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Simultaneous determination of glucocorticoids in plasma or urine by high-performance liquid chromatography with precolumn fluorimetric derivatization by 9-anthroyl nitrile

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Abstract

A new method for simultaneous determination of glucocorticoids (GCs) in plasma or urine by high-performance liquid chromatography (HPLC) with fluorimetric detection has been developed. Following extraction with ethyl acetate using a reversed-phase disposable cartridge, the six GCs [cortisol (F), cortisone (E), prednisolone (PL), prednisone (PN), 6β-hydroxycortisol (6β-OHF) and 6β-hydroxyprednisolone (6β-OHP)] and an internal standard (6β-hydroxycotortisone) were derivatized by treatment with 9-anthroyl nitrile (9-AN) in a mixture of basic catalysts (triethylamine and quinuclidine) to give the fluorescent esters through the 21-hydroxyl group. The GC derivatives so obtained were then cleaned by a straight-phase disposable cartridge and chromatographed on a straight-phase column with an isocratic HPLC technique. The fluorescence derivatives of the GCs, including the internal standard, were separated as clear single peaks and no interfering peaks were observed on the chromatograms. The lower limits of detection for F, E, PL and PN in plasma or urine were 0.1 ng/ml and those for 6β-OHF and 6β-OHP in plasma or urine were 0.5 ng/ml, at a signal-to-noise ratio of 3. The analytical recovery of known amounts of the GCs added to plasma or urine were almost 100%. This method can be applied to the determination of plasma or urinary F in renal transplant patients who received PL and can be applied for other metabolic investigations in relation to the change in blood pressure via 11β-hydroxysteroid dehydrogenase or in hepatic metabolizing via CYP3A4.

Keywords: Glucocorticoids; Cortisol; Cortisone; Prednisolone; Prednisone; 6β-Hydroxycortisol; 6β-Hydroxyprednisolone

1. Introduction

Several high-performance liquid chromatographic (HPLC) methods for quantification of glucocorticoids (GCs) in plasma or urine with UV detection have been developed and have been used for many pharmacokinetic and pharmacodynamic studies [1–3]. The HPLC methods with UV detection of cortisol (F) in biological fluid, however, are not sufficiently sensitive, because the limits of detection (LODs) are 3–5 ng/ml [4–9]. Therefore, plasma F obtained from a patient who received prednisolone (PL) could not be detected with sufficient sensitivity. However, to increase sensitivity in the lower LOD, a fluorimetric

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HPLC method for quantification of F with precolumn derivatization by dansylhydrazine has been developed [10,11]. However, by this method, PL and prednisone (PN) can not be detected, because the reaction activity of PL and PN with dansylhydrazine is very poor [12].

Recently, Goto et al. [13,14] reported that a new type of fluorescent derivatization reagent, namely, 9-anthroyl nitrile (9-AN) generated fluorescent esters through the primary hydroxyl group at the 21st position of the carbon chain in F, cortisone (E), PL and 6β-hydroxycortisol (6β-OHF) and they described respective HPLC methods with 9-AN for F, E and PL (an internal standard) in serum [13] or for 6β-OHF in urine [14]. However, there is no HPLC method for the simultaneous determination of F, E, 6β-OHF and 6β-hydroxyprednisolone (6β-OHP) in plasma or urine from a patient who has received PL.

We describe a highly sensitive and specific HPLC method of simultaneous determination of F, E, PL, PN, 6β-OHF and 6β-OHP in human plasma or urine with precolumn fluorimetric detection by 9-AN. Using this method, we determined these GCs in plasma or urine from renal transplant patients who received immunosuppressive therapy with PL, cyclosporine and azathiopurine.

2. Experimental

2.1. Materials and reagents

F, E, PL, PN, molecular sieves 4A (bees type), anhydrous acetonitrile and a solid-phase disposable column for sample preparation, Sep-Pak Plus C_{18} or Sep-Pak Plus Silica were purchased from Nacalai Tesque (Kyoto, Japan). 6β-Hydroxycortisone (6β-OHE) as an internal standard (I.S.) was purchased from Sigma (St. Louis, MO, USA). 6β-OHF and 6β-OHP were purchased from Steraloids (Wilton, NH, USA). Quinuclidine and 9-AN were purchased from Funakoshi (Tokyo, Japan). Organic solvents used for the HPLC mobile phase were of liquid chromatographic grade. All other chemicals and organic solvents were of reagent grade and an adequate amount of molecular sieves 4A was added to the organic solvents to remove moisture before use.

2.2. Preparation of standards

Reagent solutions of quinuclidine, triethylamine and 9-AN were prepared by dissolving them in anhydrous acetonitrile and adding an adequate amount of molecular sieves 4A to remove moisture to obtain final concentrations of 0.1% (w/v), 10% (v/v) and 0.02% (w/v), respectively. Stock solutions of GCs for calibration curves and the I.S. (1 μg/ml) were prepared by dissolving in ethanol, adding adequate amount of molecular sieves 4A and storing in the dark at 4°C. Plasma or urinary standard samples for the calibration curves were prepared by adding known amounts of GCs to pooled normal human plasma or urine which had been stripped of endogenous GCs using a Sep-Pak Plus C_{18} cartridge. The final ethanol contents in plasma or urinary standards were always less than 1%.

2.3. Extraction procedure

To 400 μl of plasma or urinary sample, 40 ng of 6β-OHE as an I.S. was added and then the sample mixture was loaded on a Sep-Pak Plus C_{18} cartridge which was pretreated with methanol (7 ml) and water (14 ml) before use. After successive washings with water (6 ml), 12% methanol (3 ml) and petroleum ether (3 ml), the desired fraction was obtained by elution with ethyl acetate (5 ml). The extract was dried by evaporation under a reduced pressure at 40°C. The resultant residues were used as sample extracts at the next step for derivatization with 9-AN.

2.4. Derivatization of GCs with 9-AN

To the dried residues extracted from the plasma or urinary samples, a mixture (200 μl) of 10% triethylamine–acetonitrile solution and 0.1% quinuclidine–acetonitrile solution (4:1, v/v) was added and vortexed. Then, 0.02% of 9-AN solution (200 μl) was added with a few molecular sieves 4A, and the mixture was allowed to stand at room temperature (21±1°C) for 30 min. After the evaporation under a reduced pressure at 40°C, the residue was dissolved in acetone (200 μl) and diluted with *n*-hexane (2 ml). The mixture was then loaded in a Sep-Pak Plus Silica cartridge. The cartridge was

washed with 1,2-dichloroethane (14 ml) to remove excess reagent and the desired fraction was obtained by eluting with ethyl acetate (5 ml). After the extracts were dried by evaporation under reduced pressure at 40°C, the resultant residue was reconstituted in HPLC mobile phase (200 μl) and 30–60 μl of the sample was injected into the HPLC system by an automatic sample injector.

2.5. Instrumentation and chromatographic conditions

The HPLC system consisted of an LC-10AS pump, a RF-10AXL fluorescence detector, a C-R6A chart recorder (Shimadzu, Kyoto, Japan) and a Wisp 712B automatic sample inject (Waters, Milford, MA, USA). The HPLC system was regulated by an SCL-10A system controller (Shimadzu). The column used was a straight-phase column, Cosmosil 5SL (250 mm×4.6 mm I.D., 5 μm particle size, Nacalai Tesque, Kyoto, Japan) and chromatographic separation was performed by linking two columns at ambient room temperature. The mobile phase consisted of diethylene dioxide–ethyl acetate–chloroform–*n*-hexane–pyridine (500:100:100:1400:21, v/v). The solvent was degassed before use. The flow-rate of the mobile phase was set at 1.0 ml/min for up to 45 min after sample injection and thereafter the flow-rate was increased by 0.04 ml/min until the flow-rate reached 1.2 ml /min. The column pressure reached 8.0 mPa and the column effluent was monitored at 360 nm (excitation wavelength) and 460 nm (emission wavelength). The quantification of GCs was calculated using the peak-area ratio compared with the I.S.

2.6. Clinical application for renal transplants

Four living related renal transplant patients (A, B, C, D; males, 29.5±13.6 years), who had received immunosuppressive therapy with cyclosporine, PL and azathiopurine, were enrolled into the study. Daily oral dosage of azathiopurine was maintained at 1.5 mg/kg per day throughout the monitoring periods. Daily oral dosage of PL was tapered from 50 mg/day to 10 mg/day at almost 10-day intervals. Cyclosporine was administered to patients in two divided doses with 12-h intervals, which were decreased with suitableness based on the trough level monitoring of cyclosporine. The trough levels were obtained during hospitalization once or twice in week and were measured by means of a fluorescence polarization immunoassay with monoclonal antibody. Plasma samples for GCs measurements were obtained from blood at the trough level monitoring of cyclosporine. For patient D, clinical time-courses of cyclosporine and corresponding 24 h-accumulated urinary samples were obtained at 1, 2, 4, 6 and 8 weeks after renal transplantation. Plasma or urinary GCs were measured using the HPLC method developed.

3. Results

3.1. Optimal condition for derivatization of GCs with 9-AN

It was reported that the acylation of the primary hydroxyl group by 9-AN at the 21st position in the carbon chain of GCs was affected by certain catalysts, reaction time or temperature and moisture content [9,10]. In addition, it was expected that the recovery of 9-AN derivatives of GCs would differ in individual GCs. Therefore, we examined optimal conditions for the derivatization of GCs with 9-AN (Fig. 1). The yield of 9-AN derivatives at room temperature, when 0.1% quinucline was used as a catalyst was greater than at 60°C (Fig. 1A, Fig. 1B). The reaction time for a maximum yield at room temperature, was from 30 min to 60 min, suggesting that the rate of reaction of GCs with 9-AN in the existence of only 0.1% quinuclidine differs in individuals. However, at both reaction temperatures, the fluorofore after maximum reaction time was decreased (Fig. 1A). However, the use of only 10% triethylamine as a catalyst provided constant yields of fluorofore at room temperature after 60 min (Fig. 1C). However, we found that a catalyst mixture consisting of 10% triethylamine–0.1% quinuclidine in a ratio of (4:1, v/v) increased the yields of fluorofore and shortened the maximum reaction time from 60 min to 30 min (Fig. 1D). In addition, the (4:1, v/v) mixture of 10% triethylamine–0.1% quinuclidine provided best separation of derivatives on the chromatograms. From these results, we de-

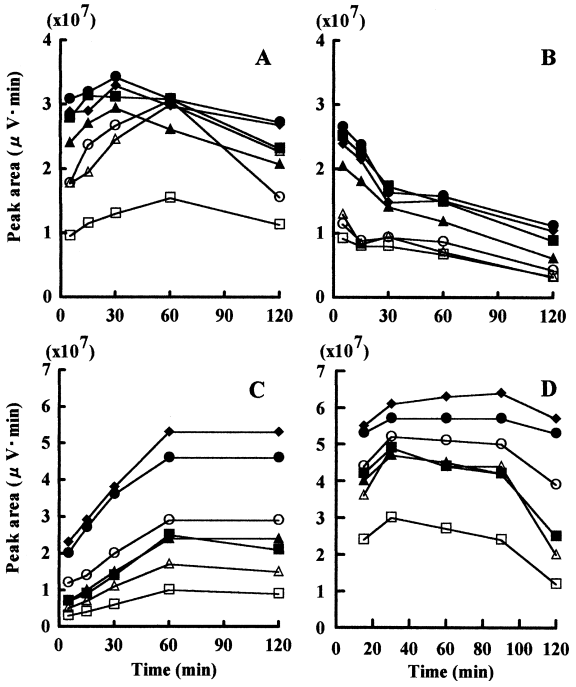

Fig. 1. Effects of reaction time, temperature and basic catalysts on derivatization of GCs with 9-AN. To each test-tube contaning 40 ng per tube of GCs, 100 μl of 10% quinuclidine in acetonitrile solution was added and the reaction was initiated by adding 100 μl of 0.01% 9-AN reagent. The samples were incubated at (A) room temperature (20±1°C) or (B) 60°C for the indicated times. To each test-tube containing 40 ng per tube of GCs, (C) 150 μl of 10% triethylamine in acetonitrile solution or (D) 150 μl mixture of 10% triethylamine and 0.1% quinuclidine in acetonitrile solution (4:1, v/v) were added and the reaction was initiated by adding 150 μl of 0.01% 9-AN reagent. The samples were incubated at room temperature (20±1°C) for the indicated times. After the reaction mixture was treated according to the clean-up method, the resultant residue was chromatographed. Each point represents the mean of three measurements. Key: ■=F; ●=E; ▲=PL; ♦=PN; □=6β-OHF; ○=6β-OHE; △=6β-OHP.

cided that the best reactive conditions for incubation temperature, time and catalyst were room temperature, 30 min, and 10% triethylamine–0.1% quinuclidine (4:1, v/v), respectively.

3.2. HPLC separation of 9-AN derivatives

Fig. 2 shows typical chromatograms for plasma or urinary samples of standard (Fig. 2A, Fig. 2D), a normal human volunteer (Fig. 2B, Fig. 2E) and a renal transplant patient (Fig. 2C, Fig. 2F). The retention times for 9-AN derivatives of F, E, PL, PN, 6β-OHF, 6β-OHP and the I.S. were 24.2, 29.7, 32.4, 40.9, 65.3, 92.6 and 87.6 min, respectively. The F level in plasma of a normal human volunteer and a renal transplant patient were 68.75 ng/ml and 0.46 ng/ml, respectively (Fig. 2B, Fig. 2C). The 6β-OHF in plasma of a normal human volunteer was found to be 3.4 ng/ml (Fig. 2B), but that of the renal transplant patient could not be detected (Fig. 2C). The 6β-OHF/F ratios in 24-h accumulated urine from a normal human volunteer and a renal transplant patient were 4.68 ng/ml and 1.83 ng/ml, respectively (Fig. 2E, Fig. 2F). The individual derivatives were separated as clear, single and symmetrical peaks on the chromatograms and no interfering peaks were detected at the retention times of the derivatives in plasma or urine.

3.3. Calibration curve, precision and reproducibility

Calibration curves for the quantification of GCs in plasma were linear and passed through the origin with a correlation coefficient of 0.997 or better in the range from 0 to 500 ng/ml for F and E and from 0 to 1000 ng/ml for PL, PN and 6β-OHP. The calibration curves for urinary F, E and 6β-OHF were also linear and passed through the origin with a correlation coefficient of 0.999 or better in the range from 0 to 500 ng/ml. However, the calibration curves for urinary PL, PN and 6β-OHP exhibited nonlinear relationships in the range from 0 to 5000 ng/ml. Therefore, a Michaelis–Menten type function was applied to these calibrations and their correlation coefficients were more than 0.999. The accuracy and precision of the methods were evaluated by analyzing in six replicates for pooled plasma or urine obtained from renal transplant patients (Table 1). The within-run C.V. and the between-run C.V. in pooled plasma calculated for the five GCs ranged from 7.76% to 9.22%, and from 8.80% to 9.93%, respectively. The within-run C.V. and the between-run C.V. in pooled urine calculated for the six GCs ranged from 5.59% to 9.51% and from 8.45% to 9.49%, respectively. To check the efficiency and the reproducibility of the extraction and derivatization

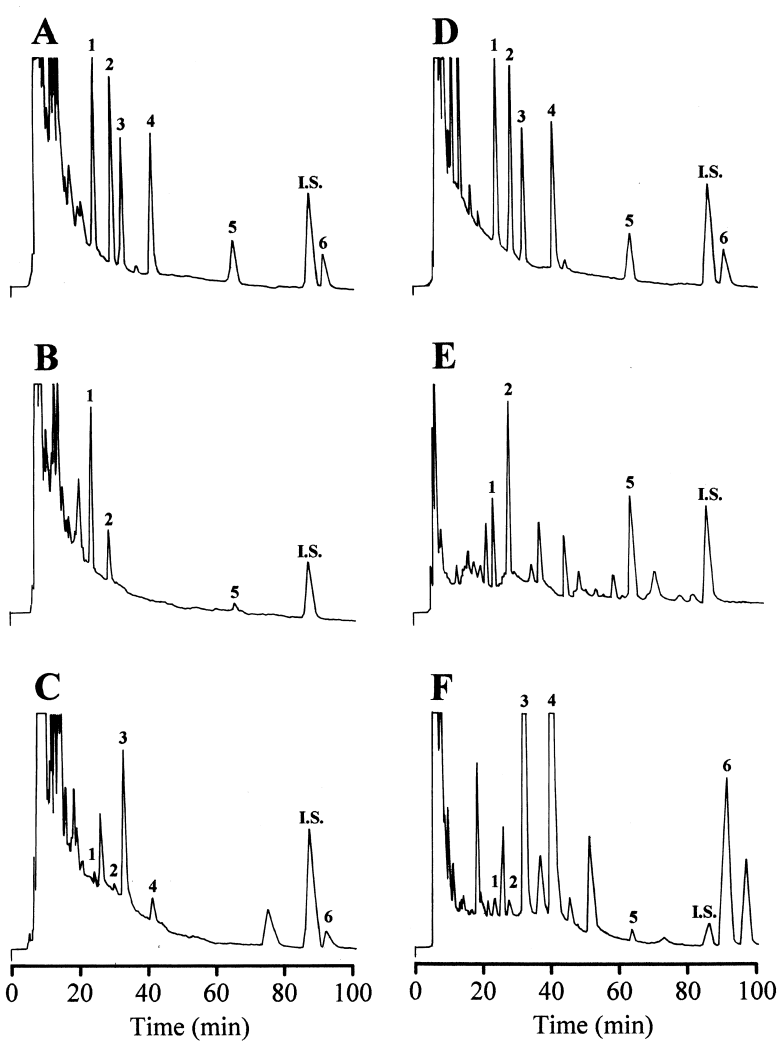

Fig. 2. Chromatograms obtained with (A) standard plasma and (D) standard urine containing 50 ng/ml of F, E, PL, PN, 6β-OHF and 6β-OHP, (B) plasma from a healthy volunteer, (C) plasma from a renal transplant patient, (E) urine from a healthy volunteer and (F) urine from a renal transplant patient. The chromatograms were pictured by a chart recorder in compliance with sample size. Peaks: 1=F; 2=E; 3=PL; 4=PN; 5=6β-OHF; 6=6β-OHP.

procedures, the analytical recovery from pooled plasma or urine of renal transplant patients by adding GCs were evaluated (Table 2). The added GCs recovered from pooled plasma and urine were from 91.62% to 106.26% and from 92.68% to 107.75%, respectively. The lower limits of detection for F, E, PL and PN were 0.1 ng/ml and for 6β-OHF and 6β-OHP were 0.5 ng/ml at a signal-to-noise ratio of 3.

3.4. Clinical adaptation for renal transplant patients

The mean results of trough level monitoring of plasma GCs for 4 renal transplant patients (A, B, C, D) during hospitalization are listed in Table 3. Despite a daily kilogram dose of PL in patient C was about 2- to 3-times higher than other patients, there was no difference in the mean levels of PL in the

Table 1
Within-run and between-run precisions of GCs in pooled plasma or urine of renal transplant patients

Compound	Within-run		Between-run	
	Found[a] (ng/ml)	C.V.[b] (%)	Found[a] (ng/ml)	C.V.[b] (%)
Plasma				
F	3.37±0.26	7.76	3.18±0.29	9.07
E	2.09±0.38	8.38	2.22±0.22	9.91
PL	100.30±8.29	8.27	102.90±9.06	8.80
PN	32.00±2.95	9.22	32.02±3.18	9.93
6β-OHP	15.64±1.41	9.01	15.78±1.53	9.68
Urine				
F	3.61±0.20	5.59	3.52±0.33	9.41
E	17.52±1.50	8.56	18.57±1.57	8.45
PL	106.29±6.99	6.58	101.6±8.97	8.83
PN	95.34±0.97	9.51	96.94±8.94	9.22
6β-OHF	99.02±8.22	8.30	95.72±9.09	9.49
6β-OHP	67.36±5.55	8.24	68.44±6.12	8.95

[a] Each value represents the mean±S.D. of six measurements.
[b] C.V. (%) represents the coefficient of variation for the concentration found.

plasma, suggesting that pediatric patients have a higher clearance of PL. The F levels in plasma during hospitalization were 50- to 100-times depressed in comparison with pretransplant F levels, however, there was no correlation between plasma PL and F. The mean F level of patient B, who had episodes of slight chronic renal rejection on and after day 180 posttransplantation, was about 2- to 40-times higher than the mean F levels of other patients who had no episodes. In contrast, there were no differences in the mean E levels among the four patients, however, that of patient B was relatively small against his mean F level. For all data, the PN/PL or E/F concentration ratios had a significant negative correlation with the systolic blood pressure (PN/PL: $r^2=0.115$, $p<0.001$, $n=72$; E/F: $r^2=0.125$, $p<0.001$, $n=72$). Table 4 shows the area under the concentration versus time curve of cyclosporine and corresponding urinary GCs after renal transplantation for patient D. There was no relationship between the daily dose of PL (mg/day) and the amount of urinary GCs. The mean 6β-OHF/F and 6β-OHP/PL ratios in urine within four weeks were relatively higher and the AUC of CsA relatively retarded during this period.

4. Discussion

We described the first HPLC method with fluorimetric determination by 9-AN for the simultaneous detection of plasma or urinary GCs. Because of higher sensitivity, this method allowed us to

Table 2
Analytical recovery of GCs added to pooled plasma or urine of renal transplant patients prior to extraction

Compound	Initial (ng/ml)	Added (ng/ml)	Expected (ng/ml)	Found[a] (ng/ml)	Recovery[b] (%)	C.V.[c] (%)
Plasma						
F	3.37	5	8.37	8.69±0.72	106.26	8.33
E	2.09	5	7.09	6.67±0.54	91.62	8.11
PL	100.30	50	150.30	149.95±13.13	93.29	8.94
PN	32.00	50	82.00	78.34±7.52	92.67	9.60
6β-OHP	15.64	10	25.64	15.64±1.41	92.14	9.67
Urine						
F	3.61	10	13.61	12.88±1.13	92.68	8.74
E	17.52	10	27.52	28.12±2.34	106.06	8.49
PL	106.29	100	206.29	200.16±19.12	93.87	9.55
PN	95.34	100	195.34	203.09±17.99	107.75	9.86
6β-OHF	99.02	10	109.02	108.52±8.49	95.06	8.49
6β-OHP	67.36	100	167.36	15.64±1.41	96.76	9.85

[a] Each value represents the mean±S.D. of six measurements.
[b] Each value represents the mean of six measurements.
[c] C.V. (%) represents coefficient of variation of analytical recovery.

Table 3
Description of mean monitoring results for individual renal transplant patients during hospitalization

Items	Patient			
	A	B	C	D
No. of points	11	20	24	17
Age (years)	44	35	12	27
PL dose (mg/kg/day)	0.56±0.19	0.44±0.15	1.27±0.50[c]	0.62±0.16
Systolic blood pressure[a] (mmHg)	146±11	153±10	128±3	164±19
Plasma PL (ng/ml)	28.82±29.54	39.79±15.56	26.44±11.23	39.13±20.82
Plasma PN (ng/ml)	3.43±3.60	5.69±2.76	5.02±2.67	4.34±1.56
Plasma 6β-OHP (ng/ml)	2.64±2.64	4.88±4.36	3.99±4.68	5.51±5.53
Plasma F (ng/ml)	1.17±0.65	2.83±3.38	0.77±0.43	1.26±1.171
Plasma E (ng/ml)	0.41±0.25	0.55±0.76	0.59±0.43	0.41±0.21
PN/PL ratio	0.15±0.08	0.17±0.07	0.20±0.08	0.14±0.44
E/F ratio	0.36±0.12	0.23±0.11	0.81±0.63	0.61±0.44
Clinical episodes	None	Chronic rejection[b]	None	None

1 mmHg=133.322 Pa.
[a] Systric blood pressure was measured in the morning.
[b] Episodes of chronic rejection were found on and after day 180 of posttransplantation.
[c] Significantly different against other patients ($p<0.01$).

measure depressed F due to PL medication. Since 9-AN generates fluorescent esters through the primary hydroxyl group at the 21st position of the carbon chain of GCs [13,14], other GCs such as dexamethasone, triamsinolon, betamethasone, 5β-dihydrocortisol, 5β-dihydrocortisone, tetrahydroxycortisol, allo-tetrahydroxycortisol and tetrahydrocortisone, which also have the 21st hydroxyl group and react with 9-AN, could have their 9-AN derivatives which were chromatographed with a minor modification of the HPLC conditions.

The acylation of GCs by 9-AN is proceeded by a basic catalyst [15]. The use of a 4:1 (v/v) mixture of 10% triethylamine–0.1% quinuclidine at room temperature provided excellent reactivity between GCs and 9-AN and it economized the working time for derivatization and provided symmetrical and well defined peaks of derivatized GCs and the I.S. without interfering peaks. However, it is noted that anhydrous conditions should be maintained during derivatization with 9-AN. Addition of molecular sieves 4A provided perfect anhydrous condition. Since sample extracts after the derivatization with 9-AN were very stable at room temperature, for at least a week, samples can be loaded into an automatic sample injector with a long run time.

Kozaki et al. [16] reported that the appearance of acute renal rejection depended on the degree of F suppression by PL. If the F levels, within 10 days after renal transplantation, are above 3 ng/ml, the

Table 4
Urinary GCs for patient D after renal transplantation

Time after transplantation	PL dose (mg/day)	Urine volume (ml/24)	Urinary GCs						6β-OHF/F ratio	6β-OHP/PL ratio	AUC of CsA[a] (ng h/ml)
			F	E	6β-OHF	PL	PN	6β-OHP			
1 week	50	2300	8.12	14.72	26.65	757.72	587.23	3320.97	3.26	4.38	3742
2 weeks	40	2520	14.85	9.88	19.39	2597.07	2444.82	21 162.81	1.31	8.15	3125
4 weeks	30	2240	12.10	21.12	37.60	920.55	571.09	1479.82	3.11	1.61	3913
6 weeks	25	2500	3.35	4.67	3.93	338.12	337.08	951.37	1.17	2.81	5178
8 weeks	20	1440	18.43	11.20	15.84	548.18	498.93	1147.15	0.86	2.09	6006

Twenty-four h urine samples were collected on the middle day of each stage of PL dose after renal transplantation.
[a] Cyclosporine.

frequency of acute renal rejection is increased. The F levels of our patient B, within 10 days after renal transplantation, was between 1.04 and 1.67 ng/ml, at this point it was expected that no acute renal rejection occurred in the future in this patient. However, patient B had episodes of chronic rejection on and after day 180 after transplantation. Taking into consideration that the mean F levels of patient B were within 3 ng/ml, and 2- to 4-times higher than that of other patients, it is suggested that the boundary line of F levels for acute or chronic renal rejection can be classified by monitoring a large population using our HPLC method.

Metabolism of F via 11β-hydroxysteroid dehydrogenase (11β-HSD), which locates aldosterone target cells, is related to hypertension [17]. During hypertension, the activity of 11β-HSD is suppressed and 11β-hydroxylation of F is prolonged [17]. Recently, a significant negative correlation was reported between the plasma E/F ratio and the systolic blood pressure in patients with essential hypertension, diabetes mellitus or chronic renal failure. In our patients, the E/F ratio and also the PN/PL ratio had a negative correlation with the systolic blood pressure, suggesting that hypertension found in renal transplant patients also depends on 11β-HSD activity. Since hypertension after renal transplantation is considered to be one of the risk factors for viability of renal transplanted [18], examining 11β-HSD activity by monitoring GCs would be useful for therapeutic management after renal transplantation.

The metabolism of F and PL by 6β-hydroxylation via CYP3A4 in liver microsomes reflects an alteration in metabolism of many other drugs which are metabolized via CYP3A4 [19]. An increase in the ratio of urinary 6β-OHF/F or 6β-OHP/PL indicates an enzyme-inducing condition [9,20], whereas a decrease in that ratio indicates an enzyme-inhibiting condition [21]. Cyclosporine is also metabolized by CYP3A4, therefore, it is considered that change in the trough levels of cyclosporine correlates with change in the ratio of urinary 6β-OHF/F or 6β-OHP/PL. In the early phase of therapy in patient D after renal transplantation, it was considered that there was an enzyme-inducing condition. However, the pharmacokinetics of cyclosporine in the early phase can not be explained solely by metabolism via CYP3A4. Nevertheless, further investigations on the relationships between the pharmacokinetics of cyclosporine and urinary GCs in a large population are required.

In conclusion, the present HPLC method with precolumn fluorimetric derivatization by 9-AN provides a highly sensitive and reliable assay procedure for plasma or urine analysis of GCs. The accuracy and reproducibility of this HPLC method were found to be satisfactory in clinical practice. Identical procedures of sample extraction and derivatization allow parallel preparation of plasma and urine. In addition, this HPLC method will be applicable for many other metabolic investigations.

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