Determination of 21-Hydroxycorticosteroids in Human Urine by High-Performance Liquid Chromatography with Fluorescence Detection

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A simple and sensitive high-performance liquid chromatographic method with fluorescence detection for the determination of nineteen 21-hydroxycorticosteroids is described. The corticosteroids are oxidized by cupric acetate to form the corresponding glyoxal derivatives. The derivatives are converted into fluorescent quinoxalines by reaction with 1,2-diamino-4,5-methylenedioxybenzene, a fluorogenic reagent for α-dicarbonyl compounds. The quinoxalines are separated within 70 min on a reversed-phase column (TSK gel ODS-120T) by stepwise elution with mixtures of methanol, acetonitrile, and 1.0 M ammonium acetate. The detection limits are 0.14—29.4 pmol at a signal-to-noise ratio of 3 in a 50-μl injection volume. This sensitivity permits precise determination of hydrocortisone, cortisone, corticosterone, and their tetrahydro derivatives in 500 μl of normal human urine.

Keywords 21-hydroxycorticosteroid; human urine; HPLC; fluorescence detection; 1,2-diamino-4,5-methylenedioxybenzene; quinoxaline derivative

21-Hydroxycorticosteroids and their major metabolites, tetrahydrocorticosteroids, occur in human urine as free and conjugated forms. Their measurement is very useful for screening of abnormalities in adrenocortical function.[1] Various methods, including column,[2] thin-layer,[3] paper,[4,5] gas (GC)[1,6-8] and high-performance liquid (HPLC)[9-12] chromatography and GC-mass spectrometry,[13] have been proposed for the determination of 21-hydroxycorticosteroid and/or their tetrahydro derivatives in human urine.

We have previously reported a fluorimetric HPLC method for the determination of hydrocortisone, cortisone and corticosterone in human plasma.[14] The method is based on the conversion of the corticosteroids into the corresponding glyoxal compounds, followed by the fluorescence derivatization of the glyoxal compounds by reaction with 1,2-diamino-4,5-methylenedioxybenzene (DMB), a fluorogenic reagent for α-dicarbonyl compounds.[15] The purpose of the present research is to establish a sensitive, simple and rapid HPLC method utilizing DMB for the determination of free and total (the sum of free and conjugated) 21-hydroxycorticosteroids in human urine. Prednisone (PRED) was used as an internal standard.

Experimental

Chemicals and Solutions All chemicals and solvents were of analytical-reagent grade, unless otherwise stated. Deionized and distilled water was passed through a Milli-QII system (Japan, Millipore Ltd., Tokyo, Japan). Aldosterone (ALD), hydrocortisone (F), PRED, cortisone (E), 18-hydroxycorticosterone (18-OHB), 18-hydroxydeoxycorticosterone (18-OHDOC), corticosterone (B), 11-deoxycortisol (S), deoxycorticosterone (DOC), 11-dehydrocorticosterone (A), 3α,5β-tetrahydroaldosterone (THALD), 3α,5β- and 3α,5α-tetrahydrocortisol (THF and allo-THF, respectively), 3α,5β-tetrahydrocortisone (THE), 3α,5β-tetrahydro-11-deoxycortisol (THS), 3α,5β- and 3α,5α-tetrahydrocorticosterone (THB and allo-THB), 3α,5β- and 3α,5α-tetrahydro-11-deoxycorticosterone (THDOC and allo-THDOC, respectively) were purchased from Sigma (St. Louis, Mo., U.S.A.). DMB was prepared as described previously;[16] it is now commercially available from Dojindo Labs. (Kumamoto, Japan).

Enzyme solution (β-glucuronidase/arylsulfatase from *Helix pomatia*) was purchased from Boehringer Mannheim-Yamanouchi Co. (Tokyo, Japan). The solution contains *ca.* 100000 Fishman units of β-glucuronidase and *ca.* 800000 Roy units of arylsulfatase/ml.

DMB solution (7.0 mM) was prepared by dissolving DMB in water containing 0.2 M β-mercaptoethanol and 0.25 M sodium hydrosulfite. The solution was stored at 4 °C in the dark; under the conditions used, it was stable for at least two weeks. Cupric acetate solution (39 mM) was prepared by dissolving 0.7 g of cupric acetate in 10 ml of water and diluting the solution with methanol to 100 ml. The solution was used within one month after preparation. PRED (internal standard) solutions were prepared in methanol; 0.60 and 4.0 μM were used for the free and total steroid assays, respectively. The solutions could be used for at least one month.

HPLC Apparatus and Conditions A Hitachi 655A high-performance liquid chromatograph was used. This was equipped with a Rheodyne 7125 syringe-loading sample injector valve (50-μl loop) and a Shimadzu RF-535 fluorescence spectromonitor fitted with a 12-μl flow-cell. It was operated at an excitation wavelength of 350 nm and an emission wavelength of 390 nm. The column was a TSK gel ODS-120T (250 × 4.6 mm i.d.; particle size, 5 μm; Tosoh Co., Tokyo, Japan). This column could be used for more than 1000 injections with only a small decrease in the theoretical plate number. The column temperature was ambient (*ca.* 20 °C). For the separation of the DMB derivatives of the corticosteroids, stepwise gradient elution with mixtures of methanol–acetonitrile–1.0 M ammonium acetate [49 : 13 : 38, 66 : 6 : 28, and 82 : 8 : 10 (v/v); mobile phases A, B, and C, respectively] was carried out with a Hitachi 833A solvent gradient device. Mobile phase A was used first for 27 min, mobile phase B for the following 22 min and mobile phase C for the next 21 min (Fig. 1). The column was equilibrated with mobile phase A for 20 min before the start of the next sample. The flow-rate was kept constant at 1.0 ml/min. Peak areas were determined by a Waters QA-1 Data System.

Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with 20-μl flow-cell; the spectral bandwidths were 5 nm for both the excitation and emission monochromators.

Urine Samples Urine (24 h) from healthy volunteers in our laboratories was collected without preservatives. The urine was frozen on dry ice immediately after collection. The urine was hydrolyzed with β-glucuronidase/arylsulfatase in the usual manner for the total corticosteroid assay.[12]

The sample solution for the determination of total corticosteroids was prepared as follows. A 500-μl aliquot of urine was mixed with 50 μl of 0.5 M acetate buffer (pH 5.0) and 40 μl of the enzyme solution, and the mixture was incubated for 24 h at 37 °C. To the resulting solution, 100 μl of the PRED solution and 6 ml of diethyl ether–dichloromethane (6 : 4, v/v) were added. The mixture was vortexed for *ca.* 2 min and centrifuged at 1000 *g* for 5 min. The organic layer (*ca.* 4 ml) was transferred into a screw-capped 10-ml vial and was evaporated to dryness under a nitrogen gas stream. The residue, dissolved in 100 μl of methanol, was used as a sample solution. For the determination of free steroids, the same procedure was carried out except that the hydrolysis with the enzyme solution was omitted.

Derivatization Procedure A 100-μl portion of the sample solution, placed in a screw-capped 1-ml vial, was mixed with 20 μl of the cupric acetate solution. The mixture was allowed to stand at room temperature for 1 h. Then, 100 μl of the DMB solution was added and the mixture was heated at 60 °C for 40 min. After cooling, the mixture was centrifuged at 1000 *g* for 5 min, and 50 μl of the supernatant was injected into the chromatograph.

The calibration graphs were prepared according to the standard procedure except that 100 μl of the PRED solution was replaced by the PRED solution containing 1.0 pmol to 30.0 nmol each of the individual cortico-

steroids. The net peak-area ratios of the individual corticosteroids and PRED were plotted against the concentrations of the corticosteroids spiked.

Results and Discussion

HPLC Conditions The best separation of the DMB derivatives of the nineteen 21-hydroxycorticosteroids of biological importance was achieved on a reversed-phase column, TSK gel ODS-120T, by stepwise elution with mixtures of methanol, acetonitrile, and 1.0 M ammonium acetate within 70 min. However, the peaks for THF and allo-THF, and DOC and THS could not be resolved successfully under any HPLC conditions tested. A typical chromatogram obtained with a standard solution of the corticosteroids is shown in Fig. 1. The change in methanol and acetonitrile concentrations actually had no effect on the fluorescence excitation (maximum, 350 nm) or emission (maximum, 390 nm) spectra or the fluorescence intensities of the derivatives of any of the corticosteroids. The individual corticosteroids gave single peaks in the chromatogram. Reproducible retention times were obtained for all the corticosteroids tested under the present HPLC conditions (Table I).

Derivatization Conditions 21-Hydroxycorticosteroids were easily oxidized by cupric acetate to form the corresponding glyoxal compounds as described previously.[14,17]

The derivatization conditions for the 9 corticosteroids (excluding tetrahydrocorticosteroids) were described previously.[14] In this study, the conditions were examined using a mixture of the 10 tetrahydrocorticosteroids. The resulting optimal conditions were similar to those for the other corticosteroids described previously.[14]

Validation of the Derivatization Procedure The relationships between the peak areas and the amounts of the nineteen individual corticosteroids were linear from 100 fmol to at least 10.0 nmol per injection volume (50 μl). The linear correlation coefficients were 0.992 or better for all the corticosteroids. The detection limits were 0.14—29.4 pmol (50 μl injection volume) at a signal-to-noise ratio of 3 (Table I). The relatively low sensitivities for 18-OHB and 18-OHDOC may be due to the following reason. The 21-aldehyde, formed after the cupric acetate oxidation of the 21-hydroxy group, or 20-carbonyl group in these compounds undergoes an intramolecular reaction with the 18-hydroxy group to form a cyclic ketal derivative, which allows only a few percent of the glyoxal to react with DMB.[14]

Urinary steroids (progesterone, androstenedione, pregnenolone, estrone, estradiol, and estriol) other than 21-hydroxycorticosteroids did not give fluorescence under the present derivatization conditions. Some α-dicarbonyl compounds such as glyoxal, methylglyoxal, pyruvic and α-ketoglutaric acids, commonly present in human urine, reacted with DMB to produce fluorescent derivatives. The fluorescence, however, was extremely weak and the retention times of the derivatives were different from those for the corticosteroids. Thus, those compounds did not interfere with the determination of the corticosteroids.

Application to Human Urine 21-Hydroxycorticosteroids in human urine were extracted with a mixture of diethyl ether–dichloromethane (6:4, v/v) in the usual manner. In the present study, a maximum and constant ratio of extraction of the corticosteroids was achieved with more than 5.0 ml of diethyl ether–dichloromethane for both the free and total corticosteroid determinations; a 6-ml aliquot was used.

Figures 2A and B show typical chromatograms obtained

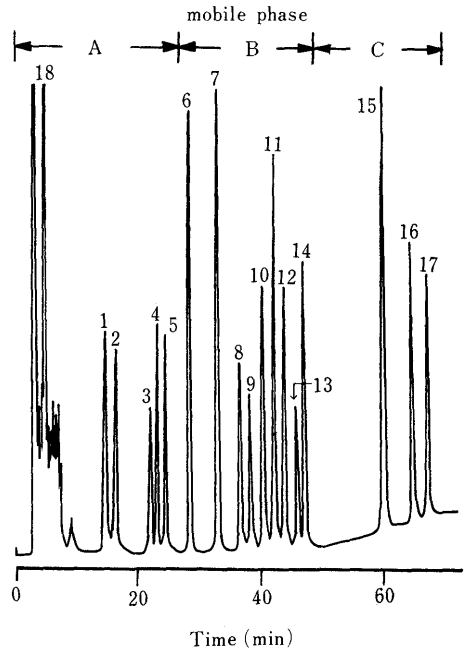

Fig. 1. Chromatogram of the DMB Derivatives of 21-Hydroxycorticosteroids

A portion (100 μl) of standard mixture of the corticosteroids (18-OHB and 18-OHDOC, 50 nmol/ml; the others, 1.0 nmol/ml each) was treated according to the standard procedure. Peaks: 1=THALD, 2=ALD, 3=F, 4=PRED, 5=E, 6=18-OHB, 7=THF (and allo-THF), 8=A, 9=18-OHDOC, 10=THE, 11=B, 12=THB, 13=S, 14=allo-THB, 15=DOC (and THS), 16=THDOC, 17=allo-THDOC, 18=blank components.

Table I. Retention Time, Detection Limit, Recovery of 21-Hydroxycorticosteroids and Within-Day Precision of the Method

Compound	Retention time (min) (mean ± S.D.)	Detection limit (pmol/50 μl)	Recovery[a] (%)	Precision[b] (R.S.D.[c] %) Free	Total
THALD	14.6 ± 0.4	0.45	92	ND[d]	ND[d]
ALD	16.1 ± 0.3	0.51	95	ND[d]	ND[d]
F	22.2 ± 0.4	0.71	100	3.8	2.3
PRED	23.4 ± 0.4	0.45	92	IS[e]	IS[e]
E	24.6 ± 0.4	0.45	102	4.3	2.3
18-OHB	28.3 ± 0.4	9.20	96	ND[d]	ND[d]
THF	33.2 ± 0.5	0.37	98	4.3[f]	2.3[f]
Allo-THF	33.2 ± 0.5	0.37	98		
A	37.7 ± 0.5	0.53	93	ND[d]	ND[d]
18-OHDOC	38.3 ± 0.5	29.40	92	ND[d]	ND[d]
THE	40.2 ± 0.5	0.35	98	4.8	2.5
B	42.2 ± 0.6	0.14	99	ND[d]	ND[d]
THB	43.9 ± 0.6	0.34	95	ND[d]	3.7
S	45.7 ± 0.7	0.69	100	ND[d]	ND[d]
Allo-THB	46.9 ± 0.7	0.30	94	ND[d]	3.4
DOC	59.7 ± 1.4	0.21	94	ND[d]	ND[d]
THS	59.7 ± 1.5	0.34	95	ND[d]	ND[d]
THDOC	64.3 ± 1.6	0.36	98	ND[d]	ND[d]
Allo-THDOC	66.9 ± 1.6	0.42	100	ND[d]	ND[d]

a) Recoveries were obtained from the hydrolyzed urine spiked with a standard mixture (see the text). *b*) Repeated determinations (n=10) of 21-hydroxycorticosteroids were carried out. *c*) Relative standard deviation. *d*) Not detected. *e*) Internal standard. *f*) F+allo-THF.

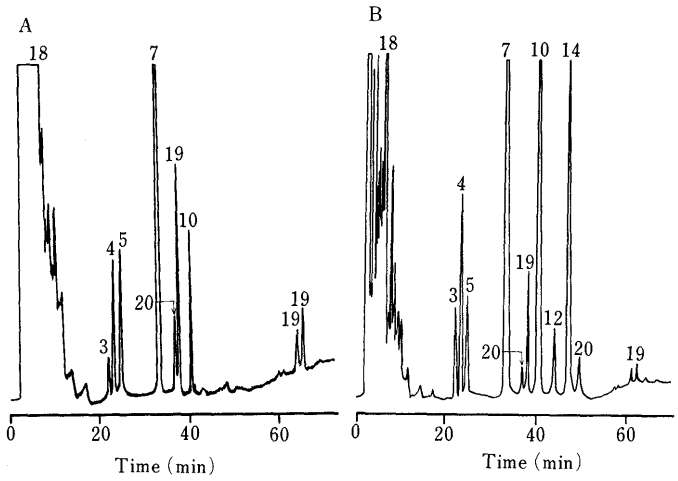

Fig. 2. Chromatograms Obtained with Healthy Human Urine (A) before and (B) after Hydrolysis

Portions (500 μl) of the urine were treated according to the standard procedure. For peaks 3—18, see Fig. 1. Peaks 19 and 20, unknown endogenous substances in human urine.

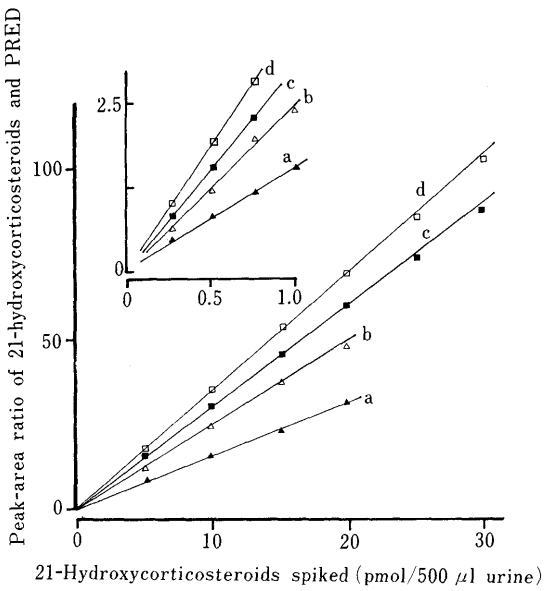

Fig. 3. Calibration Graphs of 21-Hydroxycorticosteroids for the Total Corticosteroid Assay

Curves: a, F; b, E; c, THF or allo-THF; d, allo-THB. The graphs for THE and THB are similar to curve c.

with pooled human urines before and after hydrolysis with β-glucuronidase/arylsulfatase, respectively. Peaks 3, 5, 7, 10, 12 and 14 in Fig. 2 were identified as the peaks for F, E, THF (and allo-THF), THE, THB and allo-THB, respectively, on the basis of their retention times and fluorescence excitation and emission spectra. This was achieved by comparison of the spectra with those of standard compounds, and also by co-chromatography of the standards and the urine samples with aqueous 50—100% (v/v) methanol (or acetonitrile) as the mobile phase.

Peak 19 may be due to endogenous α-dicarbonyl compounds in human urine. This was suggested by the following results. When the urine sample was replaced with water, no peaks except for blank components (peak 18) were detected in the chromatogram. Moreover, each eluate from peak 19 exhibited fluorescence excitation and emission maxima around 350 and 390 nm, almost identical with those of the DMB derivatives of the corticosteroids. Even when the oxidation step with cupric acetate was omitted, peak 19 was observed in the chromatogram. On the other hand, peak 20 appeared when the oxidation with cupric acetate was carried out. The peaks did not interfere with the determination of the corticosteroids in human urine.

In the calibration graphs, linear relationships were observed between the ratios of the peak areas of the corticosteroids to that of PRED and the amounts of the corticosteroids added in the range from 1.0 pmol to 30.0 nmol each to 500 μl of normal human urine (Fig. 3). Recovery tests were performed by adding known amounts (10.0 nmol each) of the corticosteroids to the hydrolyzed urine (500 μl). The recoveries were in the range of 94—102% for all the corticosteroids (Table I).

The within-day precision was established by repeated determinations ($n=10$) using a normal human urine (subject No. 1 in Table II). The relative standard deviations did not exceed 4.8% for both free and total corticosteroids assays (Table I).

Determination of 21-Hydroxycorticosteroids of Clinical Importance, in Human Urine The amounts of F, E, THF (and allo-THF), THE, THB and allo-THB in urine from healthy volunteers were determined by the described method (Table II). The mean values for the corticosteroids were in reasonable agreement with those obtained by other workers.[1-13]

TABLE II. Urinary Excretion (μg per 24 h) of Free and Total 21-Hydroxycorticosteroids in Urines from Healthy Volunteers

Subject	Sex	Age		F	E	THF (Allo-THF)	THE	THB	Allo-THB
1	M	22	Free	21.4	44.1	107.6	40.8	ND[a]	ND[a]
			Total	245.3	157.2	1416.5	1220.5	82.6	262.9
2	M	22	Free	27.7	71.0	32.0	90.6	ND[a]	ND[a]
			Total	97.3	365.0	1751.9	2964.0	175.9	230.8
3	M	25	Free	28.4	26.6	104.8	53.5	ND[a]	ND[a]
			Total	191.5	113.8	1247.4	1272.8	76.9	297.4
4	M	40	Free	83.0	108.5	32.0	62.4	ND[a]	ND[a]
			Total	199.4	369.2	1407.1	1848.6	124.2	348.8
5	M	31	Free	15.7	105.5	28.3	44.0	ND[a]	ND[a]
			Total	124.9	425.9	1362.8	2173.7	238.6	683.9
6	M	40	Free	35.5	85.0	57.7	90.6	ND[a]	ND[a]
			Total	91.6	259.1	2686.2	4851.6	259.6	565.1
7	M	62	Free	25.5	91.5	41.3	70.0	ND[a]	ND[a]
			Total	104.4	256.7	2259.7	2247.5	121.8	314.3
8	F	22	Free	18.0	35.4	57.8	50.2	ND[a]	ND[a]
			Total	308.8	235.0	2029.2	1460.3	238.6	583.4
9	F	23	Free	35.8	94.1	126.5	94.5	ND[a]	ND[a]
			Total	223.9	469.0	1383.7	2869.3	295.3	850.7
10	F	25	Free	17.9	46.4	97.0	111.9	ND[a]	ND[a]
			Total	169.6	307.4	2722.7	1719.2	338.0	558.8
Mean			Free	30.9	70.8	68.5	70.9	ND[a]	ND[a]
			Total	175.7	295.8	1848.7	1996.0	164.7	470.1
S.D.			Free	19.6	30.4	36.9	24.6	—	—
			Total	75.2	113.6	548.1	1220.7	89.5	207.6

a) Not detected.

The study has provided the first HPLC method with fluorescence detection for the simultaneous determination of F, E, THF (and allo-THF), THE, THB and allo-THB in human urine. The method is sensitive enough to measure

the corticosteroid in 500 μl of normal urine. The method is readily performed and could therefore be useful for evaluation of pituitary and adrenal functions for diagnostic purposes.

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