

## Diurnal Variation of 6 $\beta$ -Hydroxycortisol in Cardiac Patients

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### Summary

The 24-hour urinary excretion of 6 $\beta$ -hydroxycortisol (6 $\beta$ -OHC) and the urinary ratio of 6 $\beta$ -hydroxycortisol/cortisol (6 $\beta$ -OHC/UFC) have been proposed as noninvasive probes for human cytochrome P450 3A4 isoform (CYP3A4). In this study, we evaluated within- and between-day variability of 6 $\beta$ -OHC excretion and 6 $\beta$ -OHC/UFC ratio in nine Caucasian men with cardiac disease. Each study participant was asked to collect 24-hour urine specimens during four consecutive days in five standardized time intervals. Concentrations of UFC and 6 $\beta$ -OHC were determined by immunoassay and the high-performance liquid chromatographic (HPLC) method, respectively. The HPLC method was accurate and precise, as indicated by the recovery rate of 96.5-103.3 % and less than 5.2 % and 6.3 % of the coefficient of variation for within-run and between-run assay, respectively. In patients, diurnal variations in UFC and 6 $\beta$ -OHC excretion were parallel. Consequently, 6 $\beta$ -OHC/UFC ratio remained stable during the day. Both, 6 $\beta$ -OHC excretion and 6 $\beta$ -OHC/UFC ratio showed significant relationship between 24-hour value and values measured in corresponding collection periods with best correlations obtained from night interval (22.00-06.00,  $r = 0.86-0.91$ ). These results indicated that urinary 6 $\beta$ -OHC excretion and 6 $\beta$ -OHC/UFC ratio measured in overnight/morning urine could precisely reflect 24-hour values even in severely ill patients. In addition, a simple and sensitive HPLC method was described for determination of 6 $\beta$ -OHC in urine.

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### Key words

CYP3A4 • Cortisol • 6 $\beta$ -hydroxycortisol • Diurnal variation

### Introduction

Cytochrome CYP3A4 is the most abundant enzyme of the cytochrome P450 superfamily found in adult livers, accounting for approximately 30 % of the total hepatic cytochrome P450 (CYP450) and nearly 70 % of the CYP450 content in the intestinal mucosa (Kolars *et al.* 1992, Shimada *et al.* 1994, von Richter *et*

*al.* 2004). It appears to metabolize more than 50 % of currently marketed drugs from a spectrum of different therapeutic classes (Guengerich 1999). Clinical importance of this enzyme is therefore obvious. Numerous studies showed large interindividual variability in the expression and activity of this enzyme in humans with consequent variability in the disposition and sometimes also in the efficacy and safety of its substrates.

Two main mechanisms were described to produce a difference in CYP3A4 between humans. First, genetic factors such as inheritance of poorly active alleles and regulation by nuclear receptors are recently under more or less successful investigation (Lamba *et al.* 2002). Second, CYP3A4 is one of the enzymes which are very susceptible to induction or inhibition by endo- and xenobiotics (Guengerich 1999). As a consequence, direct assessment of actual catalytic activity using a model substrate is preferred to other methods for *in vivo* evaluation of CYP3A4 activity. In an effort to find an appropriate assay, several *in vivo* measures of CYP3A activity have been investigated, including the [ $^{14}\text{C}$  N-methyl] erythromycin breath test, the plasma clearance of midazolam, and the urinary ratio of 6 $\beta$ -hydroxycortisol to free cortisol (6 $\beta$ -OHC/UFC) (Watkins *et al.* 1989, Thummel *et al.* 1996, Kovacs *et al.* 1998). Of these probes, measurement of urinary 6 $\beta$ -OHC and free cortisol appears to be very attractive since it does not involve the administration of probe drugs and is truly noninvasive.

6 $\beta$ -hydroxycortisol, a hydrophilic metabolite formed *via* hydroxylation by CYP3A4, is the major unconjugated urinary product of cortisol accounting for approximately 1 % of the total daily cortisol secretion (Burststein *et al.* 1967). There appears to be a significant positive correlation between the urinary level of 6 $\beta$ -OHC, and both liver microsomal cortisol 6 $\beta$ -hydroxylase activity and CYP3A4 liver content (Ged *et al.* 1989). As a consequence, urinary 6 $\beta$ -hydroxycortisol excretion has been shown to be a relatively sensitive marker of CYP3A4 genetic polymorphism, induction or inhibition (Galteau and Shamsa 2003, Wang *et al.* 2005). Diurnal variation in cortisol production must be corrected either by 24-hour urine collection or by expressing results as a 6 $\beta$ -OHC/UFC ratio (Saenger 1983, Ohnhaus *et al.* 1989). However, the 24-hour urinary sampling is difficult to collect qualitatively and quantitatively. It is therefore commonly preferred to use a single-spot morning urine sample to measure both compounds and calculate 6 $\beta$ -OHC/UFC ratio. Nevertheless, most data come from studies performed in healthy volunteers. In contrast, the availability of a detailed analysis describing circadian variability in excretion of 6 $\beta$ -OHC and UFC in the group of severely ill patients is still limited.

The concentration of 6 $\beta$ -OHC in urine can be determined by various immunochemical techniques such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) (Park 1978, Zhiri *et al.* 1986). Although these methods may be highly sensitive,

they require radioactive compounds and are not entirely selective for 6 $\beta$ -OHC. Therefore, several highly sensitive gas or liquid chromatography-mass spectrometry (GC-MS, LC-MS) methods were also developed (Galteau and Shamsa 2003, Barrett *et al.* 2005). However, required equipment is not always available. Therefore, high-performance liquid chromatography methods (HPLC) may be preferred in clinical settings. Several simple, sensitive and reproducible HPLC techniques have been described (Inoue *et al.* 1994, Lee 1995). Nevertheless, only limited data are present concerning measurement of 6 $\beta$ -OHC in hospitalized patients treated concomitantly with several drugs.

The aim of the present project was to evaluate diurnal variation in urinary 6 $\beta$ -OHC excretion and urinary 6 $\beta$ -OHC/UFC ratio in nine cardiac patients receiving their regular medication to obtain detailed information about relationships between 24-hour values and data from corresponding collection periods. In addition, a sensitive and reproducible HPLC method was intended to be established for the determination of 6 $\beta$ -OHC in patients receiving several agents simultaneously.

## Methods

### *Reagents and solutions*

6 $\beta$ -hydroxycortisol (6 $\beta$ -OHC) and 6 $\beta$ -hydroxycortisone (6 $\beta$ -OHE) were purchased from Sigma Co. (St. Louis, MO, USA). The RIA immunoassay test kit for urinary cortisol (UFC) was obtained from Immunotech (Prague, Cat. no. 1841). All other chemicals and solvents were of the highest analytical-reagent grade commercially available. Sep-Pak C18 column was purchased from Waters Assoc. (Milford, Mass., USA).

### *Urine sample*

The protocol for the study was approved by the Human Ethics Committee of the Charles University Teaching Hospital in Hradec Králové and conducted in accordance with the Declaration of Helsinki. All subjects participating in the study gave a signed, written informed consent. The specimen collection was conducted at the Department of Internal Medicine in Hradec Králové under physician's care. Nine cardiac patients (only men) included in the previous study (Mičuda *et al.* 2001) were enrolled. Their age ranged from 41 to 75 years (median age, 62 years). Exclusion criteria included history of hepatic dysfunction and congestive heart failure. Twenty-four-hour urine specimens were collected during four

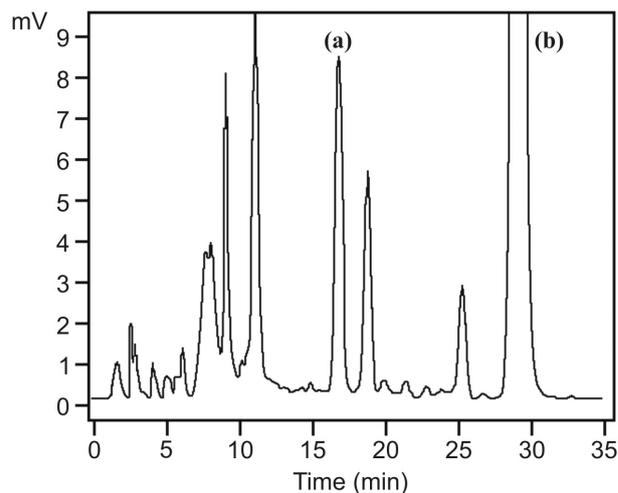
consecutive days. To study diurnal rhythm, 4-h urine specimens were collected from 06.00-10.00, 10.00-14.00, 14.00-18.00, 18.00-22.00, and 8-h specimens from 22.00-6.00. For each collection period, the total volume and pH of urine were recorded and two 20-ml aliquots were retained and frozen without preservatives at  $-20^{\circ}\text{C}$  until assayed.

#### Extraction procedure

The mixture of 4 ml of urine sample and 1 ml of internal standard (I.S., 6 $\beta$ -OHE, conc. 5  $\mu\text{g}/\text{ml}$ ) was loaded onto a preconditioned (5 ml of methanol, 5 ml of water) Sep-Pak C18 cartridge. The Sep-Pak was washed with 10 ml of water and, following removal of the aqueous phase using an air stream (2 min), the steroids were eluted with 5 ml of ethylacetate. The organic extract was washed with 2 ml of 1.0 mol/l NaOH in 20 %  $\text{Na}_2\text{SO}_4$  followed by 2 ml of 1.0 % acetic acid in 20 %  $\text{Na}_2\text{SO}_4$ . The aqueous layer was frozen (acetone bath,  $-25^{\circ}\text{C}$ ), the organic layer was transferred into another glass tube and concentrated to dryness at  $37^{\circ}\text{C}$  under a nitrogen stream. The residue was redissolved in 50  $\mu\text{l}$  of 75 % methanol and vortexed after which 200  $\mu\text{l}$  of water was added. After subsequent additional vortex-mixing, 50  $\mu\text{l}$  was used for HPLC analysis. Peak areas of 6 $\beta$ -OHC and an internal standard were measured.

#### Chromatographic analysis

Chromatography was performed with Shimadzu equipment (Kyoto, Japan) consisting of the following units: SIL-10A autosampler, LC-10AS pump, SIL-10A injector, SPD-10A detector operated at 244 nm. All units were connected to a personal computer for control as well as for collection and analysis of data (Apex Integrator version 3.1, DataApex Ltd.). The column was a Nova-Pak C18 (particle size 4  $\mu\text{m}$ , pore size 60  $\text{\AA}$ , 150 x 3.9 mm I.D., Waters) operated at 1.0 ml/min and temperature at  $30^{\circ}\text{C}$ . The stepwise gradient was used: 0-32 min 100 % of mobile phase A (MPA), 33-40 min 100 % of mobile phase B (MPB) and 41-52 min 100 % of MPA. Retention times of the steroids were approximately 16.23 min (6 $\beta$ -OHC) and 27.94 min (internal standard) in the present system. Composition of MPA was 10 mmol/l  $\text{KH}_2\text{PO}_4$  at pH 3.5 in water – methanol – acetonitril (87:9:4; v/v/v). The MPB was the same mixture but in a different volume ratio (50:34.5:15.5; v/v/v). Mobile phases were filtrated through a 0.45  $\mu\text{m}$  filter under vacuum and degassed prior to use. Concentrations of 6 $\beta$ -OHC were calculated from peak areas of the internal



**Fig. 1.** Typical chromatogram of a urine sample. Peaks: 6 $\beta$ -hydroxycortisol (a), and 6 $\beta$ -hydroxycortisone (b, I.S.). Displayed without blank subtraction.

standard and 6 $\beta$ -OHC as described previously (Lykkesfeldt *et al.* 1994).

#### Urinary cortisol assay

An assay procedure was followed, as suggested by the manufacturer. The intra-assay variation was 2.8-5.8 % and the inter-assay variation was 5.4-9.2 %.

#### Data analysis

Data are presented as mean  $\pm$  SD. In cardiac patients, the rate of urinary excretion of UFC and 6 $\beta$ -OHC (nmol/h) was determined in each urine specimen. Consequently, the 6 $\beta$ -OHC/UFC ratios were computed. To determine the stability of 6 $\beta$ -OHC/UFC ratio during the 24-hour period, data were submitted to analysis of variance (ANOVA). Simple linear regression analysis was conducted to correlate the urinary 6 $\beta$ -OHC/UFC ratios or 6 $\beta$ -OHC excretions measured in particular collecting interval with the cumulative 24-hour values obtained on the corresponding day. All statistical analyses were performed using GraphPad InStat 3.0 software (Graphpad Software, Inc., San Diego, CA, USA).

## Results

Figure 1 shows a typical chromatogram of urine sample with I.S. obtained from cardiac patient on appropriate drug therapy. The coefficient of variation (CV) of the retention times of 6 $\beta$ -OHC and I.S. was less

**Table 1.** Recovery of 6 $\beta$ -hydroxycortisol added to human urine.

Added (pmol/ml)	Found (pmol/ml)	Recovered (pmol/ml)	Recovery (%)
<i>None</i>	223	–	–
158.5	375.9	152.9	96.5
396.3	632.3	409.3	103.3
792.6	1014.0	791	99.8

**Table 2.** The diurnal rhythm of urinary 6 $\beta$ -OHC and UFC excretion in nine cardiac patients.

Collection period	6 $\beta$ -OHC		UFC		6 $\beta$ -OHC/UFC	
	(nmol/h)	CV (%)	(nmol/h)	CV (%)		CV (%)
06.00-10.00	10.97 $\pm$ 7.76	70.8	43.84 $\pm$ 31.81	72.6	4.53 $\pm$ 1.80	39.7
10.00-14.00	8.25 $\pm$ 5.68	68.8	36.27 $\pm$ 23.60	65.1	4.90 $\pm$ 1.76	36.0
14.00-18.00	6.32 $\pm$ 4.58	72.5	26.01 $\pm$ 18.13	69.7	4.80 $\pm$ 2.34	48.8
18.00-22.00	4.14 $\pm$ 3.57	86.3	18.16 $\pm$ 12.14	66.9	5.22 $\pm$ 1.97	37.7
22.00-06.00	5.50 $\pm$ 5.12	93.1	19.69 $\pm$ 11.03	56.0	4.75 $\pm$ 2.51	52.8

Data are mean  $\pm$  SD.

**Table 3.** Relationships between 24-hour values (urinary excretion of 6 $\beta$ -OHC or 6 $\beta$ -OHC/UFC ratio) and data measured in designed time intervals.

Time intervals	6 $\beta$ -OHC excretion		6 $\beta$ -OHC/UFC ratio	
	r	p	r	p
6.00-10.00	0.71	< 0.0001	0.87	< 0.0001
10.00-14.00	0.79	< 0.0001	0.81	< 0.0001
14.00-18.00	0.57	< 0.0001	0.74	< 0.0001
18.00-22.00	0.81	< 0.0001	0.91	< 0.0001
22.00-6.00	0.86	< 0.0001	0.91	< 0.0001

r - correlation coefficient.

than 0.91 % (n = 35) within series of runs and less than 1.14 % (n = 5) between series of runs. The retention times for 6 $\beta$ -OHC and I.S. in the present system were within the intervals 15.95-16.69 min (n = 80) and 27.46-28.83 min (n = 80), respectively. Linear calibration graph for 6 $\beta$ -OHC was obtained by plotting the ratio of peak area of 6 $\beta$ -OHC to that of the internal standard against the amount of 6 $\beta$ -OHC added to urine over the range of 200 - 8000 pmol/ml. The correlation coefficient of the calibration curve (r) was 0.999, and the Y-intercept did not vary significantly from zero. To check the recovery, urine samples were spiked with three different amounts of 6 $\beta$ -OHC. The results are shown in Table 1.

The recovery of 6 $\beta$ -OHC was almost complete, and was independent of the amount added. The lower limit of detection for 6 $\beta$ -OHC was 3.5 pmol/ml (mean of five determinations), at a signal-noise ratio of 3. The reproducibility of the method was assessed by repeat analysis of urine samples containing two different concentrations of 6 $\beta$ -OHC. The within-day CV was below 5.2 %, while the between-day CV was found to be below 6.3 %.

#### *Urinary excretion of 6 $\beta$ -OHC and UFC in nine cardiac patients*

The excretion of 6 $\beta$ -OHC and UFC showed a

strong diurnal rhythm with a wide interindividual variability as indicated by variation coefficients (Table 2). The variations in urinary concentrations of both compounds were relatively parallel to each other. Therefore, changes in the urinary ratio of 6 $\beta$ -OHC/UFC (Table 2) were not statistically significant during the day. The peak excretion of urinary 6 $\beta$ -OHC and UFC was detected in the morning interval (06.00-10.00), while trough was in the evening (18.00-22.00) (Table 2). The relationships between the 24-hour value and the excretion of 6 $\beta$ -OHC or 6 $\beta$ -OHC/UFC ratio in respective collecting periods are summarized in Table 3. Both the 24-hour urinary 6 $\beta$ -OHC excretions and the 24-hour urinary 6 $\beta$ -OHC/UFC ratios displayed best correlation with the corresponding values measured in the whole night/morning urine (22.00-06.00) samples. Less prominent relationship was found, when the 24-hour ratios or 6 $\beta$ -OHC excretions were compared with the ratios or excretions measured in other intervals.

## Discussion

The method described herein is a modification of the HPLC method developed by Lykkesfeldt *et al.* (1994). The method showed excellent accuracy, as indicated by the recovery study with the yield of recovery comparable to previously reported values (Ono *et al.* 1986, Lykkesfeldt *et al.* 1994). The method is also precise, as shown by coefficient of variations (CV) of the within-run and between-run assays. Lykkesfeldt *et al.* (1994) reported an equally fine reproducibility of their method. Their within- and between-day CVs were both in the range of 5-10 %. Reliability of our method was confirmed during analyses of urine specimens from nine cardiac patients. No peak interference was observed despite the broad spectrum of agents that had been used by patients (acetylsalicylic acid, amilorid, captopril, carvedilol, enalapril, hydrochlorothiazide, isosorbide dinitrate, metoprolol, molsidomine, perindopril, piracetam, simvastatin, ticlopidine). These results imply that the present method is not subject to interference by coexisting substances and is suitable for the determination of 6 $\beta$ -OHC in clinical settings.

Cortisol is well known to be excreted according to a circadian rhythm. Saenger (1983) was the first to demonstrate that 6 $\beta$ -OHC excretion paralleled cortisol excretion and was the highest between 08.00 hours and 12.00 hours and the lowest around midnight. He demonstrated in non-treated controls, as well as in

phenobarbital-treated patients, that the excretions of 6 $\beta$ -OHC and cortisol varied throughout the day, reflecting the changes in adrenal secretion. However, there was no significant diurnal variation when the 6 $\beta$ -OHC/cortisol ratio was taken into account. Since this first report, several authors have obtained similar results (Zhiri *et al.* 1986, Joellenbeck *et al.* 1992, Lee 1995) with 6 $\beta$ -OHC/cortisol ratios being relatively stable around a 24-hour period. As a consequence, several reports described good correlation between morning and 24-hour 6 $\beta$ -OHC/cortisol ratios (Ono *et al.* 1986, Nakamura and Yakata 1989, Bienvenu *et al.* 1991, Tran *et al.* 1999). Present results are in agreement with these observations. First, we found stable 6 $\beta$ -OHC/cortisol ratios during the four consecutive days with five collection periods per day. Our values of 6 $\beta$ -OHC excretion and 6 $\beta$ -OHC/cortisol ratios measured in cardiac patients were similar to those reported for healthy volunteers (Lee 1995, Zhiri *et al.* 1986, Kovacs *et al.* 1998, Ono *et al.* 1986). Second, comparison between 24-hour value and urinary measurements in designed time intervals revealed strong correlation especially with those obtained from night collection period. It could therefore be possible to use a single-spot morning urine sample to measure both 6-OHC and cortisol and to calculate the ratio 6-OHC/cortisol even in cardiac patients. Nevertheless, several reports about significant variability of 6 $\beta$ -OHC/cortisol ratio during the day also exists (Nakamura *et al.* 1997, McCune *et al.* 1998, Ohno *et al.* 2000). Ohno *et al.* (2000) have demonstrated that diurnal variations of 6 $\beta$ -OHC and of cortisol were not really parallel, and, therefore, the ratio varied from 4.3 between 09.00 hours and 13.00 hours to 12.6 between 18.00 hours and 22.00 hours. Moreover, as for hospitalized patients, cortisol secretion and metabolism could be affected by genetic and environmental factors, disease states, simultaneous drug administration etc. (Galteau and Shamsa 2003). Therefore, all these possibilities should be taken in account when evaluating CYP3A4 using endogenous cortisol as a model substrate.

In conclusion, our findings demonstrate that measurement of urinary 6 $\beta$ -OHC excretion and 6 $\beta$ -OHC/UFC ratio in overnight specimen could provide reliable information about 24-hour values even during selected disease state. This study also showed an importance of the sensitive HPLC method for determination of 6 $\beta$ -OHC to exclude possibility of an interference with simultaneously applied drugs.

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