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# Simultaneous determination of 6β-hydroxycortisol and cortisol in human urine and plasma by liquid chromatography with ultraviolet absorbance detection for phenotyping the CYP3A activity

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**Abstract**

This study describes an high-performance liquid chromatographic (HPLC)–UV method for the simultaneous determination of 6β-hydroxycortisol (6β-OHF) and cortisol (F) in human urine and plasma. The within-day relative standard deviation of the three concentrations for both analytes was less than 6.2%. Accuracy determined at three concentrations ranged between 95 and 107%. The extraction recoveries were 64.1 ± 4.3 and 88.1 ± 2.4% at three concentrations for 6β-OHF and F in urine, respectively. The extraction recoveries were 88.7 ± 1.4% at three concentrations for F in plasma. This is the first HPLC method that can simultaneously determine 6β-OHF and F in human urine and plasma and is suitable for routine assessment of the CYP3A activity expressed as 6β-hydroxylation clearance.


*Keywords:* 6β-Hydroxycortisol; Cortisol; HPLC; CYP3A

## 1. Introduction

Cytochrome P450 3A (CYP3A) enzyme catalyzes the C-6β oxidation of cortisol (F) to form 6β-hydroxycortisol (6β-OHF). The metabolite 6β-OHF is then excreted as an unconjugated form in urine [1–3]. The urinary excretion ratio of 6β-hydroxycortisol to cortisol (6β-OHF/F) is a good test to evaluate drug-metabolising enzyme inducing or inhibiting properties of drugs when the subjects are their own controls [4]. The endogenous cortisol 6β-hydroxylation clearance, calculated from the amount of urinary excreted 6β-OHF divided by the area under the plasma concentration–time curve (AUC) of cortisol can be used as a reliable index for the in vivo CYP3A phenotyping [5]. Calculation of 6β-hydroxylation clearance requires two separate analysis of F in plasma and 6β-OHF in urine.

Various methods for the simultaneous determination of 6β-OHF and F in urine have been described, such as high-performance liquid chromatographic (HPLC) with UV [6–8] and fluorescence detection [9], gas chromatography–mass spectrometry (GC–MS) [10], and liquid chromatography–tandem mass spectrometry (LC–MS–MS) [11]. Separate detection of 6β-OHF and F has been performed by radioimmunoassay (RIA) [12], enzyme-linked immunosorbentassay (ELISA) [13]. The immunochemical techniques are highly sensitive, but they lack selectivity for 6β-OHF and F due to cross-reactivity of the antibodies, resulting in higher concentration of 6β-OHF and F. Although the selectivity and sensitivity offered by GC–MS and LC–MS–MS should allow for the accurate determination of 6β-OHF and F in urine, the use of these techniques is generally limited for the routine analysis due mainly to the instrumental availability. HPLC with fluorescence detection has provided the desired selectivity and sensitivity but the extensive sample clean up, muti-step derivatization procedure, as well as time-consuming chromatography, limits its efficiency and capacity. Previous published HPLC–UV methods generally offer higher selectivity and sensitivity in measuring 6β-OHF and F, but the results are not often reproducible in different laboratories as endogenous cortisone may interference the detection

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of cortisol when the separation was achieved on a C18 bonded column. There is no published HPLC method so far that can simultaneously determine 6β-OHF and F in human urine and plasma.

In the present study, we describe a sensitive and reproducible HPLC method for simultaneous determination of 6β-OHF and F in human urine and plasma. This method was designed to be using for the characterization of polymorphic CYP3A phenotype expressed as 6β-hydroxylation clearance.

## 2. Experimental

### 2.1. Chemicals and reagents

Cortisol, 6β-hydroxycortisol and dexamethasone (DM) were purchased from Sigma (St. Louis, Missouri, USA) and were of at least 98% purity. Acetonitrile and methanol were of liquid chromatographic grade obtained commercially (Tedia company, OH, USA). All other chemicals and solvents were of analytical-reagent grade and were used without further purification. 6β-OHF was reconstituted as 0.044 mg/ml solutions in methanol. F and DM were reconstituted as 1 mg/ml solutions in methanol. All the stock solutions were stored at −20 °C.

### 2.2. High-performance liquid chromatography (HPLC)

#### 2.2.1. HPLC conditions

A Shimadzu 2010CHT high-performance liquid chromatography with an SPD-M10A photodiode array detector was used (Shimadzu, Kyoto, Japan). The separations were performed on a Inertsil PH-3 5 μm (250 mm × 4.6 mm i.d.) (GL Scineces, Tokyo, Japan), monitored by UV absorbance at 245 nm and operated at 1.0 ml/min using the following gradient of solvent mixtures acetonitrile (A) and water (B). The stepwise gradient elution program was: 12–43% A (linear) at 0–27 min. Twelve percent A at 27–35 min. The column temperature was maintained at 40 °C.

#### 2.2.2. Sample preparations for HPLC

To 2.0 ml of human urine (0.5 ml plasma) was added 0.2 ml (0.1 ml) of the solution containing known amounts of dexamethasone (2000 ng/ml) in distilled water, as internal standard. The urine or plasma samples were applied to an Oasis HLB extraction Cartridges (Waters Corporation, Milford, MA, USA) that had been pretreated with 1 ml methanol and balanced with 1 ml distilled water. The cartridges was washed with 3 ml of distilled water and then eluted with a solution of ethyl acetate-diethyl ether (4:1, v/v; 5 ml). Two milliliters of 1 M NaOH saturated with sodium sulfate was added to the organic extract, and vortex-mixed for 1 min. After centrifuging, 2.0 ml of 1.0% acetic acid saturated with sodium sulfate was added to the organic extract, and vortex-mix for 1 min. The organic extract was evaporated to dryness at 50 °C under a stream of nitrogen. The residue was reconstituted in a solution of acetonitrile-water (1:4,v/v; 100 μl). A 40 μl aliquot of the solution was injected to HPLC. Peaks of 6β-OHF, F and dexamethasone were measured.

### 2.3. Recovery

To 2 ml (0.5 ml) of solutions containing known amounts of 6β-OHF (27.5, 110, and 440 ng/ml) and F (7.8, 31.25, and 125 ng/ml) in distilled water was added 0.2 ml (0.1 ml) of the solution containing known amounts of dexamethasone (2000 ng/ml) in distilled water. Five samples of each concentration were prepared. The samples were then carried through the sample preparation procedure described above. The recoveries were calculated by comparing the peak-area of these compounds between before and after the extraction procedures.

### 2.4. Calibration graphs

To 2 ml (0.5 ml) of solutions containing known amounts of 6β-OHF (6.9, 13.75, 27.5, 55, 110, 220, 440, and 880 ng/ml) and F (1.95, 3.9, 7.8, 15.6, 31.25, 62.5, 125, and 250 ng/ml) in distilled water was added 0.2 ml (0.1 ml) of the solution containing known amounts of dexamethasone (2000 ng/ml) in distilled water. The samples were then carried through the sample preparation procedure described above. Peak areas of 6β-OHF, F and dexamethasone were measured. The calibration graphs were obtained by an unweighed least-squares linear fitting of the peak-area ratios versus the concentration of 6β-OHF and F on each analysis of the standard mixtures.

### 2.5. Accuracy and precision

The analytical accuracy and precision were examined by adding known amounts of 6β-OHF (55, 110, and 440 ng/ml) and F (15.6, 31.25, and 125 ng/ml) to urine samples containing known amounts of endogenous 6β-OHF (80.7 ng/ml) and F (13.5 ng/ml). The analytical accuracy and precision were also examined by adding known amounts of F (15.6, 31.25, and 125 ng/ml) to plasma samples containing known amounts of endogenous F (62.7 ng/ml). For intra-day assay precision, five samples of each concentration were assayed on the same day. The day-to-day inter-assay precision was not examined in this study.

### 2.6. Clinical sample collection

Twelve healthy adult volunteers (six of the volunteers are female) participated in this study. The protocol for the present study was approved by the ethics committee of School of Pharmaceutical Sciences, Central South University. All volunteers gave their written informed consent to participate in the study. No individual was receiving any medication. Blood samples (5 ml) were obtained at 08:00 AM. The heparinized blood was collected in glass tubes and centrifuged. The urine samples were collected at five different time intervals during a 24-h period: 08:00–12:00, 12:00–16:00, 16:00–20:00, 20:00–23:00, and 23:00–08:00. The volume of the urine samples was noted. The plasma and urine samples were stored at −20 °C until analysis.

Table 1
Extraction recoveries of 6β-OHF, F and dexamethasone for urine samples (2.0 ml)

| Compound | Concentration (ng/ml) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|
| 6β-OHF | 27.5 | 60.7 ± 4.0 | 6.6 |
| | 110 | 62.6 ± 3.4 | 5.4 |
| | 440 | 69.0 ± 3.8 | 5.5 |
| F | 7.8 | 85.4 ± 4.5 | 5.3 |
| | 31.25 | 89.7 ± 3.2 | 3.6 |
| | 125 | 89.2 ± 3.9 | 4.4 |
| Dexamethasone | 200 | 89.4 ± 3.0 | 3.4 |

Mean ± S.D. $n = 5$.

## 3. Results

### 3.1. Chromatography

Separation of 6β-OHF and F were examined by using two types of reversed-phase columns by gradient elute with water and acetonitrile. Typical chromatograms are shown in Fig. 1. A single peak of 6β-OHF and F were obtained on a reversed-phase phenyl column (Fig. 1b–d). 6β-OHF cannot be detected in human plasma (Fig. 1b). Dexamethasone as internal standard gave good peaks on the reversed-phase phenyl column and no interfering peaks were observed in urine or plasma samples (Fig. 1b–d). However, F was not separable from an endogenous interfering peak (probably cortisone) on a C18-bonded column (Fig. 1e).

### 3.2. Extraction recovery

6β-OHF and F were extracted from urine or plasma by using an Oasis HLB extraction cartridge, being eluted with ethyl acetate-diethyl ether (4:1,v/v). The organic extracts were then washed with alkaline and acidic solutions saturated with sodium sulfate. Extraction recoveries (mean ± S.D.) were determined at three different concentrations of 6β-OHF (27.5, 110, and 440 ng/ml) and F (7.8, 31.25, and 125 ng/ml) in water. Extraction recoveries were also determined at a fixed concentration (200 ng/ml) of the internal standard (dexamethasone). Results are displayed in Tables 1 and 2.

### 3.3. Calibration graphs

A good correlation was found between the observed peak-area ratios ($A$) and the theoretical concentration ($C$).

Table 2
Extraction recoveries of F and dexamethasone for plasma samples (0.5 ml)

| Compound | Concentration (ng/ml) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|
| F | 7.8 | 87.4 ± 4.9 | 5.6 |
| | 31.25 | 88.6 ± 3.7 | 4.2 |
| | 125 | 90.2 ± 2.8 | 3.1 |
| Dexamethasone | 200 | 88.9 ± 3.2 | 3.6 |

Mean ± S.D. $n = 5$.

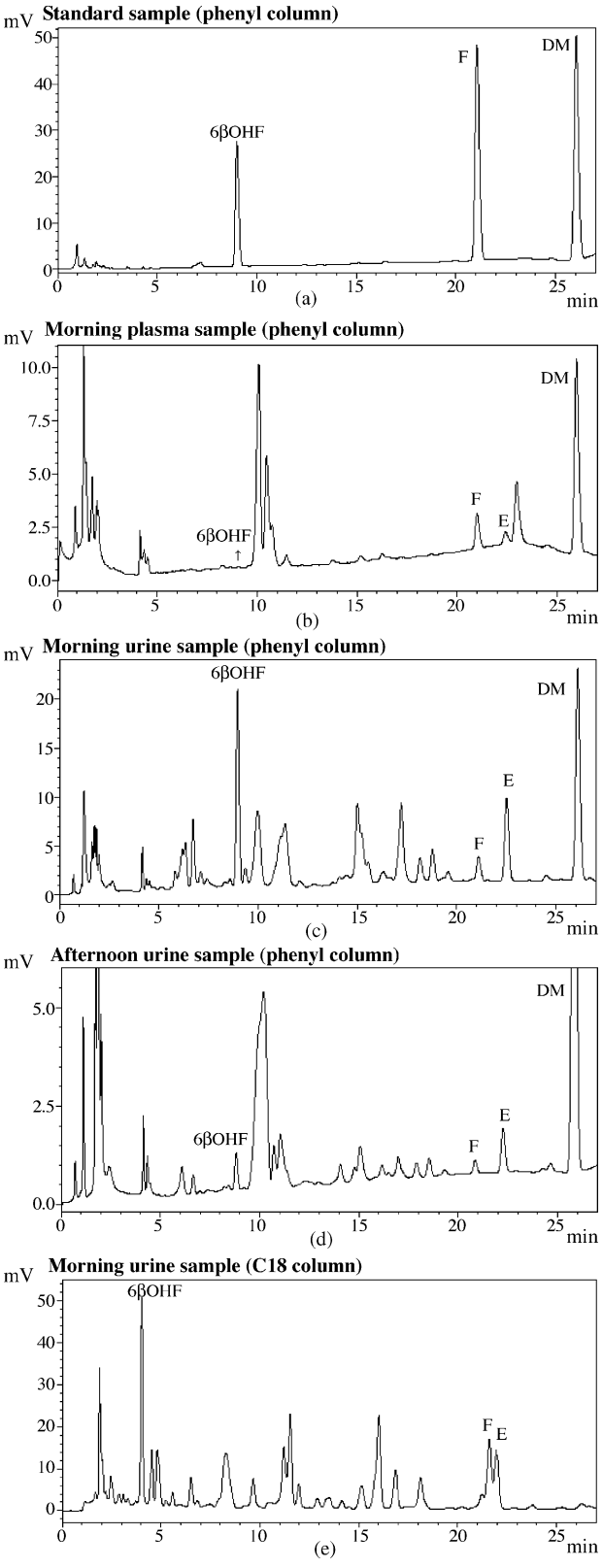


Fig. 1. High-performance liquid chromatograms of standard compounds (a); morning plasma sample using a reversed-phase phenyl column (b); morning urine sample using a reversed-phase phenyl column (c); afternoon urine sample using a reversed-phase phenyl column (d); and morning urine sample using a reversed-phase C18 column (e). 6β-OHF: 6β-hydroxycortisol. F: cortisol; DM: dexamethasone; and E: endogenous substance (probably cortisone).

Table 3
Accuracy and precision for urine samples

| Compound | Concentration Added (ng/ml) | Concentration found (ng/ml) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|---|
| 6β-OHF | 0 | 80.7 ± 2.7 | – | 3.3 |
| | 55 | 137.5 ± 8.3 | 103 | 6.0 |
| | 110 | 184.7 ± 6.1 | 95 | 3.3 |
| | 440 | 548.0 ± 26.3 | 106 | 4.8 |
| F | 0 | 13.5 ± 0.7 | – | 5.2 |
| | 15.6 | 30.2 ± 1.7 | 107 | 5.6 |
| | 31.25 | 43.5 ± 1.1 | 96 | 2.5 |
| | 125 | 139.5 ± 4.2 | 101 | 3.0 |

Mean ± S.D. $n = 5$.

### 3.3.1. Calibration graphs for urine samples

Unweighed least-squares regression analysis gave typical regression lines: $C = 320.37A - 6.505$ ($r = 0.9996$) for 6β-OHF and $C = 214.82A - 2.1343$ ($r = 0.9999$) for F. The low limit of quantitation (LOQ) was 6.9 ng/ml for 6β-OHF and 2 ng/ml for F, respectively.

### 3.3.2. Calibration graphs for plasma samples

Unweighed least-squares regression analysis gave typical regression lines: $C = 215.12A - 2.3808$ ($r = 0.9999$) for F. The low limit of quantitation (LOQ) was 7.8 ng/ml for F.

### 3.4. Accuracy and precision

The data for the validation of the within-day precision are presented in Tables 3 and 4. The results show low relative standard deviation even for low levels of 6β-OHF and F. Accuracy expressed as the ratio of compound added to that measured is also displayed in Tables 3 and 4. The results indicate that the concentration of 6β-OHF and F determined are in good agreement with the actual concentration added.

### 3.5. Application of this method

We applied this method to the plasma and urine samples collected from 12 healthy adult volunteers. Urinary excretion of 6β-OHF and F in the 12 healthy volunteers was 60.9 ± 20.0 (mean ± S.D.) μg/24 h and 21.3 ± 10.4 μg/24 h, respectively. The 24-h urinary 6β-OHF/F ratio was 3.54 ± 2.32. Circadian variation in urinary excretion of 6β-OHF and F (μg/h) and their ratio are displayed in Fig. 2. The concentrations of F in morning plasma samples of the 12 volunteers ranged between 48.8 and 151.6 ng/ml (87.9 ± 35.4).

Table 4
Accuracy and precision for plasma samples

| Compound | Concentration Added (ng/ml) | Concentration found (ng/ml) | Recovery (%) | R.S.D. (%) |
|---|---|---|---|---|
| F | 0 | 62.7 ± 2.8 | – | 4.5 |
| | 15.6 | 79.2 ± 4.9 | 106 | 6.2 |
| | 31.25 | 95.1 ± 3.0 | 104 | 3.2 |
| | 125 | 182.6 ± 5.1 | 96 | 2.8 |

Mean ± S.D. $n = 5$.

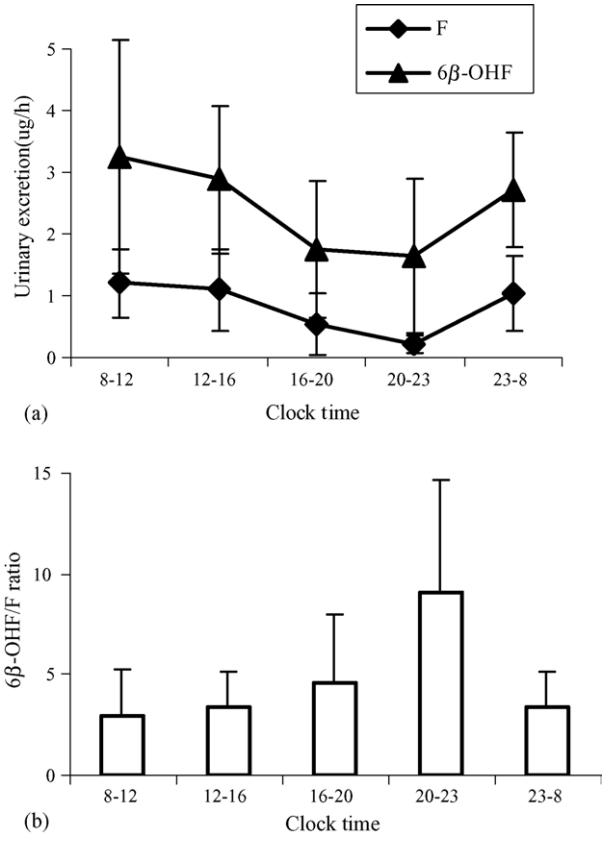


Fig. 2. Circadian variation in urinary excretion of 6β-OHF and F (a) and their ratio and 6β-OHF/F (b). The result is shown as the mean ± S.D. of 12 healthy volunteers.

## 4. Discussion

The measurement of 6β-OHF excretions has to be considered as a useful test for estimating CYP3A drug induction or inhibition in human [4]. Urinary F must be evaluated in the same patients as the ratio of 6β-OHF/F minimize the effect of circadian rhythms. Furuta et al. [5] has provided direct evidence to suggest that endogenous cortisol 6β-hydroxylation clearance is an appropriate index for the in vivo CYP3A phenotyping in humans. Determining the endogenous cortisol 6β-hydroxylation clearance requires two separate analyses of F in plasma and 6β-OHF in urine.

The HPLC method we present here is practical and permits the simultaneous determination of 6β-OHF and F in urine and plasma. Phenotyping for the CYP3A activity as well as estimating CYP3A drug induction or inhibition can be successfully performed by the present HPLC method.

In this study, a reversed-phase phenyl column was chosen to simultaneously analyze 6β-OHF and F in urine as well as F in plasma, because it produced a good chromatographic behavior. However, F was not separable from an endogenous interfering peak on the C18-bonded column. The endogenous interfering

substance is probably cortisone, as Furuta et al. [8] had found a similar interfering peak that has been confirmed as cortisone. F and cortisone share a similar chemical structure. The only difference is cortisone has an extra double bond. It is reasonable that F and cortisone can be separated better on the phenyl column than on the C18-bonded column, as phenyl phase is more selective for compounds with double bond. In order to improve the separation of F and the endogenous substance on the C18-bonded column, several gradient elution conditions were tested. However, F and the endogenous substance cannot be separated completely on the C18-bonded column while a good separation was easily accomplished on the phenyl column. F and the endogenous substance may be separable on a C18-bonded column in a certain condition. However, it seems not possbile to show a better separation than on a phenyl column considering the different properties of the two HPLC columns as disscussed above. Besides, this condition is too stirct to produce the reproducible separation results in different laboratories. As a result, referring to the measurement of 6β-OHF and F in biological fluids, a reversed-phase phenyl is recommended to produce a good chromatographic behavior.

The extraction of 6β-OHF and F in urine and plasma was based on the procedure described by Lykkesfeldt et al. [6] with minor modification. The procedure consisted of two steps; elution of the urine or plasma sample with ethyl acetate-diethyl ether (4:1, v/v) on an Oasis HLB extraction cartridge and subsequent wash of the organic extracts with alkaline and acidic solutions saturated with sodium sulfate. Lykkesfeldt et al. [6] estimated the recoveries of 6β-OHF (70.8%), F (90.6%) and dexamethasone (91.6%) as internal standard. Different extraction recoveries for the individual steroids required the corrections by the relative recoveries in the quantitation. In this study, the extraction recoveries were found to be satisfactory for F and dexamethasone. However, the extraction caused a significant loss of 6β-OHF. The low extration recovery of 6β-OHF in urine was probably caused by the relative higher water solubility of 6β-OHF compared to F and dexamethasone which could result in the significant loss of 6β-OHF in the washing steps of the sample preparation. In this study, the quantitations of 6β-OHF and F through the corrections at various concentrations were attainable by using the calibration graphs for which the standard samples including internal standard in water were extracted according to the procedure for the sample preparation. The obtained calibration graphs were linear ($r > 0.9996$) and gave good accuracy as well as within-day precision.

The chromatographic conditions of all the HPLC methods described previous [6–8] employed a mobile phase containing phosphate or/and acetate. However, phosphate and acetate were not necessary in the present study. Elution was performed with a 27 min gradient of acetonitrile 12–43% in water and it only produced negligible drift of the baseline.

The endogenous substance E cannot be confirmed as cortisone as we lack the cortisone standard. However, the present HPLC method has provide the potential possibility of determining the ratio of F and cortisone in human urine or plasma which was widely used as a reliable index for evaluating the activity of human 11β-hydroxysteroid dehydrogenase.

The 24-h urinary 6β-OHF/F ratio was $3.54 \pm 2.32$ (mean ± S.D.). The ratio presented here is similar or a little lower than previous published data ($4.8 \pm 2.6$ [7]; $5.5 \pm 1.6$ [14]; and $3.28 \pm 0.67$ [15]). The ratio of 6β-OHF/F in the period of 20:00–23:00 was significantly higher than other collection intervals ($P < 0.05$). The circadian variation in the ratio of 6β-OHF/F observed in our lab is in general agreement with that observed by Ohno et al. [16] and Barrett et al. [17]. We find out a parallel diurnal rhythm in the urinary excretion of 6β-OHF and F, with the lowest excretion rates observed in the period of 16:00–23:00 for both 6β-OHF and F, in agreement with the results obtained by Lee [18] and Barrett et al. [17].

This method is sensitive enough for accurate determination of 6β-OHF and F in human urine samples even at low levels. Although we did not apply this method to determine F in plasma samples collected at other time, the low limit of quantitation for F make this method sensitive enough for analyzing plasma samples collected at any other time considering the normal range of F in plasma. 6β-OHF cannot be detected in plasma samples as the concentration of 6β-OHF in normal human plasma is below 1 ng/ml [19].

## 5. Conclusions

The present study provides a reliable and practical technique for the simultaneous determination of 6β-hydroxycortisol and cortisol in human urine and plasma with good accuracy and precision, using dexamethasone as internal standard. The HPLC–UV method described here should be suitable for the characterization of polymorphic CYP3A phenotype expressed as 6β-hydroxylation clearance.

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