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Specific determination of urinary 6β-hydroxycortisol and cortisol by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

The urinary 6β-hydroxycortisol to cortisol ratio is believed to be a noninvasive index of cytochrome P450 3A activity. For precise assessment of the ratio in human urine, we have developed a reversed-phase high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry method. The selective method was accurate and reproducible with intra- and inter-day precision of variation coefficients of less than 8%. The 6β-hydroxycortisol to cortisol ratio ranged from 3.0 to 12.4 in healthy Japanese 24-h urine. With the recent popularization of LC–MS, our LC–MS method will be advantageous to detect human in vivo CYP3A activity for clinical investigation and routine measurement in various laboratories. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

The activity of cytochrome P450 3A (CYP3A) exhibits at least a 10-fold inter-individual variability [1,2] and is greatly changeable intra-individually due to enzyme induction and inhibition by xenobiotics including medicines [3]. Since CYP3A is responsible for numerous drugs metabolism [4] and many drug interactions, it is of great interest to have a screening method for human in vivo CYP3A activity for clinical studies. Among several tests for CYP3A activity including the erythromycin breath test, the ratio of endogenous 6β-hydroxycortisol to cortisol (6β-OHC/C) in human urine is considered the only true noninvasive indicator of CYP3A activity [5,6]. The ratio is regarded as reflecting mainly hepatic CYP3A activity, but not intestinal. In early-phase drug development the information from this indicator is useful to identify some drugs as CYP3A inducers or inhibitors. In daily drug therapy, such information could be used to exclude subjects who were in particular danger of developing side-effects.

The measurement of 6β-hydroxycortisol and cortisol in urine is based on enzyme-linked immunosorbent assay (ELISA) [7], radioimmunoassay (RIA) [8], and high-performance liquid chromatography (HPLC) [9–16]. Due to cross-reaction with other related steroids, the use of immunochemical methods

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often results in overestimation of 6β-hydroxycortisol and cortisol. Impurities often interfere in the peaks of analytes in colorimetric and fluorometric HPLC methods, producing falsely elevated values. Removal of various interferences from these steroids using HPLC was extremely difficult under the differing condition of individuals.

Thus, we tried to use liquid chromatography–mass spectrometry (LC–MS) to develop a simple, selective and precise method for simultaneous determination of 6β-hydroxycortisol and cortisol in human urine. LC–MS can give extremely reliable information of a compound by detecting both a molecular ion and a retention time different from methods used previously. Since LC–MS systems have become remarkably popular and widespread in the last few years, LC–MS is considered to be the most suitable and practical method for the specific and sensitive analysis of very small quantities of analytes in biological fluid.

2. Experimental

2.1. Reagents

6β-Hydroxycortisol (6β,11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione) was obtained from Steraloids (Wilton, NH, USA). Hydrocortisone (cortisol; 11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione) was obtained from Wako (Osaka, Japan). 6α-Methylprednisolone (6α-methyl-11β,17α-trihydroxy-1,4-pregnadiene-3,20-dione) and 6α-hydroxycortisol (6α,11β,17α,21-tetrahydroxy-pregn-4-ene-3,20-dione) were purchased from Sigma (St. Louis, MO, USA). All other solvents and chemicals were of the highest grade commercially obtainable and purchased from Nakarai Tesque (Kyoto, Japan). Stock solutions of 6β-hydroxycortisol, cortisol and 6α-methylprednisolone were prepared in absolute ethanol and stored at −20°C. For working solutions, stock solutions were diluted with distilled water.

2.2. Sample preparation

A 1.0-ml urine specimen with 6α-methylprednisolone [internal standard (I.S.), 240 ng/ml] was loaded onto an Oasis HLB cartridge (60 mg, 3 ml, Waters, Milford, MA, USA) preconditioned with methanol and water. Following rinsing with 5 ml of water, the analytes were eluted with 2.0 ml of ethyl acetate followed by 1.0 ml of ethyl ether. The eluate was evaporated to dryness at 37°C under reduced pressure. The residue was dissolved in 100 μl of water and 20 μl of ethanol, and filtered through a membrane filter (0.45 μm pore size). An aliquot (60–100 μl) of the filtrate was injected into the LC–MS system.

2.3. LC–atmospheric pressure chemical ionization (APCI)-MS conditions

LC–MS analysis was carried out using a Hitachi M-1000H connected to a Hitachi L-6200 intelligent pump with an L-4000 UV detector at 245 nm (Hitachi, Tokyo, Japan). Reversed-phase HPLC was performed on a Nova-Pak C_{18} column (4 μm, 300 mm×3.9 mm I.D., Waters) with a Sentry™ Guard column Nova-Pak C_{18} (20 mm×3.9 mm I.D., Waters). Mobile phase A consisted of 0.06% trifluoroacetic acid in ammonium acetate buffer (0.01 *M*, pH 4.8)–acetonitrile (90:10, v/v), while mobile phase B consisted of the same buffer–acetonitrile (30:70, v/v). Chromatography was achieved at ambient temperature with a flow-rate of 1 ml/min by the following gradient profile: 0–50% B (linear) for 20 min followed by 50–100% B (linear) for 10 min. An APCI interface was used in positive ionization mode for quantitative mass spectrometric detection. APCI-MS conditions were as follows: nebulizer and vaporizer temperatures at 300°C and 400°C, respectively, and drift voltage at 40 V. The selected ion monitoring (SIM) method was used for the detection of 6β-hydroxycortisol, cortisol and 6α-methylprednisolone (I.S.) with $[M+H]^+$ at (m/z) 379–380, 363–364 and 375–376, respectively.

2.4. Subjects and study design

To evaluate the validity of the first morning spot urine for extensive assessment of the urinary 6β-OHC/C ratio, 30 healthy Japanese (25 male; aged 20–45 years and five female; aged 22–40 years) collected the first morning spot urine and the following 24 h urine (24-h urine). In addition, six subjects (all male; aged 20–26 years) collected urine three

times during 2 weeks according to the same procedure above. The samples were stored without preservatives at −20°C until analysis.

All subjects had a normal biochemical profile including liver enzymes (aspartate aminotransferase; AST, alanine aminotransferase; ALT), kidney function (blood urea nitrogen; BUN, creatinine) and electrolytes. They received no drugs for at least 2 weeks prior to and during the study. The study procedure was carefully explained, especially the importance of urine collection, and they gave their consent.

Statistical analysis employed linear regression, Pearson's correlation coefficient, Fisher's exact test and a two-way analysis of variance (ANOVA) depending on the purpose. Statistical significance was defined at a P value of less than 0.05.

3. Results

Fig. 1 shows the mass spectra of 6β-hydroxycortisol, cortisol and 6α-methylprednisolone (I.S.) obtained under the recommended analytical conditions. The protonated molecular ions, $[M+H]^+$, of all compounds were observed as base peaks at m/z 379, 363 and 375, respectively. A typical UV and SIM chromatogram obtained from human urine is presented in Fig. 2. Under the conditions established for the LC–APCI-MS analysis, analytes were readily separated; the retention times of these steroids were 10.8 min (6β-hydroxycortisol), 19.5 min (cortisol) and 21.6 min (6α-methylprednisolone). 6β-Hydroxycortisol and its stereoisomer, 6α-hydroxycortisol, were completely separated from each other on the HPLC column.

All calibration curves exhibited excellent linearity, which was confirmed by plotting the ratio (y) of the analyte to the I.S. peak areas versus concentration (x) of 6β-hydroxycortisol and cortisol (Table 1). The extraction recoveries were 79.3%, 99.2% and 106.6% for 6β-hydroxycortisol (200 ng/ml), cortisol (40 ng/ml) and 6α-methylprednisolone (240 ng/ml), respectively. The intra- and inter-day precision of the method was evaluated by analyzing a control sample, which contained 120 ng/ml of 6β-hydroxycortisol and 24 ng/ml of cortisol. The samples were injected five times on the same day, and daily for 5 days,

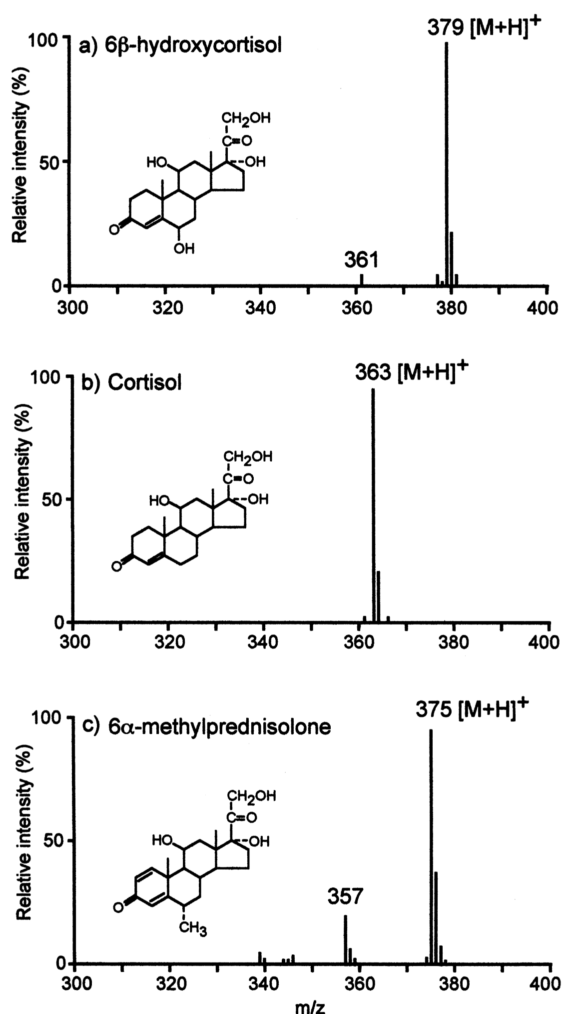

Fig. 1. APCI-mass spectra (positive ion mode) of (a) 6β-hydroxycortisol, (b) cortisol and (c) 6α-methylprednisolone and their structures.

respectively (Table 2). The findings showed good reproducibility with an overall precision of less than 7.8%. It was mandatory to control the nebulizer temperature very carefully to produce intense, stable ions for mass spectrometric monitoring of the steroids. The quantitative limits for 6β-hydroxycortisol and cortisol, at a signal-to-noise ratio of 5, were less than 50 and 10 pmol on-column, respectively.

This LC–APCI-MS method was successfully applied to determine urinary levels of 6β-hydroxycortisol and cortisol. The reference range of the urinary 6β-OHC/C ratio in 30 Japanese is summa-

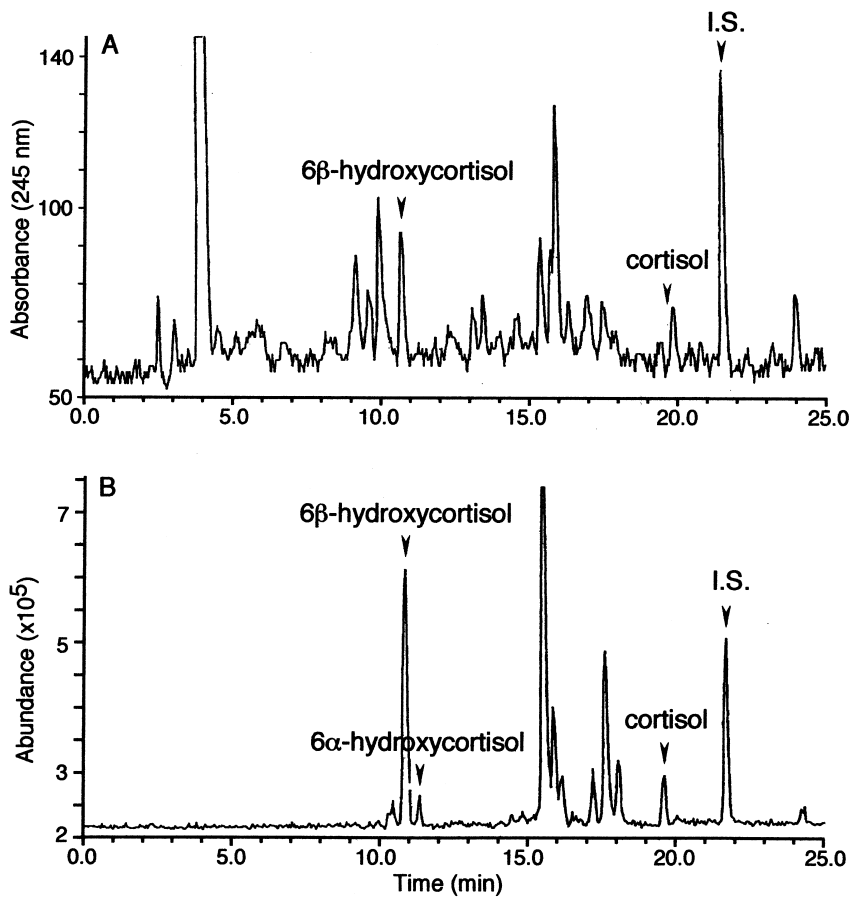

Fig. 2. Typical UV chromatogram (A) and SIM chromatogram for *m*/*z* 379–380, 363–364 and 375–376 (B) of human urine.

Table 1
Calibration data for each steroid over the linear range (n=3)

Compound	Range (ng/ml)	*r*	Intercept	Slope
6β-Hydroxycortisol	20–1600	0.991	0.019	0.002
Cortisol	4–400	0.992	−0.064	0.036

rized in Table 3. The ratio was varied from 3 to 12.4 (6.2±2.2) for 24-h urine collection and from 1.8 to 20.3 (6.8±4.6) for the morning spot urine. Inter-individual differences in the ratios among the subjects was 4.1-fold in 24-h urine and 11.3-fold in the

Table 2
Intra- and inter-day precision of 6β-hydroxycortisol, cortisol and their ratio

	Intra-day assay (n=5)		Inter-day assay (n=5)	
	Found (ng/ml)	RSD (%)[a]	Found (ng/ml)	RSD (%)
6β-Hydroxycortisol	118.5±4.97	4.2	122.1±3.96	3.2
Cortisol	24.3±1.64	6.7	24.8±1.93	7.8
6β-OHC/C[b]	4.9±0.30	6.2	4.9±0.27	5.5

[a] RSD (%) represents the relative standard deviation for the concentration found.

[b] 6β-OHC/C, the ratio of 6β-hydroxycortisol to cortisol, was calculated from the value of 6β-hydroxycortisol and cortisol in each assay. Each value represents the mean±SD.

Table 3
Reference range of urinary 6β-hydroxycortisol to cortisol ratio in Japanese subjects[a]

Sex	Age (years)		First morning spot urine	24-h urine collection
Male	20–45	n=25	6.9±4.9	6.3±2.4
Female	22–40	n=5	6.3±2.8	6.2±1.5
Total	20–45	n=30	6.8±4.6	6.2±2.2

[a] 6β-OHC/C; the ratio of urinary 6β-hydroxycortisol to cortisol. Each ratio represents the mean±SD.

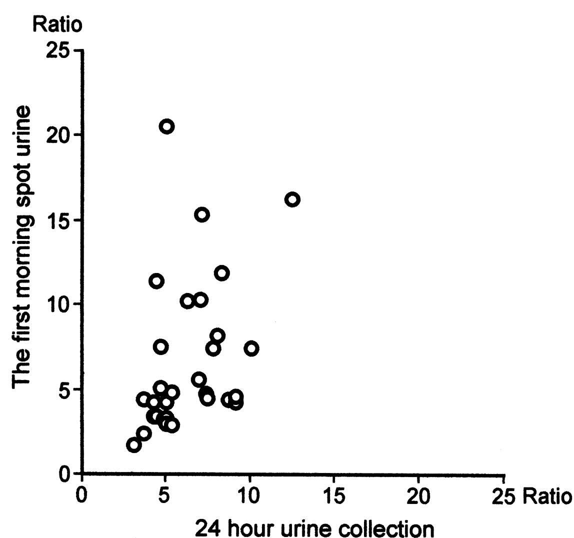

Fig. 3. Relation between the first morning spot urine and 24 h urine collection in the 6β-hydroxycortisol to cortisol ratio for 30 healthy Japanese subjects. r^2=0.156.

Table 4
Inter-day variation of the urinary 6β-hydroxycortisol to cortisol ratio

Subject	First morning spot urine (n=3)		24 h urine collection (n=3)	
	6β-OHC/C[a]	RSD (%)[b]	6β-OHC/C[a]	RSD (%)[b]
1	5.7±0.9	16.2	4.1±0.4	10.2
2	5.2±2.4	45.3	6.0±0.7	11.0
3	4.5±1.3	28.4	7.2±0.3	3.7
4	4.2±2.2	52.7	3.7±0.5	12.4
5	8.8±5.7	64.4	4.9±0.2	4.7
6	6.0±2.4	38.9	7.4±0.4	4.8

[a] 6β-OHC/C; the ratio of urinary 6β-hydroxycortisol to cortisol. Each value represents the mean±SD.
[b] RSD (%); relative standard deviation.

morning spot urine (Fig. 3). There was no significant difference between male and female. The relation between the morning spot urine and 24-h urine of the urinary 6β-OHC/C ratio was not so good as shown in Fig. 3 (r^2=0.156, n=30). The repeated urine collections from six subjects revealed that the 6β-OHC/C ratio of the first morning spot urine exhibited significant inter-day variation ($P<0.05$), although 24-h urine did not (Table 4). Intra-subject relative standard deviation (RSD) for urinary 6β-OHC/C ratio was 7.8±3.8% in 24-h urine, while it was 41.0±17.2% in the first morning spot urine. Significant inter-subject differences in the ratio were observed in 24-h urine ($P<0.001$).

4. Discussion

The proposed LC–APCI-MS method is very efficient for the simultaneous determination of urinary 6β-hydroxycortisol and cortisol. This simple and rapid method does not need laborious derivatization like the GC–MS and fluorometric HPLC methods [15,16]. In contrast with UV detection, this method can achieve the perfect and certain detection of analytes with extremely high intensity in a short time. Urinary excretion of 6α-hydroxycortisol is estimated to be usually less than 5–10% of the total 6-hydroxycortisol in healthy subjects and to be increased greatly under metabolic disorders [17]. Our LC–APCI-MS conditions allowed us specific quantification of both 6α- and 6β-hydroxycortisol. Thus, no concern is necessary about frequent overestimation of 6β-hydroxycortisol and cortisol in human urine using the selective quantitative analysis proposed. This reliable and practical LC–APCI-MS method is clearly advantageous for the assessment of the urinary 6β-OHC/C ratio in clinical situations.

The LC–APCI-MS conditions were optimized to

obtain high intensity of cortisol whose urinary concentration was usually lower than 6β-hydroxycortisol. The sensitivity of this method is satisfactory to assess the 6β-OHC/C ratio in only 1 ml of urine, because usual levels of 6β-hydroxycortisol and cortisol in human urine are greater than 20 ng/ml and 5 ng/ml, respectively. The choice of the Oasis™ HLB cartridge for solid-phase extraction resulted in very stable recovery and reproducibility. Two-step elution from the solid-phase cartridge improved selective extraction of 6β-hydroxycortisol, cortisol and 6α-methylprednisolone (I.S.).

A major problem in assessing the 6β-OHC/C ratio has been the occasional overestimation of urinary 6β-hydroxycortisol and/or cortisol. Selective analysis of those steroids in human urine was very difficult using immunochemical and HPLC methods. Interfering peaks of analytes often appeared on HPLC chromatograms due to the effects of various foods, medicines and physiological conditions. Therefore, it is important to develop a specific and practical analytical procedure for 6β-hydroxycortisol and cortisol analysis before assessing the 6β-OHC/C ratio of patients under various conditions. The superiority of the present method was confirmed by the successful analysis of urine samples which had previously been unable to determine the concentrations of 6β-hydroxycortisol and/or cortisol by HPLC, ELISA and RIA. With this LC–APCI-MS method, 6β-hydroxycortisol and cortisol were clearly separated from other substances and their concentrations selectively determined (data not shown).

The findings of the present study indicated that the individual 6β-OHC/C ratio in the first morning spot urine was highly variable from day to day. Consequently, the validity of the first morning spot urine for extensive assessment of the 6β-OHC/C ratio is doubtful although it is convenient to collect. However, the 6β-OHC/C ratio in 24-h urine showed significant inter-subject differences, but no inter-day variation during the 2-week period of the present study. Therefore, the ratio in 24-h urine should represent the intrinsic value of each subject. In healthy Japanese, the 6β-OHC/C ratio of 24-h urine exhibited a four-fold inter-individual variability as shown in Fig. 3, which might reflect inter-individual variability of hepatic CYP3A activity. Taking all things into consideration, the 24-h urinary 6β-OHC/C ratio would be appropriate for clinical use on various occasions.

In the present study, no correlation was found between the first morning spot urine and 24-h urine as reported by Bienvenu et al. [18] (r=0.899). A reason for the discrepancy is that the large day-to-day variation of the 6β-OHC/C ratio in the first morning spot urine affects its relation to the ratio in 24-h urine. A detailed questionnaire of our volunteers suggested that the difference of variation might be caused by life habits of individuals including sleeping time, and urine sampling intervals. There might be a circadian change in the 6β-OHC/C ratio of healthy subjects considering the enormous intra-day variation in the ratio in a dexamethasone loading study [19], although several reports described no intra-day variation of the ratio [6,20].

In conclusion, this study introduced the specific, sensitive and rapid determination of urinary 6β-hydroxycortisol and cortisol using an LC–APCI-MS method. The present method confirmed its remarkable reliability and usefulness for clinical study. The obvious advantage of the present method is the easy reproducibility in other laboratories for the precise assessment of the urinary 6β-OHC/C ratio: a noninvasive index of hepatic CYP3A activity. Owing to its high selectivity, the method is applicable to various conditions, such as multi-drug therapy, pregnancy and metabolic disorders, where the urinary 6β-OHC/C ratio is difficult to evaluate. Increasing awareness of the evaluation of human CYP3A activity, this reliable method will be a valuable strategy not only in early-phase drug development, but also in drug therapy after marketing.

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