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

Takashi Furuta*, Chieko Mori, Atsushi Suzuki, Hiromi Shibasaki, Akitomo Yokokawa, Yasuji Kasuya

Department of Medicinal Chemistry and Clinical Pharmacy, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan

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Abstract

This study describes a high-performance liquid chromatographic (HPLC) method for the simultaneous determination of 6β-hydroxycortisol (6β-OHF) and cortisol in human urine using either methylprednisolone or beclomethasone as internal standard. Separation was achieved on a reversed-phase phenyl column by a gradient elution of 0.05 M KH_2PO_4–0.01 M CH_3COOH (pH 3.77) and 0.05 M KH_2PO_4–0.01 M CH_3COOH with acetonitrile (4:6, v/v). 6β-Hydroxycortisol and cortisol were monitored by UV absorption at 239 nm. The lower quantitation limits of the present HPLC method were 21.5 ng/ml for 6β-OHF and 5.0 ng/ml for cortisol in urine. The within-day reproducibilities in the amounts of 6β-OHF and cortisol determined were in good agreement with the actual amounts added, the relative error being less than 1.59%. The inter-assay precisions (R.S.D. values) were less than 7.91% for 6β-OHF and cortisol. The method was compared with the GC/MS method by measuring 6β-OHF in the same urine samples. A good correlation was found between the amounts determined by the two methods. The regression equations for the HPLC (y) and GC/MS (x) methods were: $y = 1.0701x + 17.389$ ($r = 0.9772$) for methylprednisolone as internal standard and $y = 1.0827x + 6.1364$ ($r = 0.9794$) for beclomethasone as internal standard.

Keywords: Phenotyping; Cortisol; 6β-Hydroxycortisol; CYP3A activity

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) has been extensively used as a useful non-invasive index for evaluating the in vivo CYP3A activity induced by many xenobiotics including drugs [3–8]. Recently, we have demonstrated that the urinary ratio 6β-OHF/F does not always reflect the in vivo CYP3A activity, because the ratio is varied with the cortisol renal clearance [9,10]. It is also suggested that 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 [9,10]. Calculation of 6β-hydroxylation clearance requires two separate analyses of cortisol in plasma and 6β-OHF in urine.

The measurements of cortisol in biological fluids have been extensively investigated and well established by using selective chromatographic techniques such as high-performance liquid chromatography (HPLC) [11], gas chromatography-mass spectrometry (GC/MS) [12–14], liquid chromatography-mass spectrometry (LC/MS) [11,15,16], and liquid chromatography-tandem mass spectrometry (LC/MS/MS) [17,18]. The concentration of 6β-OHF in urine is usually based on the immunochemical [19–22] and HPLC techniques [23–30]. We have recently devel-

* Corresponding author. Tel.: +81-426-76-5803; fax: +81-426-76-8969.
E-mail address: furutat@ps.toyaku.ac.jp (T. Furuta).

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oped a reliable capillary GC/MS method for determining 6β-OHF in urine by using stable isotope-labelled analogue as internal standard [31,32]. Methods based on the LC/MS technique have been also proposed by other investigators [33,34]. Although the selectivity and sensitivity offered by GC/MS and LC/MS should allow for the accurate determination of 6β-OHF in urine, the use of these techniques is generally limited for the routine analysis due mainly to the instrumental availability. The immunochemical and HPLC techniques may be appropriate for the routine analysis in many clinical laboratories.

The immunochemical techniques such as radioimmunoassay (RIA) [19–21] and enzyme-linked immunosorbent assay [22] are highly sensitive, but they lack selectivity for 6β-OHF due to cross-reactivity of the antibodies, resulting in higher concentrations of 6β-OHF [35–37]. The HPLC method generally offers higher selectivity in measuring urinary 6β-OHF [23–30], but the results are not often reproducible in quantitation of 6β-OHF in urine, because of polar interferences and low recoveries of 6β-OHF.

In this study, an HPLC method was designed to be used for the characterization of the polymorphic CYP3A phenotype. Its performance reflected an improvement in the chromatographic resolution and the analyte extraction procedure based on the HPLC method reported by Lykkesfeldt et al. [27]. The HPLC-UV method described here provided good accuracy and precision to measure 6β-OHF in urine, and was then compared with our GC/MS method [31]. Simultaneous measurement of cortisol with 6β-OHF was also examined by using HPLC, which can be used for study of evaluating the cortisol renal clearance affecting the conventional index, urinary ratio 6β-OHF to cortisol.

2. Experimental

2.1. Chemicals and reagents

6β-Hydroxycortisol and 6α-hydroxycortisol were purchased from Steraloids (Wilton, NH, USA). 6β-Hydroxytestosterone was commercially obtained from Ultrafine (Manchester, England). Cortisol, cortisone, 6β-hydroxycortisone, dexamethasone, methylprednisolone, and beclomethasone were purchased from Sigma (St. Louis, MO, USA). Acetonitrile for the HPLC mobile phase was of liquid chromatographic grade obtained commercially (Kanto Chemicals, Tokyo, Japan). 6β-Hydroxy-[1,1,19,19,19-$^2H_5$]cortisol was synthesized in our laboratory [38]. All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

2.2. Preparation of standards

Stock solutions of 6β-hydroxycortisol (chemical purity >99%; 5.370 ng/μl), cortisol (>99%; 1.005 ng/μl), cortisone (99%; 1.021 ng/μl), dexamethasone (>98%; 1.017 ng/μl), methylprednisolone (96%; 1.025 ng/μl), beclomethasone (>99%; 1.009 ng/μl), and 6β-hydroxytestosterone (>99%; 0.964 ng/μl) were prepared in methanol. The chemical purities were determined by HPLC.

2.3. High-performance liquid chromatography (HPLC)

2.3.1. HPLC conditions

A Shimadzu LC-VP high-performance liquid chromatograph with an SPD-M10A photodiode array detector was used (Shimadzu, Kyoto, Japan). The separations were performed on a Synergi 4 μ Polar-RP 80A (250 mm × 4.6 mm i.d.) (Phenomenex, CA, USA), monitored by UV absorbance at 239 nm and operated at 0.8 ml/min using the following gradient of solvent mixtures (A) 0.05 M KH_2PO_4–0.01 M CH_3COOH (pH 3.77) and (B) 0.05 M KH_2PO_4–0.01 M CH_3COOH:acetonitrile (4:6, v/v). The stepwise gradient elution program was: % solvent A/solvent B; 73/27 at 0–6 min, 50/50 at 8 min, 47/53 at 17 min, 42/58 at 23 min, and 20/80 at 30–40 min.

2.3.2. Sample preparation for HPLC

To 1.0 ml of human urine was added a solution of methylprednisolone (205.0 ng) and beclomethasone (201.8 ng) dissolved in distilled water (2.0 ml), as internal standards. The urine samples were applied to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 10 ml of distilled water and then eluted with a solution of ethyl acetate-diethyl ether (4:1 (v/v), 5 ml). To the organic extract was added 2.0 ml of 1.0 M NaOH saturated with Na_2SO_4, and votex-mixed for 0.5 min. After centrifuging, 2.0 ml of 1.0% acetic acid saturated with $Na_2SO_4$was added to the organic extract, and votex-mixed for 0.5 min. The organic solvent was evaporated to dryness at 60 °C under a stream of nitrogen. To the residue was added 0.05 M KH_2PO_4–0.01 M CH_3COOH (100 μl) and acetonitrile (20 μl). A 50-μl portion of the solution was subjected to HPLC. Peak areas of 6β-OHF, cortisol, methylprednisolone, and beclomethasone were measured.

2.4. Recovery

To each of five standards containing known amounts of 6β-OHF (21.48, 107.4, 268.5, 537.0, and 805.5 ng) and cortisol (5.00, 20.10, 50.25, 100.5, and 201.0 ng) dissolved in water (1 ml) was added a solution of methylprednisolone (205.0 ng), beclomethasone (201.8 ng), 6β-hydroxytestosterone (192.8 ng), and dexamethasone (203.4 ng) in distilled water (2.0 ml). 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.

2.5. Calibration graphs

To each of six standards containing known amounts of 6β-OHF (21.48, 53.70, 107.4, 268.5, 537.0 and 805.5 ng)

and cortisol (5.00, 10.05, 20.10, 50.25, 100.5 and 201.0 ng) dissolved in methanol, 205.0 ng of methylprednisolone and 201.8 ng of beclomethasone as internal standards were added. Each sample was prepared in triplicate. After evaporation of the solvent to dryness, the samples were dissolved in 3.0 ml of distilled water. The sample was worked up according to the urine sample purification for HPLC. To the extract thus obtained was added 0.05 M KH_2PO_4–0.01 M CH_3COOH (100 μl) and acetonitrile (20 μl). A 50-μl portion of the solution was subjected to HPLC. Peak areas of 6β-OHF, cortisol, methylprednisolone, and beclomethasone were measured. The calibration graphs were obtained by an unweighed least-squares linear fitting of the peak-area ratios versus the concentrations of 6β-OHF and cortisol on each analysis of the standard mixtures.

2.6. Accuracy and reproducibility

Two sets of urine samples were prepared for determining the accuracy and precision using 1.0-ml aliquots of pooled urine containing endogenous 6β-OHF (309.3 ± 7.72 ng/ml) and cortisol (33.01 ± 2.61 ng/ml). To one set of the urine sample ($n = 6$) were added known amounts of 6β-OHF (53.70 ng) and cortisol (20.10 ng), and to another set ($n = 6$) were not. Methylprednisolone (205.0 ng) and beclomethasone (201.8 ng) were used as internal standards. After preparation of the sample for HPLC as described above, the peak-area ratios (6β-OHF/methylprednisolone or beclomethasone and cortisol/methylprednisolone or beclomethasone) were measured.

2.7. Administration of the macrolide antibiotic clarithromycin in a healthy subject for comparison of HPLC and GC/MS methods for measuring urinary 6β-hydroxycortisol

Clarithromycin (200 mg) was administered to a healthy volunteer (aged 25, male, 80 kg in weight) every 12 h at 10:00 and 22:00 for 5 days and finally at 10:00 on day 6. The subject was not receiving any other medication. The study was approved by the Tokyo University of Pharmacy and Life Science Human Subjects Review Board and written informed consent was obtained from the subject. Urine samples were obtained at a timed period of 2 h (0–2, 2–4, 4–6, 6–8, 8–10, and 10–12 h) on days 0, 1, 2, 3, 4, 5, 6, 13, and 68. The volume and pH of the urine samples were noted. The sample was stored at −20 °C. Urinary concentrations of 6β-hydroxycortisol (6β-OHF) on days 0, 4, and 13 were determined by the present HPLC and our GC/MS methods [31].

3. Results

3.1. Chromatography

Separation of 6β-OHF and cortisol was examined by using two types of reversed-phase columns by gradient elution with 0.05 M KH_2PO_4–0.01 M CH_3COOH (pH 3.77) and 0.05 M KH_2PO_4–0.01 M CH_3COOH with acetonitrile (4:6, v/v). Typical chromatograms of the two analytes after processing from urine are shown in Fig. 1. A single peak of 6β-OHF was obtained at the retention time of 12.66 min on an ether-linked phenyl phase column (Fig. 1A) and at 12.11 min on a octadecyl (C_{18}) bonded column (Fig. 1B), respectively. A single peak of cortisol (F) was also obtained at 23.18 min on the phenyl phase column (Fig. 1A). However, cortisol (F; t_R 18.92 min) was not completely separable from cortisone (E; t_R 19.50 min) on the C_{18} bonded column (Fig. 1B). 6β-Hydroxytestosterone (6β-OHT; t_R 22.31 min), methylprednisolone (MP; t_R 27.45 min), dexamethasone (DM; t_R 28.80 min), and beclomethasone (BM; t_R 31.77 min) as potential candidates for internal standard gave good peaks on the phenyl phase column (Fig. 2A). However, interfering peaks were observed in some urine samples at t_R 22.31 min for 6β-hydroxytestosterone (6β-OHT)

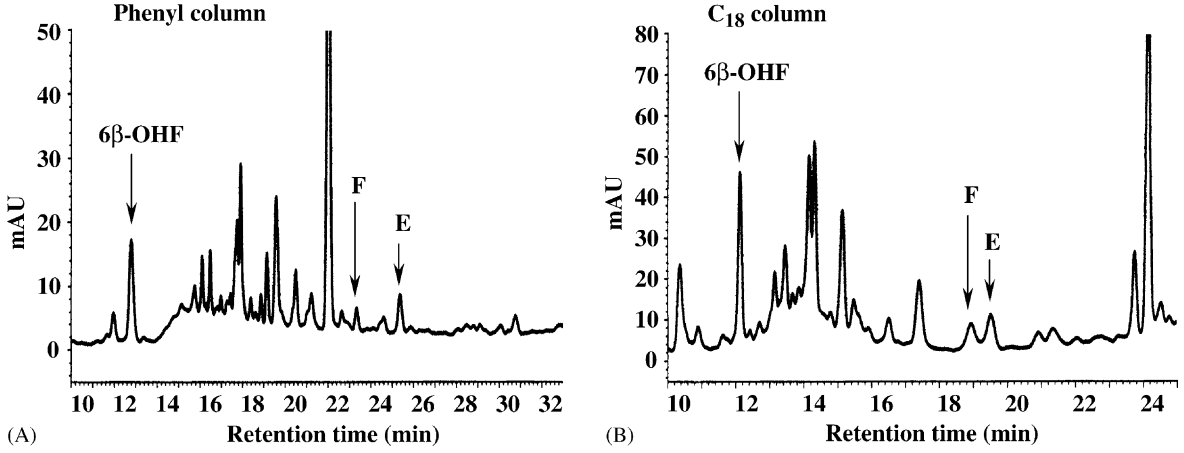

Fig. 1. High-performance liquid chromatograms of 6β-hydroxycortisol (6β-OHF), cortisol (F), and cortisone (E) after processing from 1.0 ml of a normal human urine sample using an ether-linked phenyl reverse-phase column (A) and an octadecyl (C_{18}) reverse-phase column (B).

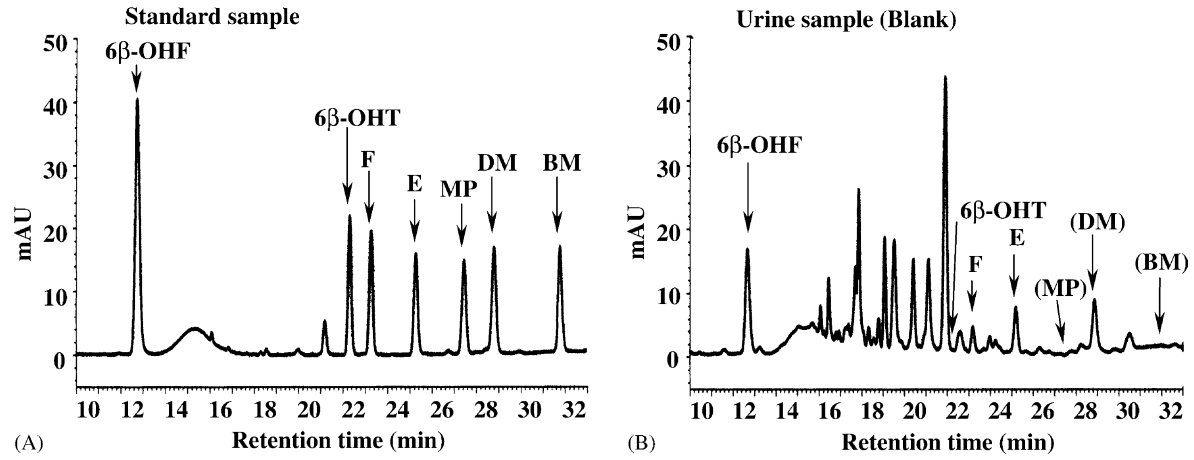

Fig. 2. High-performance liquid chromatograms of standard compounds (A) and blank urine samples (B) using an ether-linked phenyl reverse-phase column. A 50-μl portion of the solution (100 μl of 0.05 M KH_2PO_4–0.01 M CH_3COOH and 20 μl of acetonitrile) after processing from water (A) or urine (B) was subjected to HPLC. (A) 6β-OHF; 6β-hydroxycortisol (805.5 ng/ml; 12.66 min), 6β-OHT; 6β-hydroxytestosterone (192.8 ng/ml; 22.31 min), F; cortisol (201.0 ng/ml; 23.18 min), E; cortisone (204.2 ng/ml; 25.30 min), MP; methylprednisolone (205.0 ng/ml; 27.45 min), DM; dexamethasone (203.4 ng/ml; 28.80 min), and BM; beclomethasone (201.8 ng/ml; 31.77 min). (B) Endogenous 6β-OHF; 6β-hydroxycortisol (309.4 ng/ml; 12.66 min), F; cortisol (32.86 ng/ml; 23.18 min), and E; cortisone (84.15 ng/ml; 25.30 min).

and at t_R 28.80 min for dexamethasone (DM) as shown in Fig. 2B.

3.2. Extraction

6β-OHF and cortisol were extracted from urine by using a Sep-Pak 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 Na_2SO_4. Recoveries were determined at five different concentrations of 6β-OHF (21.48–805.5 ng) and cortisol (5.00–201.0 ng) in water, being 51.5–78.3% for 6β-OHF and 85.1–100.5% for cortisol, respectively. Recoveries (mean ± S.D.) were also determined at a fixed concentration (ca. 200 ng; $n = 5$) of four candidates for the internal standard, being 89.0 ± 3.2% (6β-hydroxytestosterone), 83.7 ± 0.8% (methylprednisolone), 84.1 ± 7.4% (dexamethasone), and 90.9 ± 2.8% (beclomethasone). The extraction procedure resulted in a significant loss of 6β-OHF, while it offered a satisfactory efficiency for all the other analytes in question including cortisol.

3.3. Calibration graphs

The amounts of urinary 6β-OHF and free cortisol are usually measured by using the 24-h urine collected. The normal ranges for the 24-h urinary excretion of 6β-OHF and free cortisol are approximately 50–600 μg and 10–100 μg, respectively [6,14,24,25,27]. Since the volume of 24-h urine is the range 800–2000 ml, it seems reasonable to think that the concentrations of urinary 6β-OHF and cortisol could be the ranges 25–750 and 5–125 ng/ml, respectively. Calibration graphs were then prepared in the ranges 21.48–805.5 ng/ml of 6β-OHF and 5.00–201.0 ng/ml of cortisol with methylprednisolone (205.0 ng/ml) and beclomethasone (201.8 ng/ml) as internal standards for the HPLC assay. The mixture was dissolved in water (1 ml), and then carried through the urine sample preparation procedure as described above. The peak-area ratios were plotted against the concentrations of 6β-OHF and cortisol to either methylprednisolone or beclomethasone as internal standard. A good correlation was found between the observed peak-area ratios (y) and the concentrations (x). Unweighed least-squares regression analysis gave typical regression lines: $y = (3.489 \times 10^{-3})x - 0.0219$ ($r = 0.9998$) for 6β-OHF and $y = (5.402 \times 10^{-3})x - 0.0056$ ($r = 0.9999$) for cortisol (methylprednisolone as internal standard), and $y = (3.276 \times 10^{-3})x - 0.0247$ ($r = 0.9999$) for 6β-OHF and $y = (5.081 \times 10^{-3})x - 0.0071$ ($r = 0.9999$) for cortisol (beclomethasone as internal standard). The lower limit of quantitation (LLOQ) was 21.48 ng/ml for 6β-OHF and 5.00 ng/ml for cortisol, respectively (Fig. 3).

3.4. Accuracy and reproducibility

The accuracy of measurements was determined for 6β-OHF and cortisol added to 1.0-ml aliquots of pooled urine containing endogenous 6β-OHF (309.3 ± 7.72 ng/ml) and cortisol (33.01 ± 2.61 ng/ml). To 1.0-ml aliquots of pooled urine were added fixed amounts of methylprednisolone (205.0 ng) and beclomethasone (201.8 ng) as internal standards. To another set of the urine sample were added known amounts of 6β-OHF (53.70 ng) and cortisol (20.10 ng), and fixed amounts of methylprednisolone (205.0 ng) and beclomethasone (201.8 ng). The within-day reproducibility shown in Table 1 indicates that the amounts of 6β-OHF and cortisol determined are in good agreement with the actual amounts added, the relative errors being −0.72% for both 6β-OHF and cortisol (methylprednisolone as internal standard), and −1.09% for 6β-OHF and −1.59% for cortisol

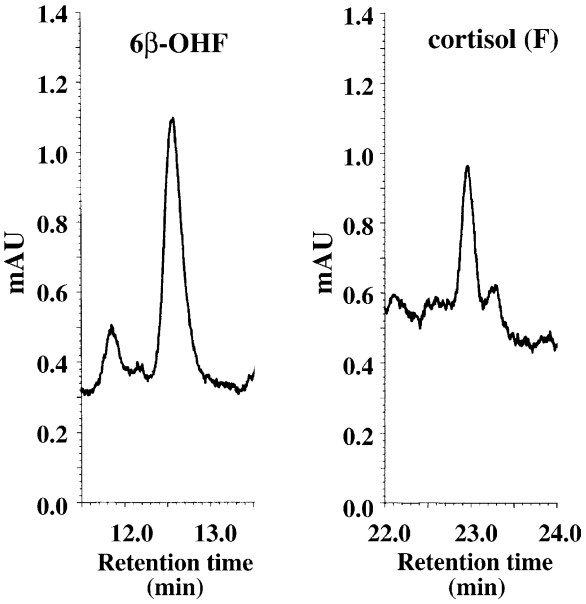

Fig. 3. High-performance liquid chromatogram for a calibration graph standard at the lower limit of quantitation (LLOQ), 21.48 ng/ml for 6β-hydroxycortisol (6β-OHF) and 5.00 ng/ml for cortisol (F). A 50-μl portion of the solution (100 μl of 0.05 M KH_2PO_4–0.01 M CH_3COOH and 20 μl of acetonitrile) after processing from water was subjected to HPLC.

(beclomethasone as internal standard). The assay precisions (R.S.D. values) for 6β-OHF and cortisol were less than 7.91% using methylprednisolone and 6.64% using beclomethasone as internal standards, respectively. Calibration graphs are prepared in each analytical run for measuring cortisol and 6β-OHF in urine. The day-to-day inter-assay precision was not examined in this study. There was no significant difference between the peak-area and -height measured for determining cortisol and 6β-OHF in urine.

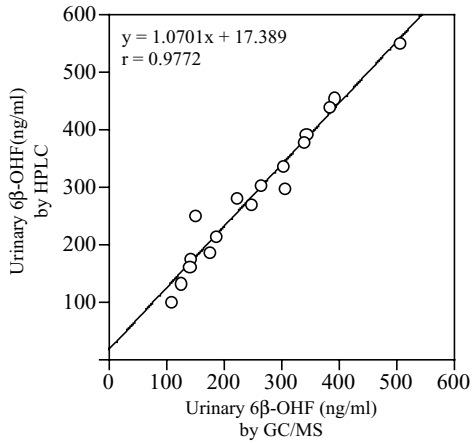

Fig. 4. Relationship of the HPLC and GC/MS methods for measuring 6β-OHF in the human urine samples on days before (day 0), during (day 4) and after (day 13 after a 7-day washout period) administration of clarithromycin in a healthy volunteer.

3.5. Comparison of HPLC and GC/MS methods for measuring urinary 6β-hydroxycortisol

Clarithromycin (200 mg), a macrolide antibiotic, was administered to a healthy volunteer every 12 h at 10:00 and 22:00 for 5 days and finally at 10:00 on day 6 [10]. Amounts of 6β-OHF excreted in urine obtained at 2 h-intervals from 10:00 to 22:00 on days before (day 0), during (day 4) and after (day 13 after a 7-day washout period) administration of clarithromycin were measured by the present HPLC. The results were compared with those determined by the GC/MS method using stable isotope-labelled internal standard, 6β-hydroxy-[1,1,19,19,19-$^2H_5$]cortisol [31]. Fig. 4 shows the relationship of the HPLC (y) and GC/MS (x) methods for measuring 6β-OHF in the same urine samples ($n = 17$). Unweighed least-squares regression analysis gave typical

Table 1
Accuracy of HPLC determination of 6β-hydroxycortisol and cortisol in human urine

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
		Individual values[a]						Mean ± S.D.		
(A) methylprednisolone (internal standard)										
6β-OHF										
–	–	307.5	307.8	309.8	307.6	299.8	323.4	309.3 ± 7.72	–	2.50
53.70	363.0	368.7	369.8	355.3	350.6	362.6	355.3	360.4 ± 7.88	−0.72	2.19
Cortisol										
–	–	30.91	32.09	34.09	32.30	30.92	37.77	33.01 ± 2.61	–	7.91
20.10	53.11	53.48	54.98	51.86	51.64	52.47	51.95	52.73 ± 1.28	−0.72	2.43
(B) beclomethasone (internal standard)										
6β-OHF										
–	–	304.8	302.9	304.1	305.6	297.8	311.7	304.5 ± 4.49	–	1.47
53.70	358.2	361.3	361.2	347.5	344.9	358.5	352.1	354.3 ± 7.12	−1.09	2.01
Cortisol										
–	–	30.84	31.77	33.65	32.27	30.91	36.57	32.67 ± 2.17	–	6.64
20.10	52.77	52.50	53.79	50.82	50.90	51.98	51.59	51.93 ± 1.11	−1.59	2.14

[a] Each individual value is the mean of triplicate measurements.

regression lines $y = 1.0701x + 17.389$ ($r = 0.9772$) for methylprednisolone as internal standard (Fig. 4) and $y = 1.0827x + 6.1364$ ($r = 0.9794$) for beclomethasone as internal standard (figure not shown).

4. Discussion

In phenotyping by using a probe drug, the clearance of the drug should provide the best estimate of the in vivo catalytic activity of the enzyme of interest [39]. Since cortisol has multiple metabolic pathways, the fractional metabolic clearance specific for the 6β-hydroxylation should be an appropriate measure for the CYP3A phenotyping [9,10]. On the other hand, the urinary ratio 6β-OHF/F is valid as the index reflecting the in vivo CYP3A activity only when there are no intra- and inter-individual variations in the renal clearance of cortisol [9,10], since the ratio 6β-OHF/F consists of a function of 6β-hydroxylation clearance (CLm(6β)) and the renal clearance of cortisol (CLr(F)) as follows: $6\beta\text{-OHF/F} = \mathrm{CLm}(6\beta)/\mathrm{CLr(F)}$. However, there are no detailed studies investigating how the cortisol renal clearance changes and affects the urinary ratio 6β-OHF/F in humans. Determining the renal clearance as well as the 6β-hydroxylation clearance requires two separate analyses of plasma cortisol and urinary cortisol and 6β-OHF.

The HPLC method we present here is practical and permits the simultaneous determination of 6β-OHF and cortisol in urine. Phenotyping for the CYP3A activity, as determined by the fractional metabolic clearance of cortisol to 6β-OHF, can be successfully performed by measuring cortisol in plasma by our GC/MS method [12,13] and 6β-OHF and cortisol in urine by the present HPLC method.

In this study, an ether-linked phenyl phase column was chosen to simultaneously analyze 6β-OHF (t_R 12.66 min) and cortisol (t_R 23.18 min), because it produced the good chromatographic behavior (Fig. 1A). The extraction of 6β-OHF and cortisol in urine was based on the procedure described by Lykkesfeldt et al. [27]. The procedure consisted of two steps; elution of the urine sample with ethyl acetate-diethyl ether (4:1, v/v) on a Sep-Pak cartridge and subsequent wash of the organic extracts with alkaline and acidic solutions saturated with Na_2SO_4. Lykkesfeldt et al. [27] estimated the recoveries of 6β-OHF (70.8%), cortisol (90.6%), and dexamethasone (91.6%) as internal standard. Different overall recoveries for the individual steroids required the corrections by the relative recoveries in the quantitations. In this study, the extraction efficiencies (%) (mean ± S.D., $n = 5$) were found to be satisfactory for dexamethasone (84.1 ± 7.4%; R.S.D., 8.8%), methylprednisolone (83.7 ± 0.8%, R.S.D., 1.0%), and beclomethasone (90.9 ± 2.8%; R.S.D., 3.1%) as candidates for internal standard. Satisfactory recoveries (92.0 ± 6.7%; R.S.D., 7.3%) were also obtained for cortisol, which were determined at five different concentrations in the range 5.00–201.0 ng/ml. However, the extraction caused a significant loss of 6β-OHF, and the recoveries largely varied (64.1 ± 10.5%; R.S.D., 16.4%) in the range 21.48–805.5 ng/ml tested. This indicates that the recoveries obtained at one concentration cannot be used appropriately for the corrections in quantitating 6β-OHF. In this study, the quantitations of 6β-OHF and cortisol 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 urine sample preparations. The calibration graphs were prepared at six different concentrations in the ranges 21.48–805.5 ng/ml for 6β-OHF and 5.00–201.0 ng/ml for cortisol, respectively. The obtained calibration graphs were linear ($r > 0.9998$) and gave good within-day reproducibilities in simultaneously measuring 6β-OHF and cortisol in human urine using either methylprednisolone or beclomethasone as internal standard (Table 1).

We tested several compounds for use as internal standards such as 6β-hydroxytestosterone, dexamethasone [26,27], methylprednisolone [33], and beclomethsone for the HPLC analysis of 6β-OHF and cortisol. Dexamethasone has been widely used as the internal standard [26,27], but an interfering peak was observed at t_R 28.80 min derived from urine (Fig. 2B). 6α-Hydroxycortisol (t_R 13.33 min), 6β-hydroxycortisone (t_R 15.83 min), and 6β-hydroxytestosterone (t_R 22.31 min) are inappropriate as internal standard, because these compounds are endogenously present in human urine. Methylprednisolone (t_R 27.45 min) and beclomethasone (t_R 31.77 min) had no interfering peaks and were suitable for internal standards to simultaneously measure 6β-OHF (t_R 12.66 min) and cortisol (t_R 23.18 min) in urine (Fig. 2).

We previously developed a sensitive and reliable GC/MS technique for the simultaneous determination of 6β-hydroxycortisol (6β-OHF), 6α-hydroxycortisol (6α-OHF), and 6β-hydroxycortisone (6β-OHE) in urine with good accuracy and precision, using the corresponding stable-isotope-labelled analogues as internal standards, i.e. 6β-hydroxy-[1,1,19,19,19-$^2H_5$]cortisol, 6α-hydroxy-[1,1,19,19,19-$^2H_5$]cortisol, and 6β-hydroxy-[1,1,19,19,19-$^2H_5$]cortisone [31]. Stable isotope dilution mass spectrometry is widely accepted as the most accurate and specific method for estimation of the small amounts of endogenous and synthetic steroids in biological fluids [40]. The method, therefore, serves as the reference to validate the revised and different analytical techniques as the *comparator*. The present HPLC-UV method was then compared with the GC/MS technique using 6β-hydroxy-[1,1,19,19,19-$^2H_5$]cortisol as internal standard by measuring 6β-OHF in the urine samples obtained on days before, during, and after clarithromycin treatment in a healthy volunteer. A good correlation was found between the amounts determined by the two methods (Fig. 4). There was no significant difference between methylprednisolone and beclomethasone as internal standards to measure urinary 6β-OHF by HPLC.

When a macrolide antibiotic, clarithromycin, was administered to a healthy volunteer in a dose of 200 mg every 12 h for 6 days, the inhibitory effects of clarithromycin on the in vivo CYP3A activity were clearly seen by the 6β-hydroxylation clearance of endogenous cortisol but not by the urinary ratio 6β-OHF/F. The urinary ratio 6β-OHF/F does not always reflect the in vivo CYP3A activity, because the ratio is varied with the cortisol renal clearance [9,10]. Evidence for the validity of cortisol 6β-hydroxylation clearance as a new index for in vivo CYP 3A phenotyping in humans has recently been reported by Furuta et al. [10].

5. Conclusions

The present study provides a reliable and practical technique for the simultaneous determination of 6β-hydroxycortisol (6β-OHF) and cortisol in urine with good accuracy and precision, using either methylprednisolone or beclomethasone as internal standard. The HPLC-UV method described here should be suitable to measure 6β-OHF and cortisol in urine routinely, with a particular interest in evaluating the C-6β oxidation of cortisol catalyzed by CYP3A in vivo.

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