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Automated on-line SPE LC–MS/MS method to quantitate 6beta-hydroxycortisol and cortisol in human urine: Use of the 6beta-hydroxycortisol to cortisol ratio as an indicator of CYP3A4 activity

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Abstract

A sensitive method for quantitation of urinary 6beta-hydroxycortisol (6beta-HC) and cortisol using on-line SPE and LC–MS/MS was developed and validated. Human urine samples were injected directly onto an on-line solid phase extraction apparatus, Prospekt-2, followed by HPLC separation and electrospray triple quadrupole LC–MS/MS detection. The inter-day precision for the 6beta-HC:cortisol ratio was 7–9%. The lower limit of quantitation was 1 and 0.2 ng/mL for 6beta-HC and cortisol, respectively. Using the method we observed a diurnal variation on the 6beta-HC:cortisol ratio in healthy volunteers with the maximal ratio observed in the 2–10 pm urine collection period.

Keywords: 6beta-Hydroxycortisol; Cortisol; LC–MS/MS; CYP3A4; Human urine

1. Introduction

CYP3A4 is known to be responsible for the metabolism of numerous drugs [1]. Among several tests for CYP3A4 activity, including the erythromycin breath test, midazolam clearance, the ratio of endogenous 6beta-hydroxycortisol (6beta-HC) to cortisol (6beta-HC:cortisol) in human urine is currently considered the only true noninvasive and endogenous indicator of CYP3A4 activity [2,3]. Studies of the correlation between the erythromycin test or the midazolam clearance and the 6beta-HC:cortisol ratio have given mixed results [4–7], suggesting that the erythromycin test, the midazolam clearance test and the 6beta-HC:cortisol ratio test may reflect the activity of different isoforms of hepatic CYP3A4 or CYP3A. Although the ideal CYP3A activity probe is not yet identified, in drug development, 6beta-HC:cortisol can be used to indicate whether a drug is a CYP3A4 inducer or inhibitor. In general, an increase in 6beta-HC:cortisol indicates that the drug may be a potential CYP3A4 inducer and a decrease in the ratio indicates that the drug may be a CYP3A4 inhibitor.

Several methods for determinations of 6beta-HC:cortisol in human urine have been published based on enzyme linked immunosorbent assay (ELISA) [8], Radioimmunoassay (RIA) [9], high performance liquid chromatography (HPLC) [10–17] and liquid chromatography-tandem mass spectrometry (LC–MS/MS) [18]. Immunochemical results have been shown to overestimate both 6beta-HC and cortisol concentrations due to antibody cross-reactivity with other structurally similar steroids [8]. HPLC methods with colorimetric and fluorometric detection have been shown to give falsely high cortisol and or 6beta-HC values caused by interfering substances with similar retention times as those of cortisol and/or 6beta-HC [17]. Ohno et al. addressed most of these problems with their atmospheric pressure chemical ionization (APCI) LC–MS/MS method [18]. However, the method employed a labor intensive manual liquid/liquid

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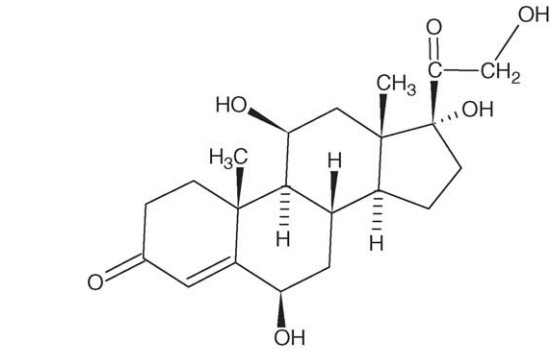
extraction procedure, which required 1.0 mL of specimen and the lower limit of quantitation (LLQ) of the assay was quite high (20 ng/mL for 6beta-HC and 4.0 ng/mL for cortisol).

In order to improve the assay sensitivity and automate the extraction procedure, a method using an on-line SPE system for sample extraction and quantitative LC–MS/MS was developed and validated.

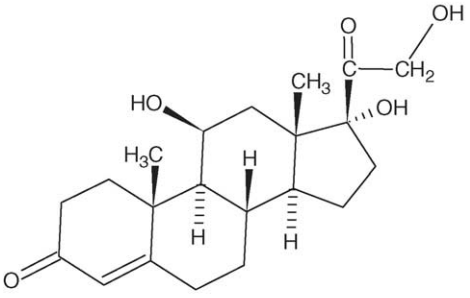
2. Experimental

2.1. Chemicals

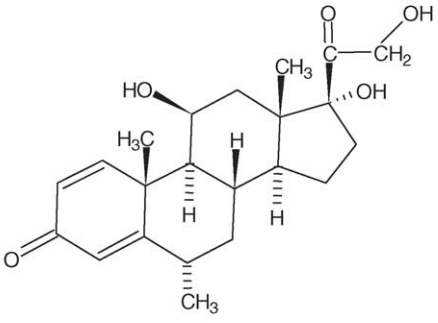
6beta-Hydroxycortisol, FW 378.5, cortisol (hydrocortisone), FW 362.5, 6α-methylprednisolone, the internal standard (IS), FW 374.5 (Fig. 1), boric acid were from Sigma, St. Louis, MO; formic acid, ammonium hydroxide (NH_4OH), were from EM Science, Gibbstown, NJ; *o*-phosphoric acid was from Fisher Chemical, Fair Lawn, NJ; phosphate buffered saline tablets from Sigma Chemical Co., St. Louis, MO. To prepare the assay buffer, one tablet of phosphate buffer saline (PBS) was dissolved in 200 ml of HPLC grade water to yield 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4 at 25 °C. 6beta-HC and cortisol and IS were dissolved in 100% methanol to prepare 1.00 mg/mL initial stock standards.



6beta-Hydroxycortisol



Cortisol



IS (6 alpha-Methylprednisolone)

Fig. 1. Chemical structure of 6beta-hydroxycortisol, cortisol and IS.

2.2. Calibration curve and sample preparation

Calibration standard solutions were prepared by diluting solutions of 6beta-HC and cortisol in 50/50 (v/v) of methanol/water with assay buffer (0.01 M phosphate buffer, pH 6). The final ratio of methanol to assay buffer was less than 5:95 (v/v). The range of the calibration standard solutions was 1.00–500 ng/mL and 0.200–100 ng/mL for 6beta-HC and cortisol, respectively.

Three levels of validation samples were prepared using a human urine pool obtained from apparently healthy volunteers. The pooled urine (endogenous concentration) was used to create the LOW sample. The MID and HIGH samples were prepared by spiking the pooled urine with 6beta-HC and cortisol at concentrations of 50 and 10 ng/mL (MID), respectively, and 400 and 80 ng/mL (HIGH), respectively.

One hundred microlitres of study samples was pipetted using a Tecan Genesis RSP 100 into a 96-well assay block containing 100 μL of assay buffer in each well (0.01 M phosphate buffer, pH 6), followed by addition of 100 μL assay buffer and 25 μL of IS. The assay block was sealed and vortexed to ensure proper mixing of solutions. Samples were then injected into the SPE LC–MS/MS system where the analytes were purified by an on-line SPE, separated on an HPLC and detected by MS/MS.

2.3. Chromatographic conditions

The on-line SPE system consisted of a Prospect-2^{TM} on-line solid phase extraction apparatus (Spark Holland Inc., Plainsboro, NJ) using HySphere C18, HD 7 μm SPE cartridges (Spark, Holland Inc.). The SPE cartridges were solvated by methanol and equilibrated by water prior to sample application. The cartridges were washed using 2% NH_4OH in water then 10% methanol in water. The analytes were eluted with HPLC pump gradient for 3 min at flow rate of 0.3 mL/min.

The HPLC system consisted of two Shimadzu LC-$10AD_{VP}$ mobile phase delivery pumps (Piscataway, NJ), an on-line degasser and a Perkin Elmer Series 200 autosampler (Shelton, CT). The HPLC analytical column was a Symmetry Shield RP_{18} (2.1 × 100 mm, 3.5 μm) from Waters Corporation (Morristown, NJ). HPLC separation was achieved by applying linear gradients with 0–50% B for 1.5 min, followed by 50–80% B for 4.5 min, then 100% B for 1.5 min. 100% A

was applied for 1.5 min for column equilibration. ($A = 0.1\%$ formic acid in water, $B = 0.1\%$ formic acid in methanol.) The HPLC column temperature was set at 60 °C. The flow rate was set at 0.3 mL/min. The autosampler injection volume was set at 10 μL.

2.4. LC–MS/MS conditions

Mass spectrometry was carried out using a SCIEX API 3000 triple quadrupole from Applied Biosystems MDS/SCIEX (Foster City, CA) equipped with a Turbo Ion-Spray as LC–MS interface. The turbo spray temperature was maintained at 500 °C and the turbo gas flow was set at 6 L/min with nebulizing gas (N_2), curtain gas (N_2) and collision gas (N_2) set at flow rates of 1.46, 1.25 and 1.25 L/min, respectively. The ion spray voltage was set at −3000 V. The declustering and focusing potentials were set at −33 and −160 V, respectively, for 6eta-HC; −29 and −135 V, respectively, for cortisol; and −30 and −130 V, respectively for the IS. The entrance potential and collision energy were set at 10 and −25 V, respectively for all analytes. The electrospray ionization spectra were acquired in negative ion mode with multiple reaction monitoring. The mass transitions were: 6beta-HC, m/z 423.5 → 347.5; cortisol, m/z 407.5 → 331.5; and 6α-methylprednisolone (IS), m/z 419.5 → 343.5 The data were acquired with a dwell time of 250 ms for all analytes. The calibration curves were fitted to a $1/x^2$ quadratic regression using Analyst software version 1.1 (SCIEX).

2.5. Validation parameters

The method LLQ was evaluated by repeat measurements of six independently prepared samples in buffer at concentrations of 1.0 and 0.2 ng/mL of 6beta-HC and cortisol, respectively.

Samples for accuracy (analytical recovery) were prepared by spiking 18, 38 and 56 ng/mL of 6beta-HC and cortisol each into urine specimens from four apparently healthy volunteers to yield 12 samples. The endogenous concentrations of 6beta-HC and cortisol were also measured. The expected concentration was calculated as endogenous + spiked concentrations and the percent recovery = 100 × found concentration/expected concentration.

The intra-assay precision was assessed by six repeated measurements of spot urine specimens from six apparently healthy volunteers in a single analytical run. The inter-assay precision was assessed by assaying the three validation samples (LOW, MID and HIGH) in duplicates in six analytical runs over 6 days.

Matrix parellelism of 6beta-HC and cortisol were assessed by diluting a urine specimen from an apparently healthy volunteer 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128 (v/v) with assay buffer prior to analysis.

Stability of 6beta-HC and cortisol in human urine during storage in the presence or absence of boric acid as well as after repeated freezing and thawing was assessed using spot urine specimens from 6 to 12 apparently healthy volunteers. For assessing the effects of boric acid, samples were collected and then immediately spiked with final concentrations of 0, 5, 10 and 20 mg of boric acid per mL urine. Urine aliquots were then stored at room temperature (25 °C), 4 °C and −70 °C. The 4 °C and 25 °C samples were stored for 8 and 24 h, then frozen at −70 °C until assayed. All samples were assayed in one analytical run.

The potential ion suppression by boric acid was evaluated by spiking boric acid at concentrations of 0, 5, 10 and 20 mg/mL freshly collected spot urine from three apparently healthy volunteers. All samples were assayed immediately in one analytical run.

2.6. Circadian variation

Assessment of circadian variation in the urinary excretion of 6beta-HC, cortisol, and in the 6beta-HC:cortisol ratio was conducted in study of five apparently healthy volunteers (three males and two females). The specimens were collected at three different time intervals during a 24-h period: 0600–1400 h (morning), 1400–2200 h (afternoon) and 2200–0600 h (night). The specimens were stored on ice packs during collection without preservatives. Additional spot urine specimens were collected from another twenty apparently healthy volunteers (10 males aged 25–45 and 10 females aged 28–46) at 0700–1100 h (morning) and 1500–1800 h (afternoon) on the same day. Urinary creatinine was measured in all samples using a modified Jaffe' method [19]. In this method, creatinine forms a color complex in the presence of picric acid, which is measured photometrically using a Roche P module autoanalyzer (Roche Diagnostics, Indianapolis, IN).

2.7. Reference intervals

Reference intervals for 24 h urinary excretion of 6beta-HC, cortisol and the 6beta-HC:cortisol ratio were established in 42 healthy males and 4 females participating in two clinical trials by obtaining 24-h pre-dose urine samples. Both clinical trials were approved by the local Institutional Review Board (IRB) and written consents were obtained from all volunteers before they entered into clinical trials. The health status was determined by medical history, physical examination, and vital signs. Subjects' ages ranged from 19 to 46 years.

3. Results and discussion

3.1. Validation of the method

3.1.1. LLQ

6beta-HC and cortisol were measured from 51 healthy volunteers and the minimum and maximum concentrations of 6beta-HC and cortisol were 17–300 and 2–56 ng/mL, respectively. We estimated that a 10-fold reduction in the urinary concentration of 6beta-HC or cortisol would be sufficient to

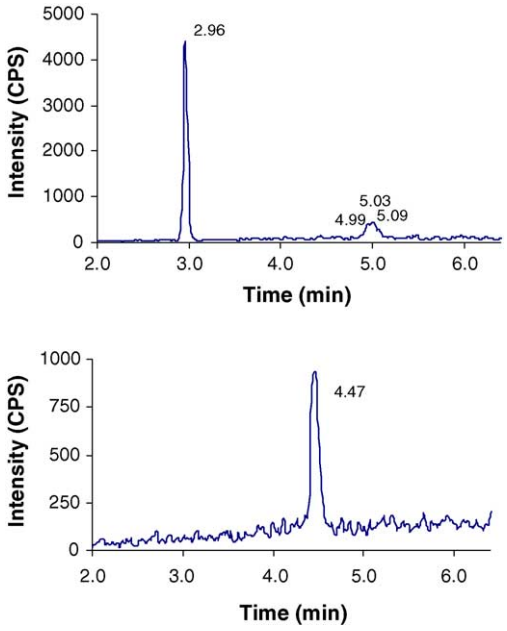


Fig. 2. Mass chromatograms of 6beta-HC and cortisol at LLQ (6beta-HC (1.0 ng/mL top), cortisol (0.2 ng/mL bottom).

cover pharmacodynamic effects on CYP3A4 activity, and we therefore evaluated a method LLQ of 1 ng/mL for 6beta-HC and 0.2 ng/mL for cortisol. At these concentrations, deviations from nominal values and assay imprecision were all below 10%. A typical MRM chromatogram at the LLQ concentration is shown in Fig. 2. The signal-to-noise ratios for 6beta-HC and cortisol were 42:1 and 10:1, respectively, suggesting that the actual LLQ values for both analytes are lower than those being claimed.

3.1.2. Calibration curve

As 6beta-HC and cortisol are endogenous in nature, construction of the calibration curve was evaluated in both PBS buffer (no cortisol) and rat urine (low in cortisol). Similar signals were observed between the rat urine calibration curve points and the buffer calibration curve points when 6beta-HC and cortisol concentrations were greater than 1 ng/mL (results not shown). Below 0.8 ng/mL, the cortisol signal continued to decrease linearly with concentration in buffer matrix, while the signal decrease flatted out in rat urine matrix, due to the presence of low but existing endogenous cortisol. As a sensitivity of 1 ng/mL cortisol may not be sufficient to measure cortisol reduction in some of the healthy donors, also the rat urine matrix is difficult to procure and the lot to lot consistency is difficult to maintain, we focused our assay development on using calibration curve prepared in buffer.

A 10-point calibration curve ranging from 1 to 500 ng/mL for 6β-HC and from 0.2 to 100 ng/mL for cortisol was evaluated in six different analytical runs on 6 different days. The calibration curves were constructed by plotting the analyte/IS peak area ratio (y) against analyte concentrations (x) and the curves were fitted using quadratic regression models, weighted by $1/x$ in analyst® software. The mean equations were $y = -1.13e-07x^2 + 0.0013x + 0.00019$ for 6beta-HC ($R = 0.9996$) and $y = -1.13\,ex^2 + 0.00256x + 0.000195$ for cortisol ($R = 0.9992$). The calibration curve read-back accuracy, defined as %measured/nominal, was 92–104% for 6β-HC and 97–102% for cortisol, while the imprecision of the individual standards ranged from 3 to 7% CV for 6β-HC and 2 to 9% CV for cortisol.

3.1.3. Clinical reportable ranges

Clinical reportable range is defined as the range of analyte that as method can report as a quantitative result, allowing for sample dilution, concentration, or other pretreatment used to extend the direct analytical measurement range [20]. The %change of 6beta-HC and cortisol at 1/2–1/128 dilution of a volunteer's urine sample compared with neat ranged from 3–13% and 4–17%, respectively. The clinical reportable range is therefore extended to 1.0–64,000 ng/mL for 6β-HC and 0.2–12,800 ng/mL for cortisol. Results of the assay accuracy (analytical recovery) and inter- and intra-assay precision evaluation are presented in Tables 1 and 2, respectively. In summary, the analytical recovery ranged from 100–120% (mean 108%) for 6beta-HC, and 97–126% (mean 108%) for cortisol. The intra-assay coefficients of variation were 2–4%, 2–3%, and 2–6% for 6beta-HC, cortisol, and the 6beta-HC:cortisol ratio, respectively, while the inter-assay coefficients of variation were 7–10%, 2–7%, and 7–9% for 6beta-HC, cortisol, and the 6beta-HC:cortisol ratio, respectively.

3.1.4. Freeze–thaw stability

Clinical specimens from six apparently healthy volunteers were frozen at −70 °C and then thawed at room temperature for 1, 2 and 3 times before they were compared with fresh samples (never frozen). The average changes from the 6 donor samples were 5 and −6% (6beta-HC and cortisol, respectively) following one freeze–thaw cycle, 12 and −1% ((6beta-HC and cortisol, respectively) following two freeze–thaw cycles and 12 and −1% (6beta-HC and cortisol, respectively) following three freeze–thaw cycle. An ANOVA based mixed model statistical analysis was performed using the SAS software v.9.1 to test the null hypothesis between freeze–thaw cycles of 0, 1, 2 and 3. A p-value of 0.746 was obtained, suggesting that no statistically significant changes were observed during three cycles of repeated freezing and thawing.

3.1.5. Short-term storage stability

Boric acid is recommended to be used as a preservative for urinary cortisol analysis in clinical practice to reduce bacterial action for samples not analyzed within 2 h of collection [21]. To ensure that the boric acid did not cause ion suppression in LC–MS to a degree that would affect the measured MS signal intensity (analyte/IS), different amounts of boric acid were compared with that without boric acid added. The mean %change of 6beta-HC signal intensity from 3 volunteer samples was 2, 2 and 4%, respectively, at boric

Table 1
Analytical recovery of 6beta-HC and cortisol from human urine

$n = 2$	Measured (ng/mL)	Donor 1% recovery	Measured (ng/mL)	Donor 2% recovery	Measured (ng/mL)	Donor 3% recovery	Measured (ng/mL)	Donor 4% recovery
6beta-HC analytical recovery								
Endogenous	139		42		159		274	
Endo + 18	158	101	69	114	213	120	314	108
Endo + 38	186	105	90	113	225	114	326	104
Endo + 56	195	100	106	108	237	110	343	104
Grand mean (%)	108							
Cortisol analytical recovery								
Endogenous	9		5		8		20	
Endo + 18	28	104	25	107	33	126	38	101
Endo + 38	50	106	44	104	56	124	55	97
Endo + 56	69	105	62	100	78	121	76	100
Grand mean (%)	108							

acid concentrations of 5, 10 and 20 mg/mL, while the mean %change of cortisol signal intensity was 6, 3 and 3%, respectively, suggesting boric acid has no significant impact in analyte MS signal.

For 6beta-HC and cortisol stability, without preservatives, urine samples stored at 4 °C for up to 24 h showed a mean change of −0.4% (individual change ranged from −11 to 12%) and 3% (individual change ranged from −5 to 14%) in the concentration of 6beta-HC and cortisol, respectively. No samples showed a concentration decrease of greater than 20%. After storing samples at 25 °C for 24 h, 3 of the 12 samples for 6beta-HC and 2 of 12 samples for cortisol showed a decrease in the concentration of greater than 20% from baseline (fresh sample), suggesting that degradation may be occurring. After storing urine specimens at 25 °C for 24 h in the presence of boric acid (5–20 mg/mL), only one out of 12 samples showed a change greater than 20% and the mean changes were less than 7% for both 6beta-HC and cortisol. We therefore recommend that boric acid be used when samples are to be stored at room temperature for longer than 8 h.

3.1.6. Long-term storage stability

When 6beta-HC and cortisol were measured at baseline (fresh sample), and after 2, 4, 16, 26 and 40 weeks, the mean change was 18, 4, −4, 6 and 7%, respectively, for 6beta-HC and −9, −6, −9, −5 and −7%, respectively, for cortisol. This suggests that both 6beta-HC and cortisol are relatively stable in human urine for up to 40 weeks (∼9 months) when stored below −70 °C.

3.2. Applications

3.2.1. Reference Intervals

The reference intervals, defined as central 95% interval bounded by the 2.5 and 97.5% percentile, were determined using a non-parametric method and based on a sample

Table 2
Intra- and inter-day assay variation of 6beta-HC, cortisol and the ratio

	Intra-day variation						Inter-day variation		
	Donor						Sample		
	1	2	3	4	5	6	1	2	3
6beta-HC (ng/mL)									
Mean	118	147	122	48.3	292	305	7.70	59.78	402
S.D.	3.66	4.59	3.94	0.925	5.16	10.7	0.517	4.20	38.5
%CV	3.10	3.12	3.24	1.92	1.77	3.53	6.72	7.02	9.58
n	6	6	6	6	6	6	12	12	12
Cortisol (ng/mL)									
Mean	26.9	9.37	10.3	5.54	12.9	18.6	0.464	10.1	75.4
S.D.	0.836	0.320	0.207	0.155	0.352	0.637	0.029	0.183	5.27
%CV	3.10	3.41	2.00	2.81	2.73	3.42	6.15	1.82	6.99
n	6	6	6	6	6	6	12	12	12
6beta–HC:cortisol ratio									
Mean	4.38	15.7	11.8	8.73	22.6	16.4	16.7	5.94	5.33
S.D.	0.087	0.931	0.511	0.237	0.721	0.664	1.48	0.409	0.352
%CV	1.99	5.91	4.34	2.72	3.19	4.05	8.88	6.89	6.60
n	6	6	6	6	6	6	12	12	12

Table 3
AM and PM urinary excretion of 6beta-HC, cortisol and the ratio

		6beta-HC/creatinine (μg/g)	Cortisol/creatinine (μg/g)	6beta-HC:cortisol ratio
AM (700–1100 h)	Mean	373	57.7	9.5
	S.D.	153	50.0	6.7
	Min	175	11.4	2.1
	Max	682	224	30.9
	N	20	20	20
PM (1500–1800 h)	Mean	175	14.1	13.4
	S.D.	84	7.0	4.3
	Min	6.3	0.4	6.6
	Max	378	28.3	24.3
	N	20	20	20

population of 46 healthy subjects. The reference intervals were 32.2–328 μg/24 h for 6beta-HC, 5.5–38.8 μg/24 h for cortisol and 3.1–13.5 for the ratio. The ratio reference range observed in our laboratory is in good agreement with Ohno et.al. (3–12.4) [18] determined by APCI LC–MS/MS in 30 Japanese subjects. Other groups reported reference ranges that seem to be somewhat different from our range, e.g. Lykkesfeldt et al. reported the ratio reference range of 2.7–26.9 in 11 subjects [14] and Lee reported a ratio reference range of 2.48 ± 0.94 (mean ± S.D.) determined in 7 subjects [15]. Both studies used HPLC to quantitate 6beta-HC and cortisol. The differences were possibly biased by the small numbers of subjects used in these studies and/or the use of the HPLC technology, which is known to be less specific than LC–MS/MS.

3.2.2. Circadian effect

The ratio of 6beta-HC:cortisol appeared to have a circadian variation with the highest value observed in the afternoon (1400–2200 h) for all five subjects (mean = 19.7) comparing to morning (mean = 12.0) and night (mean = 12.6), although both urinary cortisol and 6beta-HC excretion, expressed per mg creatinine, was lowest in the afternoon (Fig. 3). The ratio of urinary 6beta-HC:cortisol in the morning and the night sample was 59% (range: 38–85%) and 65% (range: 41–82%), respectively, of that in the afternoon sample. The twenty apparently healthy volunteer spot urine samples collected during 700–1100 h or 1500–1800 h, confirmed that the urinary excretion of both 6beta-HC and cortisol were higher in the morning but the ratio of 6beta-HC:cortisol was higher in the afternoon (Table 3).

The circadian variation in the 6beta-HC:cortisol ratio observed in our lab is in general agreement with that observed by Ohno et al, however, the Ohno et al. [18] showed that the urinary excretion of 6beta-HC was lowest at night (2100–900 h), while we observed it to be lowest in the afternoon. Lee [15] reported a parallel diurnal rhythm in the urinary excretion of 6beta-HC and cortisol by HPLC, with the lowest excretion rates observed in the afternoon for both cortisol and 6beta-HC, in agreement with our observation. However, Lee reported the ratio to be fairly constant throughout the day, which differs from our observations.

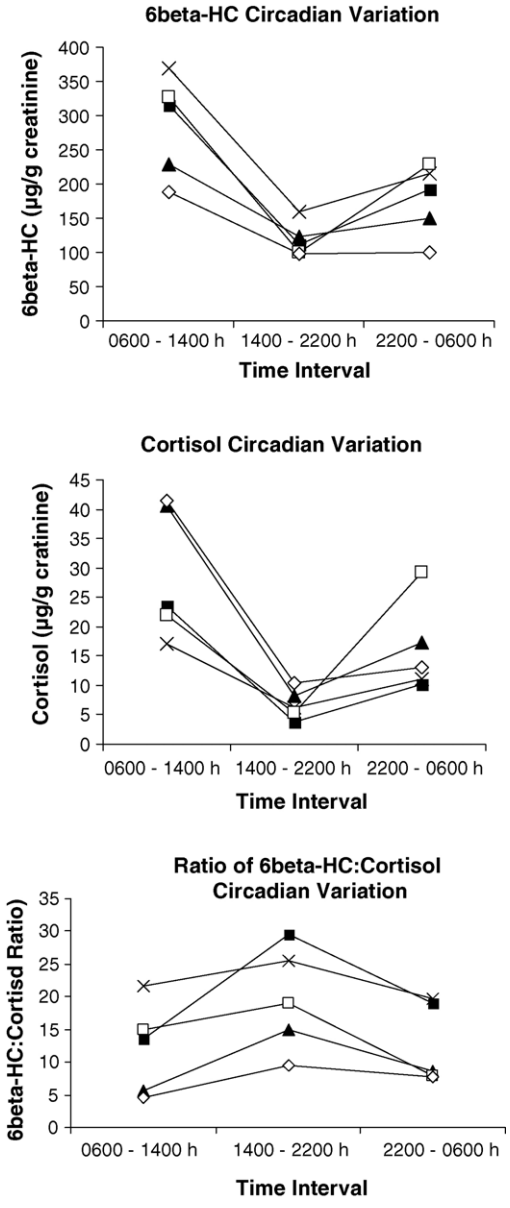


Fig. 3. Circadian variation of excreted 6beta-HC, cortisol and the ratio.

4. Conclusion

A sensitive, reproducible and fully automated on-line SPE LC–MS/MS method for quantitation of 6beta-HC and cortisol in human urine was developed. This method is able to determine the ratio of urinary 6beta-HC:cortisol and shows that the ratio, as a CYP3A4 indicator, is able to cover the entire dynamic range with adequate sensitivity and reproducibility. In addition, the method is fully automated with only minor operator intervention. The preservative, boric acid, may not be needed when urine specimens are collected and stored at 4 °C for up to 24 h. However, it is recommended that boric acid is added whenever urine specimens are to be stored at room temperature for longer than 8 h. A circadian variation in 6beta-HC:cortisol ratio was observed, with a higher ratio in the 1400–2200 h period compared to the 0600–1400 h and 2200–0600 h periods, suggesting the need to design studies in such a way that urine collections before and after a therapeutic intervention are matched to the time of the day. A 24-h urine collection would be optimal to avoid circadian variation although it may be difficult to collect in practice. The circadian effect of 6beta-HC to cortisol ratio may also limit its application for investigating the diurnal effect of the CYP3A4 activity [6,7]. The large inter subject variation of 6beta-HC:cortisol ratio (3.1 to 13.5) suggests that the individual should be used as his/her own control when evaluating the 3A4 induction or inhibition, which can also eliminate the cortisol metabolism variation caused by patients' other pathological states [5].

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