		34 011±2270	6.7	114 931±1043	1.0	93 787±5657	6.0
Liquid extraction with ethyl acetate	Blank	11 228±1091	9.7	20 887±978	4.6		
(Method 1A)	A	62 403±2249	3.6	108 037±3926	3.6	70 790±5049	7.1
	B	46 121±1175	2.5	101 028±7930	7.8	69 743±5083	7.8
		74%[b]		94%		98%	
SPE with methanol elution	Blank	11 182±3145	28	39 262±8407	21		
(Method 2A)	A	44 015±3488	7.9	210 720±8314	4.0	100 345±4722	4.7
	B	32 431±8048	25	156 302±33 717	22	65 978±17 482	26
		74%		74%		66%	
SPE with ethyl acetate elution	Blank	14 824±2653	18	27 142±3058	11		
(Method 2B-1)	A	52 334±6569	13	127 908±5598	4.4	70 230±7892	11
	B	56 761±3675	6.5	139 698±19 254	14	53 745±15 868	30
		107%		109%		76%	
SPE with ethyl acetate elution	Blank	8427±476	5.6	21 637±1116	5.2		
followed by washing	A	46 192±1349	2.9	132 528±2246	1.7	63 816±4618	7.2
(Method 2B-2)	B	36 063±2023	5.6	118 085±4611	3.9	54 025±984	1.8
		78%		89%		85%	

[a] In treatment A, 50 μl of 2 μ*M* standard solution was added to extract from 0.5 ml blank urine. In treatment B, same amount of standard solution was spiked to 0.5 ml blank urine followed by extraction.
[b] Extraction efficiency.

4. Discussion

As described herein, the selectivity offered by the MRM experiment greatly simplified the procedure of sample clean-up for the measurement of 6β-OHC and C in urine. Although extraction and concentration was required, due to the low analyte concentration and the presence of abundant other steroid analogs in the urine, a one step of ethyl acetate extraction was sufficient to minimize interference from endogenous components. Further washing with basic and acidic solutions did not improve the

Table 2
Intra-day precision and accuracy of 6β-OHC and C determination in Rhesus monkey urine[a]

Nominal concentration (μ*M*)	6β-OHC			C		
	Calculated concentration[b] (μ*M*)	Accuracy[c] (%)	RSD (%)	Calculated concentration (μ*M*)	Accuracy (%)	RSD (%)
0.25	0.253	101	7.2	0.265	106	3.8
0.5	0.499	99.8	3.8	0.459	91.8	4.0
1.0	1.03	103	6.8	0.994	99.4	10
2.5	2.59	104	1.6	2.57	103	8.9
5.0	4.82	96.4	5.0	4.66	93.3	2.7
7.5	7.76	104	5.2	7.51	100	5.4
10.0	10.09	101	4.1	10.3	103	2.8

[a] Method 2A was employed for this study.
[b] From the linear least-squares regression of the calibration curves using all points ($n=3$) at all concentrations.
[c] Calculated as (mean calculated concentration)/(nominal concentration)×100%.

Table 3
Inter-day precision and accuracy of 6β-OHC and C determination in Rhesus monkey urine

Analyte	Nominal concentration (μM)	Calculated concentration[a] (μM)	Accuracy[b] (%)	RSD (%)
6β-OHC	0.80	0.89±0.08	111	8.8
	2.00	2.01±0.14	101	6.8
	8.00	7.69±0.64	96.0	8.4
C	0.80	0.82±0.05	103	6.1
	2.00	1.91±0.13	95.6	3.9
	8.00	8.87±0.19	111	2.1

[a] $n=3$ days (five replicates per day).
[b] Calculated as (mean calculated concentration)/(nominal concentration)×100%.

separation and caused a loss of the analytes. Regardless of two interfering peaks appearing in the channel for 6β-OHC, they were adequately separated from the 6β-OHC peak. The presence of a cluster of peaks detected in the channel of ion pair for C might interfere the quantitation of C, but baseline separation was obtained by optimizing the chromatography.

SPE was tested in order to adapt the method to a 96-well format. However, similar to the reports of others [18], ethyl acetate elution was found to be superior to methanol elution with respect to the level of interfering components. Much higher background was encountered in the samples eluted with methanol than those eluted with ethyl acetate, and, therefore, resulted in poor separation of the analytes from the interfering peaks. In light of this observation, SPE with ethyl acetate without further washing is suitable for LC–MS–MS assay of urinary 6β-OHC and C in the 96-well format, but validation is needed. However, for the medium sized samples ($n<200$), ethyl acetate extraction appears to be the ideal choice.

The selectivity offered by the MRM experiment also allowed for the determination of urinary 6β-OHC and C using a rapid chromatographic method. Unlike other conventional HPLC–UV and HPLC–fluorescence methods, which usually required 40–60 min for each run [18,20], it took only 8 min for each sample with the current method.

In summary, the simplicity of sample preparation, rapidity of chromatography and selectivity of MRM methodology described in the current method greatly improved the efficiency and capacity of the assay for simultaneously determining 6β-OHC and C in urine

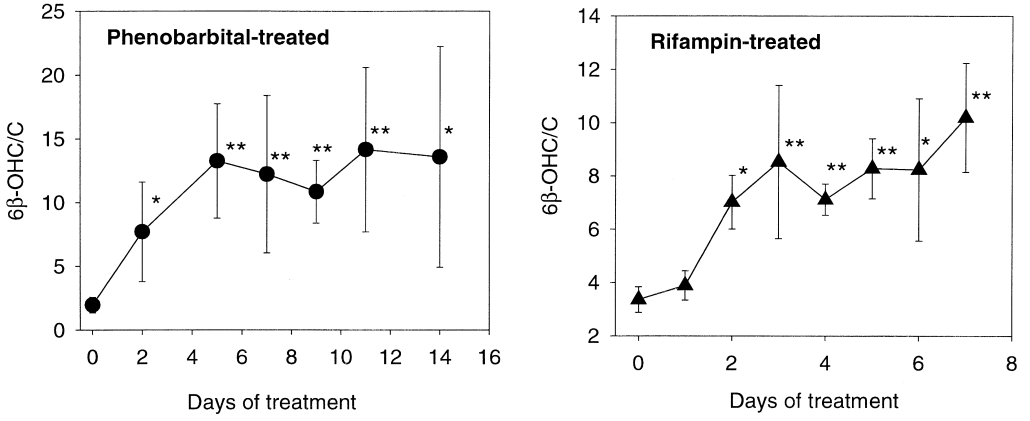


Fig. 4. Changes in urinary 6β-OHC/C ratio of Rhesus monkeys treated orally with phenobarbital (30 mg/kg per day) for 14 days and rifampin (13 mg/kg per day) for 7 days. Significant differences from pre-administration values are indicated: * ($P<0.05$); **($P<0.01$).

samples. This method successfully detected the induction of CYP3A in monkeys treated orally with two clinically relevant inducers and demonstrated that the Rhesus monkey can be used as an animal model for evaluating the potential for induction of CYP3A in man. This method may be applicable in early phases of drug development for the screen of drug candidates as potential CYP3A inducers.

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