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High-Performance Liquid Chromatographic Determination of 6β-Hydroxycortisol in Urine

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A simple, rapid, sensitive and reproducible reversed-phase high-performance liquid chromatographic (HPLC) method was developed for the quantitative determination of urinary 6β-hydroxycortisol (6β-OHF) as a non-invasive indicator of drug-metabolizing enzyme activity in man. The urine (10 ml) containing 6β-hydroxycortisone as an internal standard was loaded onto a Sep-Pak C_{18} column and eluted with ethyl acetate. The samples were analyzed on a Radial-Pak Nova-Pak C_{18} column, and the compounds were eluted with a mixture of 0.01 M potassium dihydrogen phosphate–acetonitrile–methanol (935 : 55 : 10) containing 0.005% trichloroacetic acid as a mobile phase and quantified by ultraviolet absorbance measurement at 243.5 nm. The analytical recovery of added 6β-OHF was almost complete. The reproducibility assessed by repeated analysis was satisfactory, that is to say, the coefficient of variation ranged from 2.06 to 2.25% (within-run) and from 2.82 to 3.11% (between-run). In healthy adult subjects (23—41 years), the mean excretion rate of 6β-OHF was 214 μg/d (S.D. = 41.4; $n = 10$) and the mean ratio of 6β-OHF to 17-hydroxycorticosteroids was 0.0310 (S.D. = 0.00662; $n = 10$).

The present HPLC method was found to be applicable to the routine assay of urinary 6β-OHF in clinical studies.

Keywords——6β-hydroxycortisol; 17-hydroxycorticosteroid; HPLC; mixed-function oxygenase; anticonvulsant

The hepatic mixed-function oxygenases are responsible for the metabolism of many drugs, and therefore changes in the activities of these enzymes may lead to changes in drug action and toxicity.[1] Assessment of factors that influence the activity of these enzymes is therefore an important part of our overall understanding of drug action and may help to avoid drug toxicity. Many methods for the determination of the activity of the drug-metabolizing enzymes have been developed for use in animal models and for *in vitro* studies, but only a few of these methods are applicable to clinical studies in man.[2-3]

6β-Hydroxycortisol (6β-OHF) is a polar, unconjugated metabolite of cortisol. It is formed in the endoplasmic reticulum of hepatocytes by the mixed-function oxygenases, which are also responsible for the metabolism of many drugs, and is excreted by the kidney.[4] Changes in urinary 6β-OHF, in relation to total 17-hydroxycorticosteroids (17-OHCS), have been suggested to be a non-invasive indicator of drug-metabolizing activity,[2] and a good correlation has been found between changes in the urinary excretion of 6β-OHF and the pharmacokinetics of antipyrine as a model drug.[5] To date, extensive investigations of urinary 6β-OHF have been restricted by the complexity and the insensitivity of the methods available for measurement.

The purpose of this study is firstly to describe a method which permits rapid, simple, sensitive and reproducible determination of urinary 6β-OHF by reversed-phase high-performance liquid chromatography (HPLC), and secondly to report the normal values for urinary 6β-OHF excretion and the ratio of 6β-OHF to 17-OHCS in healthy adult subjects.

Experimental

Materials——6β-OHF and 6β-hydroxycortisone (6β-OHE) were purchased from Sigma Chemical Co., Ltd., St. Louis, Mo., U.S.A., β-glucuronidase type I (13000 unit/ml) was from Tokyo-zoki Chemical Co., Ltd., Tokyo, Japan, and acetonitrile, methanol, ethanol and methylene chloride (special grade for liquid chromatography) were from Nakarai Chemicals Co., Ltd., Kyoto, Japan. All other chemicals and reagents were of analytical grade. Sep-Pak C_{18} column was purchased from Waters Assoc., Milford, Mass., U.S.A.

Urine samples were obtained from healthy volunteers (aged 23—41 years) who had not taken any drugs for at least four weeks prior to the study and from patients on anticonvulsant drug therapy. The samples were stored frozen at −20 °C, and assayed after centrifugation at 4000 rpm for about 30 min.

Apparatus and Chromatographic Conditions——The chromatograph assembly (Shimadzu Corp., Kyoto, Japan) consisted of a liquid chromatograph (model LC-4A) equipped with a sample injection system (model SIL-2AS) and a variable-wavelength detector (model SPD-2AS). The column was a Radial-Pak Nova-Pak C_{18} column (particle size 5 μm, 100 × 8 mm i.d., Z-Module radial compression system) obtained from Waters Assoc. The mobile phase adopted was a mixture of 0.01 M potassium dihydrogen phosphate–acetonitrile–methanol (935 : 55 : 10) containing 0.005% trichloroacetic acid, and the flow rate was 4.5 ml/min. The solvent was ultrasonicated before use. The column effluent was monitored at 243.5 nm. HPLC analysis was performed at ambient temperature.

A μ-Bondapak C_{18} column (particle size 8—10 μm, 300 × 3.9 mm i.d.) obtained from Waters Assoc. and a Cosmosil 5SL column (particle size 5 μm, 150 × 4.6 mm i.d.) from Nakarai Chemicals Co., Ltd., besides the Radial-Pak Nova-Pak C_{18} column, were used for determination of the relative phase capacity ratio of 6β-OHF.

Assay Procedure of 6β-OHF——To 10 ml of the urine sample was added 0.3 ml of a 35 μg/ml aqueous solution of the internal standard, 6β-OHE. The mixture was loaded onto a Sep-Pak C_{18} column, and the column was washed by passing 10 ml of distilled water. 6β-OHF and the internal standard were eluted from the column with 5 ml of ethyl acetate. The eluent was washed successively with 2 ml of 1.0 N sodium hydroxide containing 20% sodium sulfate and 2 ml of 1.0% acetic acid containing 20% sodium sulfate, and evaporated to dryness at 37 °C under reduced pressure. The resultant residue was dissolved in 0.2 ml of ethanol, an aliquot of the solution was injected into the HPLC column, and the peak area ratio of 6β-OHF to the internal standard was measured.

Assay Procedure of 17-OHCS——17-OHCS (17,21-dihydroxy-20-ketosteroids; Porter-Silber chromogens) were determined by the method of Nishikaze and Furuya[6] which is an improvement of the original Porter–Silber method.[7] β-Glucuronidase was used for hydrolysis of glucuronides.

Results and Discussion

Typical chromatograms of normal human urine samples with and without the internal standard and of urine samples from epileptic patients on anticonvulsant drug therapy are shown in Figs. 1 and 2. We chose 6β-OHE as an internal standard because this compound had a suitable retention time and no interfering peak was observed at the retention time of this compound. As for 6β-OHF, a single peak was observed and was identified from its retention time. Confirmation was obtained by the method of Goto *et al.*[8] The eluate corresponding to the peak on the chromatogram was collected and, after the addition of the internal standard, was subjected to HPLC under four different conditions. As shown in Table I, the relative phase capacity ratios (k') of 6β-OHF in urine were identical with those of the authentic sample. Moreover, the peak area ratios of 6β-OHF to the internal standard were almost identical under the three different HPLC conditions. These results imply that the present method is not subject to interference by coexisting substances and is suitable for the determination of 6β-OHF in urine.

Standard solutions containing known amounts of 6β-OHF (0.15—5.0 μg/ml) were analyzed by the assay procedure. A calibration curve was obtained by plotting the ratio of peak area of 6β-OHF to that of the internal standard against the amount of 6β-OHF. The calibration curve was linear over the concentration range examined and passed through the origin.

To check the recovery, urine samples spiked with known amounts of 6β-OHF were analyzed by the assay procedure. The results are shown in Table II. The recovery of 6β-OHF added to urine was almost complete, and was independent of the amount added. The reproducibility of the method was also assessed by repeated analysis of urine samples

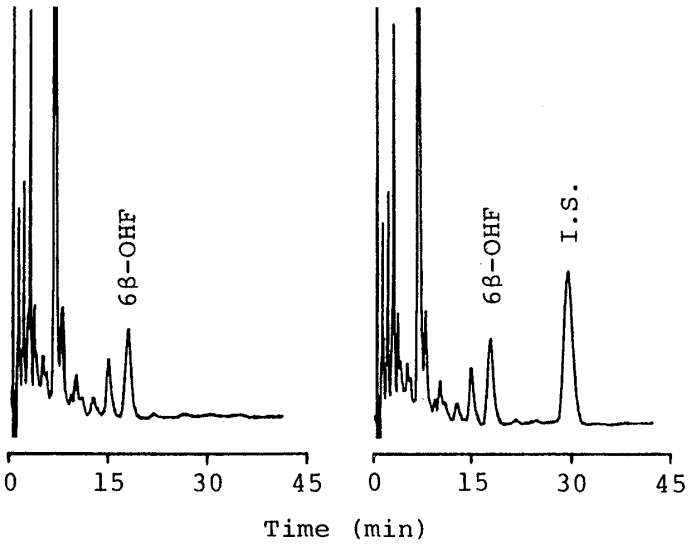

Fig. 1. Chromatograms of Normal Human Urine Samples with and without Internal Standard (I.S.)

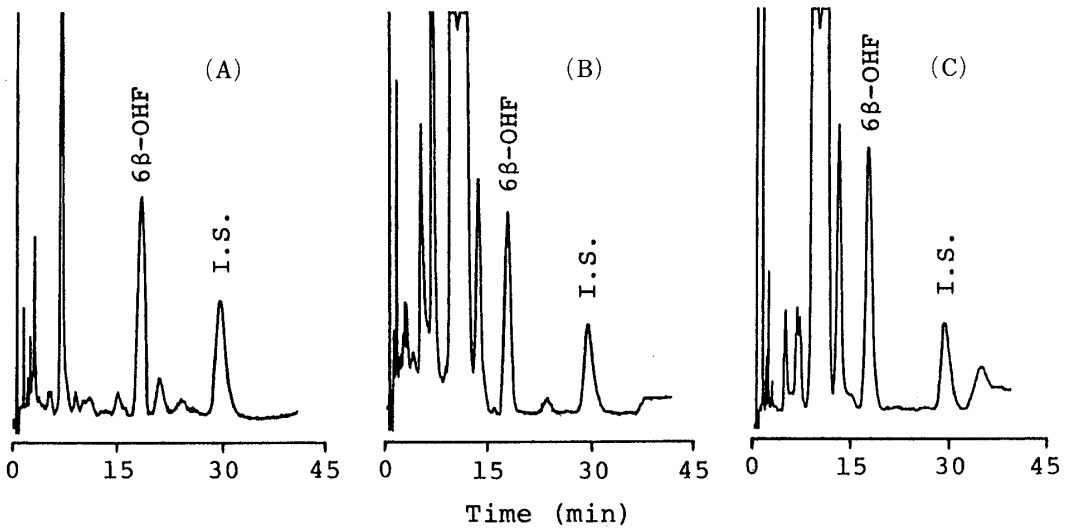

Fig. 2. Typical Chromatograms of Urine Samples from Epileptic Patients on Chronic Therapy with PB, PHT, VPA, CZP and AZM (A), with CBZ, PHT and NZP (B), and with CBZ, ESM, PB and PHT (C)

CBZ, carbamazepine; ESM, ethosuximide; PB, phenobarbital; PHT, phenytoin; VPA, valproic acid; CZP, clonazepam; NZP, nitrazepam; AZM, acetazolamide.

TABLE I. k' Values and Peak Area Ratios of 6β-OHF in Urine Relative to Internal Standard

	k' value				Peak area ratio			
	A	B	C	D	A	B	C	D
6β-OHF	0.61	0.60	0.78	1.87				
Urine sample	0.61	0.60	0.78	1.87	—	0.84	0.85	0.84

Conditions: A) Radial-Pak Nova-Pak C_{18}, 0.01 M potassium dihydrogen phosphate–acetonitrile–methanol (935 : 55 : 10) containing 0.005% trichloroacetic acid, 4.5 ml/min; B) μ-Bondapak C_{18}, acetonitrile–water (1 : 9), 1 ml/min; C) μ-Bondapak C_{18}, methanol–water (2 : 3), 0.5 ml/min; D) Cosmosil 5SL, methylene chloride–methanol–ethanol–water (928 : 40 : 20 : 12), 0.5 ml/min.

TABLE II. Recovery of 6β-OHF Added to Urine

Added (μg/ml)	Urine No.	Found (μg/ml)	Recovered (μg/ml)	Recovery (%)	Mean ± S.D.
None	1	0.248			
	2	0.332			
	3	0.456			
	4	0.581			
	5	0.664			
0.2	1	0.452	0.204	102.0	
	2	0.537	0.205	102.5	
	3	0.646	0.190	95.0	98.4 ± 3.6
	4	0.774	0.193	96.5	
	5	0.856	0.192	96.0	
0.6	1	0.829	0.581	96.8	
	2	0.943	0.611	101.8	
	3	1.084	0.628	104.7	100.6 ± 3.3
	4	1.166	0.585	97.5	
	5	1.276	0.612	102.0	
2.0	1	2.283	2.035	101.8	
	2	2.297	1.965	98.3	
	3	2.401	1.945	97.3	99.7 ± 3.1
	4	2.663	2.082	104.1	
	5	2.606	1.942	97.1	
4.0	1	4.204	3.956	98.9	
	2	4.454	4.122	103.1	
	3	4.340	3.884	97.1	101.1 ± 3.2
	4	4.629	4.048	101.2	
	5	4.862	4.198	105.0	

TABLE III. Reproducibility in the Determination of Urinary 6β-OHF

Added (μg/ml)	Within-run ($n=5$)		Between-run ($n=5$)	
	Found[a] (μg/ml)	C.V. (%)	Found[a] (μg/ml)	C.V. (%)
None	0.267 ± 0.006	2.25	0.280 ± 0.008	3.11
0.3	0.579 ± 0.013	2.21	0.574 ± 0.016	2.82
0.6	0.878 ± 0.018	2.06	0.882 ± 0.026	2.99

a) Values are given as means ± S.D.

containing three different concentrations of 6β-OHF. As shown in Table III, within-run variation ranged from 2.06 to 2.25% and between-run variation from 2.82 to 3.11%. Thus, the method was proved to be highly reproducible.

Daily excretion of 6β-OHF by 10 healthy volunteers is shown in Table IV. In addition, 17-OHCS excretion was measured for calculation of the ratio of 6β-OHF to 17-OHCS. The mean excretion rate of 6β-OHF was 214 μg/d (S.D. = 41.4) and the coefficient of variation (C.V.) was 19.3%. The mean ratio of 6β-OHF to 17-OHCS was 0.0310 (S.D. = 0.00662) and the C.V. was 21.4%.

In general, the 24-hour urinary output of 6β-OHF is measured to eliminate the error introduced by the diurnal variation in adrenal cortisol production. Recently, it was reported

TABLE IV. 6β-OHF and 17-OHCS Excretions and Their Ratio in 24-h Urine Samples from Healthy Volunteers

Subject No.	Age	Sex	6β-OHF (μg/d)	17-OHCS (μg/d)	6β-OHF / 17-OHCS
1	28	M	180	8890	0.0202
2	23	M	295	12140	0.0243
3	25	M	266	10900	0.0244
4	34	M	189	7010	0.0270
5	41	M	200	6380	0.0313
6	29	M	195	5730	0.0340
7	34	M	234	6690	0.0350
8	30	M	176	4910	0.0358
9	23	M	174	4680	0.0372
10	32	M	233	5780	0.0403
Mean			214	7310	0.0310
S.D.			41.4	2530	0.00662
C.V. (%)			19.3	34.6	21.4

TABLE V. Ratio of 6β-OHF to 17-OHCS in Random Urine Samples from Healthy Volunteers

Subject No.	Age	Sex	6β-OHF / 17-OHCS	Subject No.	Age	Sex	6β-OHF / 17-OHCS
1	23	M	0.0209	8	32	M	0.0388
2	25	M	0.0227	9	28	M	0.0429
3	34	M	0.0272	10	29	M	0.0447
4	30	M	0.0272				
5	34	M	0.0280	Mean			0.0318
6	36	M	0.0318	S.D.			0.00816
7	41	M	0.0335	C.V. (%)			25.7

that the diurnal variation in the excretion of 6β-OHF parallels the diurnal variation in the excretion of 17-OHCS.[9] As shown in Table V, the mean ratio of 6β-OHF to 17-OHCS in randomly obtained urine specimens from healthy volunteers was found to be 0.0318 (S.D. = 0.00816; $n=10$) and to be in good agreement with that in the 24-h urine samples mentioned above, though the variation was slightly higher.

Many techniques have been described for 6β-OHF determination in urine. The normal values for 6β-OHF obtained by paper chromatography (PC),[10-12] thin layer chromatography (TLC),[3,13-16] and column chromatography (CC)[17,18] appeared to be higher than those by gas-liquid chromatography (GLC),[19] HPLC[20] and radioimmunoassay (RIA),[21,22] since the fluorometric or colorimetric reactions used were not specific enough. Moreover, the methods based upon PC, TLC and CC are complicated and time-consuming. GLC and RIA also have disadvantages, that is to say, GLC requires prior derivatization of the sample and RIA requires highly specific antiserum. As regards HPLC, three methods have been developed by Roots *et al.*,[20] Lodovici *et al.*[23] and Goto *et al.*[8] The method of Goto *et al.* is sensitive and reproducible, but is somewhat complicated because it requires derivatization of the sample and removal of the excess reagent. Our proposed method has advantages over the methods of Roots *et al.* and Lodovici *et al.*, mainly due to the considerably higher recovery than the values of 75% (S.D. = 4.4; $n=20$) reported by Roots *et al.* and 91.5% (S.E.M. = 0.8;

$n=16$) by Lodovici *et al.* Moreover, Lodovici *et al.* have not investigated the applicability of their method to urine samples from patients on drug therapy, and indeed we observed interfering chromatographic peaks at the retention time of 6β-OHF when urine samples from patients on carbamazepine therapy were analyzed by their method. In contrast, there was no interference when urine samples from patients receiving any of carbamazepine, ethosuximide, phenobarbital, phenytoin, valproic acid, clonazepam, nitrazepam and acetazolamide were analyzed by our method. Furthermore, the Sep-Pak C_{18} column extraction method adopted is simpler and faster than those of Roots *et al.* and Lodovici *et al.*

In conclusion, our newly developed method is applicable to the routine assay of urinary 6β-OHF in clinical studies.

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