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Simultaneous high-performance liquid chromatographic determination of 6β-hydroxycortisol and cortisol in urine with fluorescence detection and its application for estimating hepatic drug-metabolizing enzyme induction

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Abstract

A simple and sensitive high-performance liquid chromatographic method is described for the simultaneous determination of 6β-hydroxycortisol (6β-OHF) and cortisol (F) in urine. Urine (1 ml) containing fludrocortisone as the internal standard is extracted with ethyl acetate. The extract is washed successively with sodium hydroxide solution and water, and subsequently dried under a stream of nitrogen. The residue is redissolved in methanol. The 6β-OHF, F and fludrocortisone in the methanol solution are oxidized by cupric acetate and the resulting glyoxal compounds are converted into fluorescent derivatives with 1,2-diamino-4,5-methylenedioxybenzene (DMB). The DMB derivatives of the corticosteroids are separated within 70 min on a reversed-phase column, L-Column ODS, using stepwise elution with methanol–acetonitrile–0.5 *M* ammonium acetate and detected fluorimetrically at 350 nm (excitation) and 390 nm (emission). The lower limits of detection for 6β-OHF and F are 1.8 pmol (680 pg) and 2.4 pmol (950 pg)/ml urine (0.6 pmol and 0.8 pmol/100 μl injection volume), respectively, at a signal-to-noise ratio of 3. This method can be applied to the determination of urinary 6β-OHF, and the ratio of 6β-OHF to F in humans and in rhesus monkeys treated orally with phenobarbital as a hepatic drug-metabolizing enzyme inducer.

1. Introduction

Cortisol (F) is converted by 6β-hydroxylase in hepatic microsomes to 6β-hydroxycortisol (6β-OHF), which is then excreted as a polar, unconjugated metabolite in urine [1–3]. Certain drugs with hepatic drug-metabolizing enzyme induction activity have been reported to cause an increase in the urinary excretion of 6β-OHF in humans [4–6]. It was suggested, therefore, that urinary excretion of 6β-OHF could be used as a non-invasive indicator of enzyme induction [7]. However, since urinary 6β-OHF concentrations are variable, the urinary excretion of 6β-OHF should be expressed as the ratio of 6β-OHF to F, to correct for daily and inter-individual varia-

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tions in adrenal cortisol production [8]. Recently, Saenger [9] reported the absence of circadian changes in 6β-OHF/F ratios in humans while Bienvenu et al. [10] found that in humans the 6β-OHF/F ratio can be determined from a morning spot urine sample instead of from a 24-h urine collection. Thus, the urinary 6β-OHF/F ratio appears to be of greater significance than the 6β-OHF value in assessing enzyme induction.

Several methods for the determination of urinary 6β-OHF by high-performance liquid chromatography (HPLC) with UV [11–13] and fluorescence detection [14], or radioimmunoassay (RIA) [15–17] have been developed to assess the drug-metabolizing capacity of human or animal liver. Although the RIA methods are very sensitive, they require radioactive compounds and are not very selective for 6β-OHF. The HPLC methods using UV absorbance detection lack sufficient sensitivity. In contrast, RIA methods have been widely used for the determination of urinary free F. However, concentrations of F are generally overestimated due to cross-reaction of the antiserum with many other urinary constituents, including drugs [19,20]. HPLC with UV absorbance detection is suitable for the specific determination of urinary free F [10], but is not very sensitive.

There are only a few reports describing the simultaneous determination of urinary 6β-OHF and F by HPLC. The method described by Nakamura and Yakata [18] involved chromatography of urinary extracts on a Zorbax-silica column and rechromatography of the eluents on a Zorbax-CN column. In the method developed by Bienvenu et al. [10], urinary extracts were divided into two parts for 6β-OHF and F determination, one part being chromatographed on an ultrabase column for the assay of 6β-OHF, and the other part on an ODS column for the analysis of F. However, these HPLC methods are rather complicated and have limited sensitivity and reproducibility.

The present research was aimed at establishing a sensitive, reproducible and simple HPLC method for the simultaneous determination of urinary 6β-OHF and F. The method is based on the conversion of the corticosteroids to their corresponding glyoxal compounds, followed by derivatization to fluorescent glyoxal compounds by reaction with 1,2-diamino-4,5-methylenedioxybenzene (DMB), a fluorogenic reagent for α-dicarbonyl compounds [21]. Using this method we determined the urinary 6β-OHF and F levels and the 6β-OHF/F ratio in healthy male volunteers and in rhesus monkeys treated orally with sodium phenobarbital (30 mg/kg/day) for a 14-day period. Fludrocortisone, a steroid with properties similar to those of 6β-OHF and F, was used as internal standard to control assay variability.

2. Experimental

2.1. Reagents and solutions

Fludrocortisone (F-E) and F were purchased from Sigma (St. Louis, MO, USA). 6β-OHF was obtained from Steraloids (Wilton, NH, USA). DMB was prepared as described previously [22] and is now commercially available from Dojindo Laboratories (Kumamoto, Japan). All other chemicals and solvents were of analytical-reagent grade or special grade for liquid chromatography. DMB solution (1 mg/ml) was prepared by dissolving DMB in distilled water containing sodium hydrosulphite (50 mg/ml) and β-mercaptoethanol (50 μl/ml). Copper(II) acetate solution (39 m*M*) was prepared by dissolving 77.9 mg of copper(II) acetate in 1 ml of ultra-pure water and diluting the solution with methanol to a total volume of 10 ml. The internal standard (I.S.) solution of F-E (5 μ*M*), was prepared in methanol.

2.2. Urine sample

Twenty-four hour urine collections were obtained from 16 healthy male volunteers, or from normal rhesus monkeys (24 males, 12 females). The total number of urine samples from humans, male monkeys and female monkeys was 48, 39 and 21, respectively. The samples were stored without preservatives at −30°C prior to analysis.

2.3. Extraction procedure

Each urine specimen was centrifuged at 1000 *g* for 30 min to remove insoluble matter. To the resulting urine sample (1 ml), 200 μl of the F-E solution (I.S.) and 6 ml of ethyl acetate were added. The mixture was vortex-mixed for ca. 3 min and the ethyl acetate extract was washed with 1 ml of 1 *M* sodium hydroxide, and subsequently with 2 ml of ultra pure water. After a brief centrifugation at 1000 *g* for ca. 3 min, 4.5 ml of the extract were evaporated to dryness under a flow of nitrogen at 37°C. The residue, dissolved in 100 μl of methanol, was used as a sample solution.

2.4. Fluorescence derivatization procedure

Derivatization to fluorescent corresponds was performed according to the method of Yoshitake et al. [21]. A 100-μl volume of the sample solution, placed in a screw-capped vial, was mixed with 20 μl of the copper(II) acetate solution. This mixture was allowed to stand at room temperature for 1 h, and then 100 μl of the DMB solution was added. After heating at 60°C for 40 min, the mixture was cooled in ice-water and filtered through a membrane filter (0.45-μm pore size). A 100-μl aliquot of the filtrate was injected to the HPLC system.

2.5. Chromatography

We used a TOSOH liquid chromatographic system (Tokyo, Japan) consisting of a CCPM multi pump, an AS-8010 autosampler and an FS-8010 fluorescence spectromonitor. An excitation wavelength of 350 nm and an emission wavelength of 390 nm were used for fluorescence detection. Separations were performed on an L-Column ODS (250 × 4.6 mm I.D.; particle size, 5 μm; Chemicals Inspection and Testing Institute, Tokyo, Japan) at ambient temperature with a flow-rate of 1.0 ml/min. The mobile phase was composed of solvent A (methanol–acetonitrile–0.5 *M* ammonium acetate, 30:15:55, v/v), and solvent B (methanol–acetonitrile–0.5 *M* ammonium acetate 45:15:40, v/v). First solvent A was used for 25 min, then solvent B for 45 min. The column was washed for 20 min with 80% methanol and then equilibrated with solvent A for 20 min before the next injection.

2.6. Quantification

To establish a calibration curve, 200 μl of the F-E (I.S.) solution were replaced with I.S. solution (200 μl) containing 6β-OHF and F (10–500 pmol each). The net peak-height ratios of the individual steroids and F-E were plotted against the concentrations of steroids added.

2.7. Treatment of rhesus monkeys with phenobarbital

Sodium phenobarbital was dissolved in distilled water (30 mg/ml) for oral dosing via a catheter. Three male and one female rhesus monkeys obtained from a commercial supplier (CSK Research Park, Japan) were administered PB (30 mg/kg per day) orally for a 14-day period. Twenty-four hour urine samples were collected just before dosing and on days 2, 5, 8, 11 and 14 after administration.

2.8. Preparation of hepatic microsomes

Monkey livers were removed after necropsy and weighed. Each liver was perfused with 1.15% (w/v) KCl and homogenized in 4 volumes of 50 m*M* Tris-HCl buffer (pH 7.4) containing 1.15% (w/v) KCl, 1 m*M* EDTA and 10 μ*M* phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 10 000 *g* for 20 min and the resulting supernatant was recentrifuged at 100 000 *g* for 1 h. The microsomal pellet was suspended in 0.1 *M* Tris-HCl buffer (pH 7.4) containing 1 m*M* EDTA. Microsomal protein was determined with a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA), with bovine serum albumin as the standard.

2.9. Measurement of cytochrome content and NADPH-cytochrome c reductase activity

Cytochrome P-450 and cytochrome b_5 were assayed by the method of Omura and Sato [23]. NADPH-cytochrome c reductase activity was determined by the method of Phillips and Langdon [24].

2.10. Statistics

The difference between the values for 6β-OHF and F in 24-h urine before and after treatment was evaluated statistically by the paired t-test. The level of significance chosen was $p < 0.05$.

3. Results and discussion

3.1. Derivatization conditions

The conditions for the oxidation reaction with cupric acetate and fluorescence derivatization with DMB for corticosteroids (excluding 6β-OHF) were described previously [21]. In this study, the conditions for 6β-OHF were examined. The resulting optimal conditions were similar to those for the other corticosteroids described previously [21].

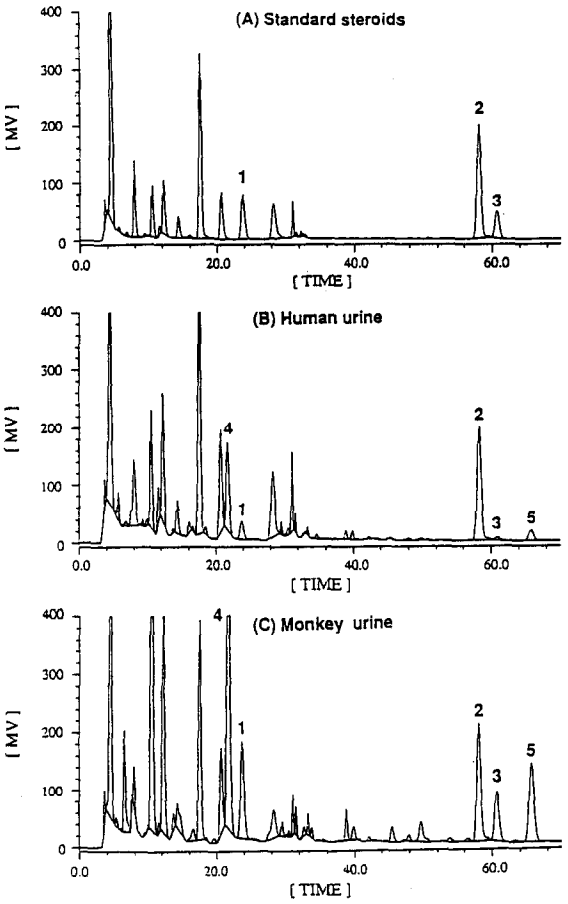

Fig. 1. Chromatograms obtained with (A) standard steroids, (B) human urine, and (C) monkey urine. Peaks: 1 = 6β-OHF; 2 = F-E; 3 = F; 4 = unknown urinary component; 5 = cortisone; others = reagent blank components.

3.2. Clean-up of urine and HPLC separation

Complete separation of 6β-OHF, F-E, F and reagent-blank components was achieved on a reversed-phase column, L-Column ODS, by stepwise gradient elution with mixtures of methanol–acetonitrile–0.5 *M* ammonium acetate. A typical chromatogram obtained with a standard mixture of the three corticosteroids is shown in Fig. 1A. The retention times for 6β-OHF, F-E and F were 23.8, 58.2 and 60.7 min, respectively. The individual steroids gave single, symmetrical peaks in the chromatogram.

Figs. 1B and 1C show typical chromatograms obtained from human and monkey urine specimens, respectively; the HPLC elution pattern of human urine resembled that of monkey urine.

Two methods for corticosteroid extraction from urine have been reported previously. One is an organic solvent extraction [11,12,18] and the other involves a solid-state cartridge extraction procedure [3,13,19]. The cartridge method offers a rapid means of steroid extraction, but it was not suitable for our fluorimetric HPLC method because of interferences caused by other unknown urinary fluorescent substances and because of low reproducibility. A high recovery

of the steroids from urine and a high reproducibility were obtained using ethyl acetate extraction. However, when the organic layer was used without clean-up, large and broad peaks from urinary contaminants appeared at retention times between 10 and 30 min in the chromatograms. These contaminants (except for peak 4 in Figs. 1B and 1C), were removed by successively washing the ethyl acetate extract with 1 *M* sodium hydroxide (1 ml) and water (2 ml). Peak components were identified by comparison of their retention times with those of the standard compounds, and co-chromatography with the standards using aqueous 10–100% (v/v) methanol (or acetonitrile) as the eluent. When the oxidation step with copper(II) acetate was omitted, peaks 1–5 disappeared (Figs. 1B and 1C). These results indicated that peaks 1–3 in Figs. 1B and 1C are attributable to 6β-OHF, F-E and F, respectively, and that peak 4 might be an unknown urinary corticosteroid. Furthermore, peak 5 in Figs. 1B and 1C could be ascribed to urinary cortisone; when authentic cortisone was treated according to the described procedure the peak due to cortisone had a retention time identical to that of peak 5.

3.3. Interference by endogenous steroids and drugs

The urinary corticosteroids, 18-hydroxycorticosterone, 18-hydroxydeoxycorticosterone, corticosterone, 11-deoxycortisol, deoxycorticosterone, 11-dehydrocorticosterone, 3α,5β-tetrahydroaldosterone, 3α,5β- and 3α,5α-tetrahydrocortisol, 3α,5β-tetrahydrocortisone, 3α,5β-tetrahydro-11-deoxycortisol, 3α,5β- and 3α,5α-tetrahydrocorticosterone, 3α,5β- and 3α,5α-tetrahydro-11-deoxycorticosterone, also reacted with DMB to give fluorescent derivatives. However, these compounds were not eluted within 80 min under the conditions employed, but were eluted by the 20 min-washing with 80% (v/v) methanol. Phenobarbital, rifampicin and antipyrine have been used in previous investigations of drug-metabolizing enzyme induction, however, they gave no fluorescent derivatives and therefore did not interfere with the present determination of 6β-OHF and F in urine.

3.4. Calibration curve, recovery, detection limit and reproducibility

Linear calibration graphs for 6β-OHF and F were obtained when the peak-height ratios of the respective corticosteroids to F-E were plotted against the amounts of the steroids added to urine over the range 10–5000 pmol/ml. The correlation coefficients of the calibration curves were ≧0.998 for both steroids.

Recoveries were determined for three different concentrations of the standard steroids added to human urine (Table 1). Recoveries for 6β-OHF and F of 87–105% and 97–106%, respectively, were obtained when 3 concentrations of the 2 steroids were analysed in urine and compared with the injection of appropriate controls (Table 1).

The lower limits of detection for 6β-OHF and F were 1.8 pmol (680 pg) and 2.4 pmol (950 pg)/ml urine (0.6 pmol and 0.8 pmol/100 μl injection volume), respectively, at a signal-to-noise ratio of 3.

The within-run and between-run variability in the assay of human urinary 6β-OHF and F is shown in Table 2. The within-run coefficients of variation ($n = 7$) were 1.0% and 2.7% for 6β-

Table 1
Recovery of 6β-hydroxycortisol and cortisol added to human urine

Added (pmol/ml)	Found (pmol/ml)	Recovered (pmol/ml)	Recovery (%)
6β-Hydroxy cortisol			
None	272.7	–	–
125	382.3	109.6	87.7
250	534.6	261.9	104.8
500	772.8	500.1	100.0
Cortisol			
None	42.9	–	–
62.5	105.1	62.2	99.5
125.0	174.9	132.0	105.6
250.0	285.4	242.5	97.0

Table 2
Reproducibility in the determination of human urinary 6β-hydroxycortisol (6β-OHF) and cortisol (F)

Compound	Within-run ($n = 8$)		Between-run ($n = 8$)	
	Found (mean ± S.D.) (pmol/ml)	C.V. (%)	Found (mean ± S.D.) (pmol/ml)	C.V. (%)
6β-OHF	268.0 ± 2.6	1.0	248.7 ± 0.8	6.4
F	40.3 ± 1.1	2.7	33.3 ± 0.8	2.4

Table 3
Changes in microsomal protein, cytochrome contents and NADPH-cytochrome c reductase activity of rhesus monkeys treated orally with phenobarbital sodium [phenobarbital (30 mg/kg/day)] for a 14-day period

	Animal No.[a]					Mean ± S.D.
	Control ($n = 9$) (M, 6; F, 3)	106 (M)	113 (M)	117 (M)	508 (F)	
Microsomal protein (mg/g of liver)	13.7 ± 3.9	31.9	33.8	32.0	33.9	32.9 ± 1.0
Cytochrome P-450 (nmol/mg of microsomal protein)	0.85 ± 0.40	2.45	2.34	2.60	3.13	2.63 ± 0.17
Cytochrome b5 (nmol/mg of microsomal protein)	0.47 ± 0.18	0.68	0.70	0.73	1.15	0.82 ± 0.19
NADPH-cytochrome c reductase activity (nmol/mg of microsomal protein/min)	158 ± 51	114	183	192	153	160 ± 31

[a] M, male; F, female.

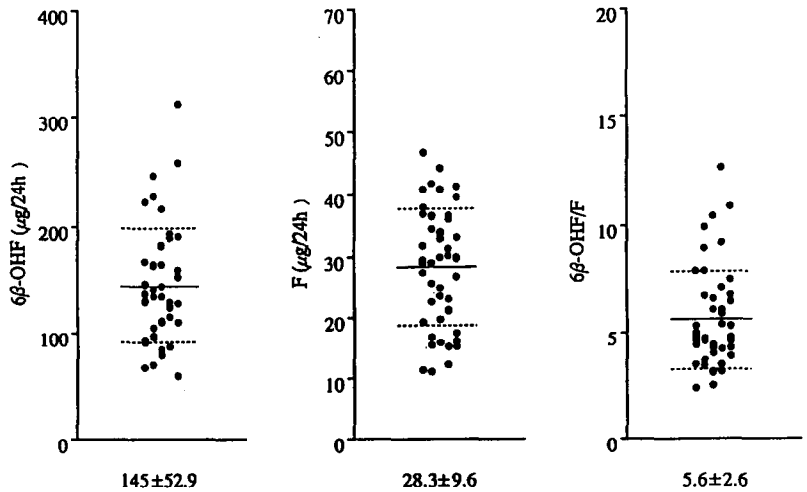

Fig. 2. Urinary 6β-hydroxycortisol (6β-OHF) and cortisol (F) and their ratio (6β-OHF/F) in healthy male volunteers. Results are mean ± S.D. ($n = 47$).

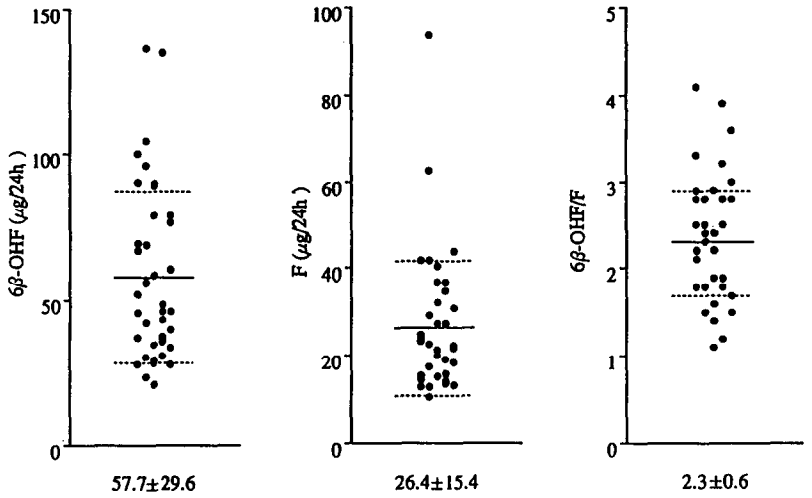

Fig. 3. Urinary 6β-hydroxycortisol (6β-OHF) and cortisol (F) and their ratio (6β-OHF/F) in normal male rhesus monkeys. Results are mean ± S.D. ($n = 37$).

OHF and F, respectively, while between-run ($n = 8$) coefficients of variation of 6.4% and 2.4% were calculated for 6β-OHF and F, respectively.

3.5. Urinary excretion of 6β-OHF and F, and the 6β-OHF/F ratio in urine from humans and rhesus monkeys

Urinary excretion of 6β-OHF and F, and the 6β-OHF/F ratio for humans and rhesus monkeys are shown in Figs. 2–4. The mean urinary excretion of 6β-OHF and F and the 6β-OHF/F ratio in healthy male volunteers were 145 μg/day, 28.3 μg/day and 5.6, respectively. In healthy male rhesus monkeys these values were 57.7 μg/day, 26.4 μg/day and 2.3, respectively, while in normal female rhesus monkeys, values of 32.7 μg/day, 14.3 μg/day and 2.4, respectively, were found. The values of urinary 6β-OHF and F

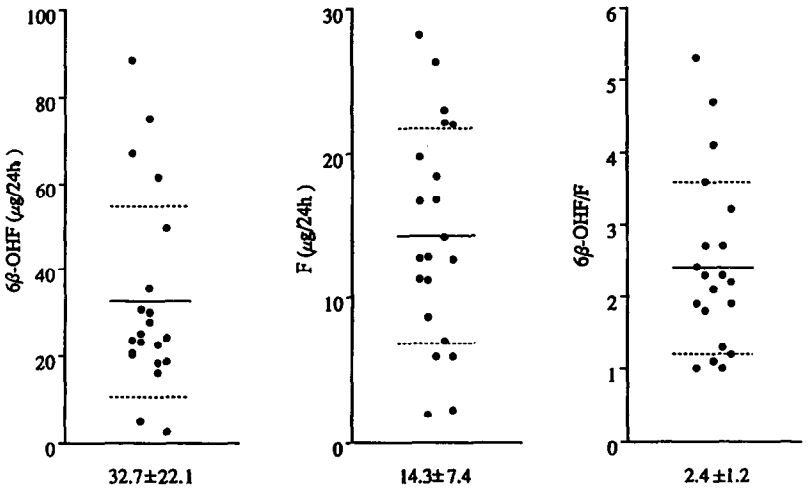

Fig. 4. Urinary 6β-hydroxycortisol (6β-OHF) and cortisol (F) and their ratio (6β-OHF/F) in normal female rhesus monkeys. Results are mean ± S.D. ($n = 20$).

obtained by the present HPLC method for healthy male volunteers were similar to those reported by Nakamura and Yakata [18] and Bienvenu et al. [10] using HPLC with UV absorbance detection.

3.6. Effects of phenobarbital on urinary excretion of 6β-OHF in rhesus monkeys

Phenobarbital was administered to monkeys to investigate the usefulness of the present method to evaluate enzyme induction by drugs. For these studies we used rhesus monkeys which have cortisol as a major component of their circulating corticosteroid pools. Firstly, the results shown in Table 3 confirmed that phenobarbital induced the drug-metabolizing enzyme in rhesus monkeys treated orally with phenobarbital over a 14-day period. As shown in table 3, hepatic microsomal protein, cytochrome P-450 and cytochrome b_5 increased 2.4-, 3.1- and 1.7-fold, respectively, compared with control data. The NADPH-cytochrome c reductase activity remarried unchanged.

Urinary levels of 6β-OHF and F and the 6β-OHF/F ratio in rhesus monkeys teated with phenobarbital were determined by the present method (Fig. 5). During the 3 days before phenobarbital administration, there was a large inter- and intra-animal variation in urinary excretion of 6β-OHF and F, but the 6β-OHF/F ratio in all animals ranged only from 2 to 4. In the rhesus monkeys given phenobarbital (30 mg/kg/day) the mean urinary excretion of 6β-OHF was dependent on the duration of the phenobarbital administration and showed an approximately 3-fold increase on day 11 compared with the value before treatment. The amounts of free urinary F were apparently not effected by phenobarbital administration, although there was considerable variation between animals. The 6β-OHF/F ratio in all animals was dependent on the duration of the phenobarbital administration with an approximately 4-fold increase on day 8 of dosing, as compared with pre-treatment values.

During administration of phenobarbital, the animals showed increased urinary excretion of 6β-OHF and an increased 6β-OHF/F ratio

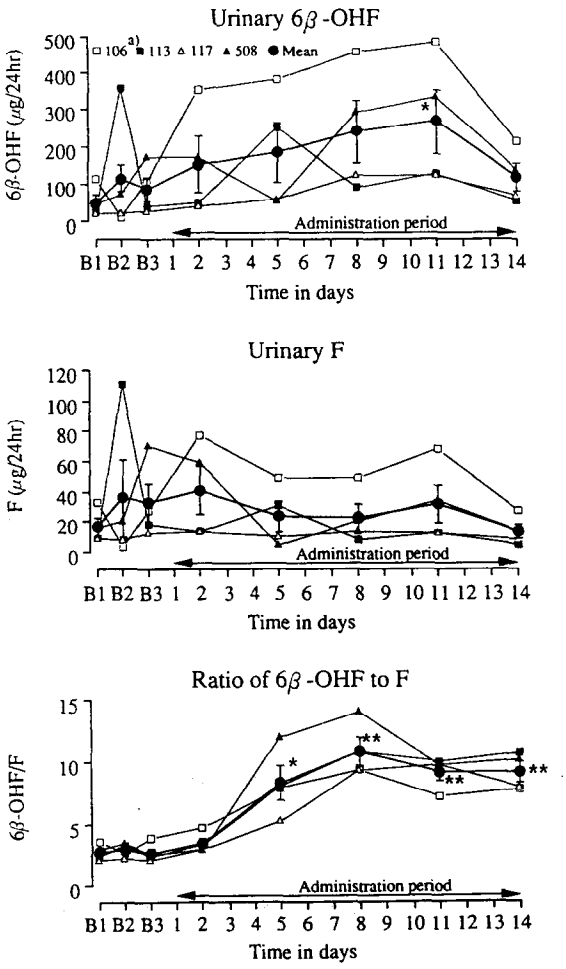

Fig. 5. Changes in urinary excretion of 6β-hydroxycortisol (6β-OHF) and cortisol (F) and in the ratio 6β-OHF/F in rhesus monkeys treated orally with phenobarbital (30 mg/kg/day) for a 14-day period. Significant differences from pre-administration (B3) values are indicated: *($p < 0.05$); **($p < 0.01$). [a]Number of animals.

because of drug-metabolizing enzyme induction (Table 1). There was a large intra-animal variation in the urinary excretion of 6β-OHF, and therefore, the 6β-OHF/F ratio was considered to be a better index of enzyme induction than the values of 6β-OHF alone. These studies demonstrated that the 6β-OHF/F ratio in urine may be used as a non-invasive indicator for monitoring the time-course of enzyme induction by drugs in rhesus monkeys.

A relatively large variation was observed in

the 6β-OHF/F ratio in humans administered phenobarbital (Fig. 2). Accordingly, it is desirable to determine the 6β-OHF/F ratio in the control of the same human subject in order to draw viable conclusions regarding enzyme induction.

4. Conclusions

This paper describes the first HPLC method with fluorescence detection for the simultaneous determination of urinary 6β-OHF and F in humans and monkeys. The method also permits the determination of urinary cortisone. Moreover, urinary corticosteroids other than the three steroids presently studied might also be measured simultaneously by appropriate modification of the HPLC method. We consider this method to be an excellent means for assessment of the enzyme-inducing activity of drugs such as phenobarbital because of the high sensitivity and capacity of the method for the simultaneous determination of urinary 6β-OHF and F.

Further, the method is potentially useful for clinical investigation of disorders such as Cushing's syndrome and diseases which give abnormal levels of urinary F and/or 6β-OHF.

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