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Simultaneous determination of prednisolone, prednisone, cortisol, and cortisone in plasma by GC–MS: Estimating unbound prednisolone concentration in patients with nephrotic syndrome during oral prednisolone therapy☆

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ABSTRACT

Individual variability of the pharmacokinetics of prednisolone based on the unbound concentration in plasma is of significant clinical consideration. The unbound concentrations of prednisolone were measured in 10 patients with nephrotic syndrome, two patients with systemic lupus erythematosus, and one patient with dermatomyositis by examining protein bindings of prednisolone on one or more occasions during prednisolone treatment. In this study, plasma concentrations of prednisolone, prednisone, cortisol, and cortisone were simultaneously analyzed by GC–MS by using stable isotope-labeled internal standards. Equilibrium dialysis was employed to accurately estimate the unbound fractions of prednisolone in plasma. The unbound fraction of prednisolone changed depending on plasma total prednisolone concentration and plasma albumin concentration. The unbound fraction of prednisolone (Y) is calculated: $Y = (-0.0101x' + 0.0736)\,x + 10.23$, where x' is the plasma albumin concentration and x is the total prednisolone concentration. The estimated concentrations of unbound prednisolone by using the above equation were in good agreement with the measured concentrations of unbound prednisolone. Since the protein binding of prednisolone did not change in the presence of prednisone (114.0 ng/ml), it appeared that prednisone produced from the therapeutic dose of prednisolone did not affect the unbound fraction of prednisolone.

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1. Introduction

Prednisolone is the most widely used oral glucocorticoid in the treatment of patients with nephrotic syndrome. Corticosteroid dosage is generally adjusted on empirical grounds, and protocols are primarily designed to reduce doses to a low maintenance level as soon as possible, without taking into consideration differences in the altered pharmacokinetics. The plasma concentrations of prednisolone depend on the intestinal absorption of oral prednisolone, distribution, elimination, and the interconversion of prednisolone to prednisone. In the treatment of nephrotic patients with prednisolone, the information on pharmacokinetics of the steroid is needed to optimally adjust the dosage regimens by changing the dosage, administration frequency and duration of the treatment.

Prednisolone binds to both corticosteroid binding globulin (CBG) and albumin [1–5]. Only the unbound form of prednisolone in plasma is thought to be subject to tissue distribution and clearance, and available for pharmacological activity [6]. Since plasma protein concentrations are low in patients with nephrotic syndrome, altered pharmacokinetics of prednisolone and thus altered pharmacological responses have to be expected. Several studies have shown that systemic plasma clearance, volume of distribution, and plasma protein binding vary in a dose-dependent manner [2,7–11]. In our recent prednisolone pharmacokinetic study in 15 patients with nephrotic syndrome, there was a marked dose-dependency in the prednisolone clearance, and a negative correlation between the prednisolone total clearance and plasma albumin concentration [12]. However, it is still unclear how unbound prednisolone concentrations are altered by the changes in plasma albumin concentration and prednisolone dose.

The measurements of glucocorticoids such as cortisol and prednisolone in biological fluids have been extensively investigated

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and well established by using selective chromatographic techniques such as HPLC [13,14], GC–MS [15–17], LC–MS [14,18,19], and LC–MS–MS [20,21]. We have previously examined an LC–MS method for the simultaneous assay of cortisol, cortisone, prednisolone, and prednisone in human plasma using their respective deuterated analogs synthesized in this laboratory as internal standards [19]. However, when the mobile phase composed of 0.05 M ammonium formate/acetonitrile (65:35,v/v) was used, incomplete separation of cortisol and prednisone was observed. Since the $[MH]^+$ ions of cortisol and [1,19,19,19-$^2H_4$]prednisone (prednisone-$^2H_4$) appear at the identical *m/z* 363 on the mass spectra, selected ion *m/z* 363 should account for the contribution of cortisol present at the $[MH]^+$ ion of prednisone-$^2H_4$ vice versa. We have also developed a GC–MS method for the simultaneous determination of endogenous and synthetic glucocorticoids, i.e., cortisol, cortisone, prednisolone, and prednisone in human plasma using deuterium-labeled internal standards, [1,1,19,19,19-$^2H_5$]cortisol (cortisol-$^2H_5$), [1,1,19,19,19-$^2H_5$]cortisone (cortisone-$^2H_5$), [1,19,19,19-$^2H_4$]prednisolone (prednisolone-$^2H_4$), and [1,19,19,19-$^2H_4$]prednisone (prednisone-$^2H_4$) [22]. The deuterium labels at C-1 and C-19 of cortisol and cortisone are chemically stable. However, ^{13}C-labeled compounds should preferably be used as internal standards for measurement of prednisolone and prednisone which have C1/C2 double bond in order to avoid the possible loss of a deuterium atom at C-1. Recently, we have synthesized ^{13}C-labeled prednisolone and prednisone (prednisolone-$^{13}C_4$ and prednisone-$^{13}C_4$) containing four ^{13}C at C-1, C-2, C-4 and C-19 with high isotopic purity (>97.3 atom%^{13}C) [23]. In the present study, we have developed a sensitive and reliable GC–MS method for the simultaneous determination of prednisolone, prednisone, cortisol, and cortisone in plasma using $^{13}C_4$-labeled prednisolone and prednisone and $^2H_5$-labeled cortisol and cortisone as internal standards. The method was then applied to the pharmacokinetic study of estimating unbound concentrations of prednisolone in 13 patients with nephrotic syndrome during oral prednisolone therapy. Plasma concentrations of other glucocorticoids (prednisone, cortisol, and cortisone) in the patients also were determined by the GC–MS method.

2. Experimental

2.1. Chemicals and reagents

Prednisolone, prednisone, cortisol, and cortisone were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Stable isotopically labeled compounds, i.e., [1,2,4,19-$^{13}C_4$]prednisolone (prednisolone-$^{13}C_4$, >97.3 atom%^{13}C), [1,2,4,19-$^{13}C_4$]prednisone (prednisone-$^{13}C_4$, >97.3 atom%^{13}C), [1,1,19,19,19-$^2H_5$]cortisol (cortisol-$^2H_5$, 98 atom%^{2}H), and [1,1,19,19,19-$^2H_5$]cortisone (cortisone-$^2H_5$, 98 atom%^{2}H) were synthesized in our laboratory (Fig. 1) [23–25]. Heptafluoro-*n*-butyric anhydride (HFBA) and paraformaldehyde were purchased from Pierce (Rockford, Illinois, USA) and Kanto (Tokyo, Japan), respectively. All other chemicals and solvents were of analytical-reagent grade and were used without further purification.

A solution of 16% paraformaldehyde in 25.7% HCl was freshly prepared by dissolving paraformaldehyde (1.68 g) in water (2.9 ml) and concentrated HCl (7.1 ml). Isotonic Krebs–Ringer buffer (4.29 g/l NaCl, 0.438 g/l KCl, 0.0876 g/l $CaCl_2$, 1.69 g/l KH_2PO_4, 0.142 g/l $MgSO_4$) was used for equilibrium dialysis. The pH was adjusted to 7.4 with 1N sodium hydroxide and the osmolarity was adjusted to 290 mOsm/l by sodium chloride.

Stock solutions of prednisolone (10.05 mg per 100 ml), prednisone (11.44 mg per 100 ml), cortisol (10.63 mg per 100 ml), cortisone (11.19 mg per 100 ml), presnisolone-$^{13}C_4$ (0.2427 mg per 100 ml), presnisone-$^{13}C_4$ (0.04228 mg per 100 ml), cortisol-$^2H_5$ (0.1582 mg per 100 ml), cortisone-$^2H_5$ (0.1491 mg per 50 ml) were prepared in methanol. All analyses were performed by diluting the stock solutions with methanol. The stock solutions were stored at 4 °C.

2.2. Study protocol

Ten patients with nephrotic syndrome (five males and five females), two female patients with systemic lupus erythematosus, and one male patient with dermatomyositis ranging in age from 18 to 76 years and eight healthy volunteers (four males and

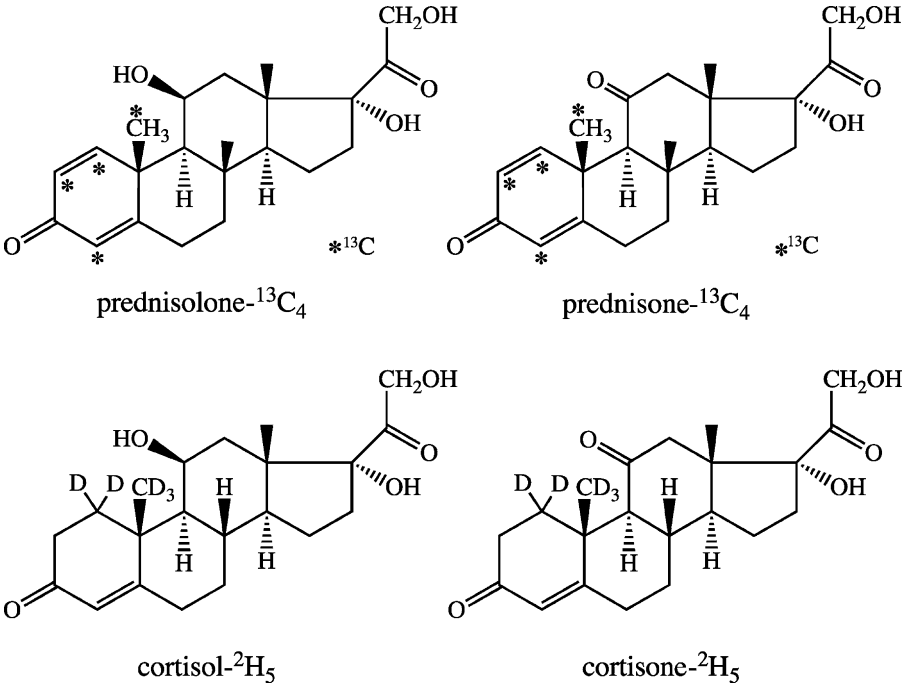

Fig. 1. Structures of stable isotopically labeled prednisolone, prednisone, cortisol, and cortisone (prednisolone-$^{13}C_4$, prednisone-$^{13}C_4$, cortisol-$^2H_5$, and cortisone-$^2H_5$).

four females) gave their informed consent to participate in the study. The study protocol was approved by the ethical committee of Kyorin University Hospital in accordance with the Declaration of Helsinki. There was no history for liver disease in any subjects and tests of liver function (transaminase, alkaline phosphatase, γ-glutamyltranspeptidase) were within the normal range. There was no patient undergoing hemodialysis.

Each patient was admitted to Kyorin University Hospital and studied on one or more occasions over a period of 1–2 months during treatment with daily doses of 20–60 mg of prednisolone (Prednisolone Tablets® (prednisolone 5 mg tablets), Takeda Pharmaceutical Company, Tokyo, Japan). On each occasion, all the prescribed drugs were given as usual and blood samples, 5 ml each, were obtained from a peripheral arm vein before the morning, noon and next morning dose. Additional samples were collected for some patients at a few time points between the noon and next morning dose. Blood samples were collected into heparinized tubes. The blood was immediately centrifuged and the plasma was frozen and stored at −20 °C. The plasma albumin concentration ranged from 1.2 to 3.7 g/dl.

2.3. GC-MS-selected ion monitoring (GC-MS-SIM)

Capillary GC-MS–SIM analysis was carried out on a QP1100EX GC–MS equipped with a data processing system (Shimadzu, Kyoto, Japan). Gas chromatography was performed on an SPB-1 fused-silica capillary column (15 m × 0.25 mm I.D.) with the stationary phase coated at a 0.25-μm film thickness (Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas at a column head pressure of 40 kPa.

A split–splitless injection system (Shimadzu SPL-14) operated in the splitless mode was used with a septum purge flow rate of 10 ml/min and a split flow rate of 40 ml/min. The purge activation time was 2 min. The initial column temperature was set at 100 °C. After the sample injection, it was maintained for 2 min and was increased at 20 °C/min to 250 °C, maintained there for 1 min, then increased at 10 °C/min to 280 °C. The temperature of the injector was 260 °C. The mass spectrometer was operated in the electron-impact mode at an energy of 70 eV and the ion source temperature was set at 280 °C.

The multiple-ion detector was focused on the molecular ions ($[M]^{+\cdot}$) at *m*/*z* 598 and 602 for the bismethylenedioxy-3-heptafluoro-*n*-butyrate (BMD-monoHFB) derivatives of prednisolone and prednisolone-$^{13}C_4$, at *m*/*z* 582 and 587 for the cortisol and cortisol-$^{2}H_5$, and at *m*/*z* 598 and 603 for the cortisone and cortisone-$^{2}H_5$, and on the fragment ions ($[M\text{-}30]^{+}$) at *m*/*z* 566 and 570 for the prednisone and prednisone-$^{13}C_4$.

2.4. Sample preparation for GC-MS-SIM

2.4.1. Extraction

To 1 ml of human plasma was added 72.80 ng (30 μl) of prednisolone-$^{13}C_4$, 42.28 ng (100 μl) of prednisone-$^{13}C_4$, 94.92 ng (60 μl) of cortisol-$^{2}H_5$, and 89.46 ng (30 μl) of cortisone-$^{2}H_5$ as the internal standards dissolved in methanol. The plasma sample was applied to a Sep-Pak C_{18} Plus short-body cartridge (Waters Assoc., Milford, MA, USA). The cartridge was washed with 10 ml of distilled water and then eluted with 4 ml of ethyl acetate into a conical centrifuge tube (100 mm × 13 mm I.D.). After evaporation to dryness at 60 °C under a stream of nitrogen, acetone (200 μl × 3) was added to the residue and the sample was transferred into a 2 ml micro product V-vial (Wheaton, Millville, NJ, USA) and then evaporated to dryness at room temperature under a stream of nitrogen.

2.4.2. Derivatization

To the residue suspended in chloroform (50 μl) were added 16% paraformaldehyde in 25.7% HCl (50 μl), and the reaction mixture was vigorously vortexed at room temperature for 20 min. The reaction mixture was extracted with chloroform (300 μl × 4) and the extracts were washed with water (300 μl × 3). The solvent was evaporated to dryness under a stream of nitrogen at room temperature. To the residue dissolved in acetone (200 μl) was added 50 μl of heptafluoro-*n*-butyric anhydride (HFBA). The reaction mixture was vortexed for 1 min and then left for 1 h at room temperature. After evaporating the excess reagent under a stream of nitrogen at room temperature, the residue was dissolved with cyclohexane (10 μl). A 1.0-μl portion of the solution was subjected to GC–MS. The derivative was stable at room temperature for at least 2 months.

2.5. Calibration graphs

To each six of standards containing known amounts of prednisolone (10.05, 50.25, 100.5, 150.75, 301.5, and 502.5 ng), prednisone (11.44, 22.88, 34.32, 57.20, 80.08, and 114.4 ng), cortisol (1.701, 8.504, 21.26, 53.15, 95.67, and 138.2 ng), and cortisone (1.790, 8.952, 22.38, 33.57, 44.76, 67.14 ng) dissolved in methanol, 72.80 ng of prednisolone-$^{13}C_4$, 42.28 ng of prednisone-$^{13}C_4$, 94.92 ng of cortisol-$^{2}H_5$, and 89.46 ng of cortisone-$^{2}H_5$ were added. After evaporation of the solvent to dryness, the samples were derivatized as described above. A 1.0-μl portion of a cyclohexane solution (10 μl) was subjected to GC–MS. The peak-area ratios (*m*/*z* 598 to 602 for prednisolone, *m*/*z* 566 to 570 for prednisone, *m*/*z* 582 to 587 for cortisol, and *m*/*z* 598 to 603 for cortisone) were determined in triplicate. The calibration graphs were obtained by an unweighted least-squares linear fitting of the peak-area ratios versus the mixed molar ratios of prednisolone/prednisolone-$^{13}C_4$, prednisone/prednisone-$^{13}C_4$, cortisol/cortisol-$^{2}H_5$, and cortisone/cortisone-$^{2}H_5$ on each analysis of the standard mixtures.

2.6. Accuracy

Accuracy was determined by assaying six preparations of 1.0 ml portions of human plasma spiked with 80.40 ng of prednisolone (72.80 ng of prednisolone-$^{13}C_4$ as internal standard), 34.32 ng of prednisone (42.28 ng of prednisone-$^{13}C_4$ as internal standard), 42.52 ng of cortisol (94.92 ng of cortisol-$^{2}H_5$ as internal standard), and 22.38 ng of cortisone (89.46 ng of cortisone-$^{2}H_5$ as internal standard). After preparation of the sample for GC-MS-SIM as described above, the peak-area ratios were measured.

2.7. Equilibrium dialysis

Total (unbound and bound) concentrations of prednisolone were analyzed by GC–MS according to the procedure described above. Equilibrium dialysis was performed to determine unbound fraction of prednisolone in plasma. Plasma samples (1 ml each) were dialyzed for 12 h against an equal volume of isotonic Krebs–Ringer buffer (pH 7.4) using Spectra/Por 1 cellulose membrane (Spectrum Laboratories, Inc.) maintained in an incubator at 37 °C. At the end of dialysis, the internal standards were added to plasma (0.8 ml) and buffer (0.8 ml), respectively. The concentrations of prednisolone in plasma and buffer solution were analyzed by the GC-MS-SIM. The unbound fraction of prednisolone was calculated by the ratio of prednisolone concentration in buffer to concentration in plasma after dialysis. The unbound prednisolone concentration was calculated from the measured total prednisolone concentration multiplied by the corresponding unbound fraction. Plasma albumin concentrations were measured by the

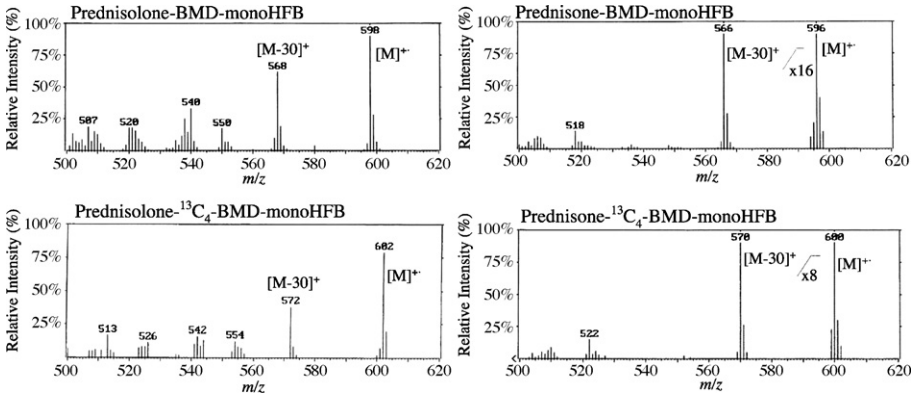

Fig. 2. Electron-impact mass spectra of BMD-monoHFB derivatives of unlabeled and labeled prednisolone and prednisone (prednisolone, prednisone, prednisolone-$^{13}C_4$, and prednisone-$^{13}C_4$).

radioimmunoassay in Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. (Tokyo, Japan).

3. Results and discussion

The mass spectrometric technique using stable isotope-labeled internal standards ensures the highest specificity and sensitivity, and a more accurate approach in determining a trace amount of endogenous and synthetic steroid compounds in biological fluids [23,26–28]. One of the major advantages of this technique is that stable isotope-labeled internal standards practically behave in almost identical manner to the analyte through all steps in the extraction and chromatographic procedures thereby allowing procedural losses to be disregarded. Furthermore, the labeled internal standards can easily be distinguished from the unlabeled compounds in the mass spectrometry. In the present study, ^{13}C-labeled prednisolone and prednisone were used as the analytical internal standards for the simultaneous determination of prednisolone and prednisone, together with cortisol and cortisone (cortisol-$^2H_5$ and cortisone-$^2H_5$ as internal standards) in human plasma. The structures of these labeled compounds are given in Fig. 1.

Fig. 2 shows the electron-impact (EI) mass spectra of the BMD-monoHFB derivatives of unlabeled and ^{13}C-labeled prednisolone and prednisone. The BMD-monoHFB derivatives gave the molecular ions $[M]^{+\cdot}$ at *m/z* 598 for prednisolone, at *m/z* 602 for prednisolone-$^{13}C_4$, at *m/z* 596 for prednisone, and at *m/z* 600 for prednisone-$^{13}C_4$.

The characteristic $[M-30]^+$ ($M-CH_2O$) fragment ions were observed for the unlabeled and labeled prednisolone and prednisone, respectively. The molecular ions $[M]^{+\cdot}$ for prednisolone and prednisolone-$^{13}C_4$ (*m/z* 598 and 602) and the fragment ions $[M-30]^+$ for prednisone and prednisone-$^{13}C_4$ (*m/z* 566 and 570) (Fig. 2) were chosen for the selected ion monitoring of the BMD-monoHFB derivatives. The molecular ions $[M]^{+\cdot}$ of the BMD-monoHFB derivatives of cortisol and cortisol-$^2H_5$ (*m/z* 582 and 587) and cortisone and cortisone-$^2H_5$ (*m/z* 598 and 603) were monitored for the analysis of cortisol and cortisone in plasma as described in our previous paper [22].

A good GC separation was observed for four steroids, i.e., prednisolone (retention time, t_R 13.3 min), prednisone (t_R 15.3 min), cortisol (t_R 13.5 min), and cortisone (t_R 14.6 min). When a signal-to-noise (S/N) ratio of 2.5 or greater was used as a criterion for a significant response, the sensitivity limit of the present GC-MS-SIM method was found to be 10 pg per injection (S/N = 2.7) for cortisone, 100 pg (S/N = 4.4) for prednisolone and 250 pg for cortisol (S/N = 2.7) and prednisone (S/N = 3.4). Calibration curves were linear from 10 to 500 ng/ml for prednisolone, from 10 to 115 ng/ml for prednisone, from 1 to 140 ng/ml for cortisol, and from 1 to 65 ng/ml for cortisone. A good correlation was found between the observed peak-area ratio (y) and the mixed molar ratio (x). Unweighted least-squares regression analysis gave the regression lines $y = 1.035x - 0.037$ ($r = 0.999$) for prednisolone, $y = 0.8717x - 0.0128$ ($r = 0.999$) for prednisone, $y = 1.307x - 0.043$

Table 1
Accuracy of GC-MS-SIM determination of prednisolone, prednisone, cortisol, and cortisone in human plasma

Added (ng/ml)	Found (ng/ml)							Relative error (%)	R.S.D. (%)
	Individual values[a]						Mean ± S.D.		
Prednisolone									
80.40	79.25	80.17	76.71	79.37	79.53	80.87	79.32 ± 1.41	−1.35	1.78
Prednisone									
34.32	34.99	34.83	35.04	35.08	35.17	34.54	34.94 ± 0.23	1.81	0.65

Added (ng/ml)	Expected (ng/ml)	Found (ng/ml)						Relative error (%)	R.S.D. (%)
		Individual values[a]					Mean ± S.D.		
Cortisol									
		71.32	72.59	67.93	70.26	70.92	69.29	70.39 ± 1.63		2.31
42.52	112.91	107.43	103.85	111.07	107.45	112.30	108.89	108.50 ± 3.00	−3.90	2.77
Cortisone									
		25.52	25.30	26.00	25.31	26.12	25.27	25.59 ± 0.38		1.48
22.38	47.97	47.81	47.62	47.45	49.06	48.31	47.35	47.93 ± 0.65	−0.08	1.35

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

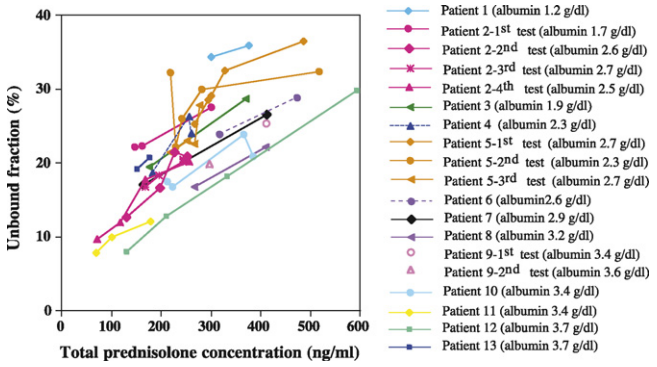

Fig. 3. Relationship between the unbound fraction of prednisolone and total plasma prednisolone concentration in 13 patients (patients 1–13). Each patient was studied during treatment with daily dose of 20–60 mg of prednisolone. Patients 2, 5, and 9 were studied on two or more different occasion over a period of 1–2 months.

($r = 0.999$) for cortisol, and $y = 1.0004x - 0.0058$ ($r = 0.999$) for cortione. The within-day reproducibility in which the amounts of prednisolone, prednisone, cortisol, and cortisone determined were in good agreement with the actual amounts added, the relative errors being less than 3.9%. The inter-assay relative standard deviations (R.S.D) were less than 2.8% for all four corticosteroids. Accuracy (Table 1) was comparable to our previously reported data [22]. The method was then applied to the measurement of plasma concentrations of prednisolone, prednisone, cortisol, and cortisone in patients with nephrotic syndrome during oral prednisolone therapy.

A total number of 19 tests were performed over a period of 1–2 months during treatment with a daily dose of 20–60 mg of prednisolone for the 13 patients. As shown in Fig. 3, the unbound fraction of prednisolone in each plasma sample of the patients was plotted against the total prednisolone concentration in plasma. The total prednisolone concentrations ranged from 69 to 593 ng/ml and the percentage of unbound prednisolone ranged from 7.8 to 36.4%. On the other hand, the total concentration of prednisone, a metabolite of prednisolone, ranged from 4.5 to 74.5 ng/ml. Since secretions of cortisol and cortisone are suppressed during prednisolone therapy, plasma concentrations of endogenous cortisol and cortisone were low, being either less than 1 ng/ml (lower limit of quantification) or 1.69–43.2 ng/ml for cortisol and either less than 1 ng/ml or 1.0–10.8 ng/ml for cortisone in each plasma sample of the patients tested.

In each individual patient tested, the unbound fraction of prednisolone at the same plasma albumin concentration became higher as the total prednisolone concentration in plasma increased (Fig. 3). Moreover, the unbound fraction tended to increase for lower plasma albumin concentrations (1.2–3.7 g/dl) (Fig. 3). The results clearly showed that the unbound fraction of prednisolone was altered by plasma total prednisolone concentration and plasma albumin concentration.

The relationship between unbound fraction and total prednisolone concentration in a total number of 57 points from 13 patients and 45 points from eight healthy volunteers at five different albumin levels (1 – 1.9; 2 – 2.9; 3 – 3.9; 4 – 4.9; and 5 – 5.9 g/dl) was examined. At each albumin level, there was a good correlation between unbound fraction of prednisolone (y) and total plasma prednisolone concentration (x); $y = 0.0541x + 12.98$, $r = 0.8480$ (albumin 1 g/dl), $y = 0.0521x + 9.59$, $r = 0.8199$ (albumin 2 g/dl), $y = 0.0390x + 7.33$, $r = 0.8746$ (albumin 3 g/dl), $y = 0.0256x + 10.69$, $r = 0.9447$ (albumin 4 g/dl), $y = 0.0218x + 10.57$, $r = 0.9586$ (albumin 5 g/dl). Unbound fraction of prednisolone also is influenced by plasma albumin concentration. The unbound fraction was then calculated from both total prednisolone and albumin concentrations. The unbound fraction of prednisolone (Y) is expressed as the following equation: $Y = (-0.0101x' + 0.0736)x + 10.23$, where x' is the plasma albumin concentration and x is the total prednisolone concentration. The Y-intercept (10.23) was determined by the best-fit linear equation: $y = 0.0385x + 10.23$, the unbound fraction (y) and total prednisolone concentration (x) in albumin levels of 1, 2, 3, 4, and 5 g/dl. The slope ($-0.0101x' + 0.0736$) was determined from the best-fit linear equation: $y' = -0.0101x' + 0.0736$, the relationship between slope (y') and albumin concentration (x').

Miller et al. [29] studied the relationship between percentage of unbound prednisolone (y) and plasma albumin concentration (x) and proposed a linear equation of $y = -17.5x + 92$. It should be noted that the Miller's equation does not take into consideration of the fact that the unbound fraction of prednisolone varies with the total prednisolone concentration as discussed above. Unbound prednisolone fractions were calculated using the above equation ($Y = (-0.0101x' + 0.0736)x + 10.23$) in five tests for two patients with nephrotic syndrome. The unbound prednisolone concentration was obtained by multiplying the total prednisolone concentration by calculated unbound fraction. The calculated unbound fraction of prednisolone ranged from 15.9 to 34.9%. The estimated unbound concentration values derived from our regression equation correlated well with the actual unbound concentration of prednisolone ($r = 0.9595$).

Prednisolone is metabolically converted to prednisone after oral administration. In this study, prednisone concentrations ranged from 4.5 to 74.5 ng/ml in each plasma sample of the patients tested. Since the protein binding of prednisolone did not change in the presence of prednisone (114.0 ng/ml), it appeared that prednisone produced from the therapeutic dose of prednisolone did not affect the unbound fraction of prednisolone. This result is consistent with reports from Boudinot and Jusko [30], and Legler and Benet [31]. In our eight healthy volunteers tested, the unbound fraction at a plasma prednisolone concentration of 200 ng ranged from 14.5% (in the presence of 25.7 ng/ml endogenous cortisol) to 16.7% (in the presence of 107.1 ng/ml endogenous cortisol), indicating that the plasma cortisol concentration did not affect the unbound concentration of prednisolone.

It has been pointed out that, due to the complicated pharmacokinetic properties of prednisolone, determining the precise doses needed to obtain a desired target concentration will be extremely difficult [11]. Changes in clinical efficacy and appearance of side effects have been related to altered unbound prednisolone concentration. Although preferable, measurement of plasma-unbound concentration is relatively uncommon in clinical practice, since ultra filtration or equilibrium dialysis is rather tedious and time consuming. Hypoalbuminemia is a common problem among persons with acute and chronic medical conditions. Hypoalbuminemia can be caused by various entities, including nephrotic syndrome, hepatic cirrhosis, heart failure, and systemic lupus erythematosus, etc. Our proposed equation for estimating the unbound prednisolone should be useful for studying the pharmacokinetics of prednisolone in the treatment of patients with hypoalbuminemia.

4. Conclusion

The present GC–MS technique provides a sensitive and reliable method for the simultaneous determination of prednisolone, prednisone, cortisol, and cortisone in plasma. The method was applied to the protein binding studies of prednisolone in patients with nephrotic syndrome for estimating the concentration of unbound prednisolone from measured values of the plasma total concentration of prednisolone and the plasma concentration of albumin. The developed equation permits a simple means of calculating the

unbound prednisolone concentration, taking into consideration of intra- and inter-individual variations in the protein binding.

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