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SIMULTANEOUS MEASUREMENT OF PREDNISONE, PREDNISOLONE AND 6β-HYDROXYPREDNISOLONE IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH A RADIOACTIVITY DETECTOR

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SUMMARY

We describe the first method for routine measurement of prednisone, prednisolone and 6β-hydroxyprednisolone concomitantly in urine. Urine (3 ml) is extracted with ethyl acetate, washed with base and separated by high-performance liquid chromatography on a silica column with a solvent system of hexane—diethyl ether—ethanol—tetrahydrofuran—glacial acetic acid (59.9:31:2.3:6.5:0.3, v/v). The steroids are detected at 254 nm. Because no conventional internal standard was found, 6β-[^{3}H]hydroxycortisol and [^{3}H]prednisolone are added to urine prior to extraction; ^{3}H is monitored by a radioactivity detector coupled with the chromatograph. The assay exhibits linearity from 200 to 7500 ng and an inter-day variability of < 11.4% (C.V.).

INTRODUCTION

Metabolic studies of prednisolone, the most widely used synthetic glucocorticoid, require specific analytical techniques. The radioimmunoassay technique for prednisolone suffers from poor specificity [1] and poor reproducibility [2]. Recently, specific and reproducible high-performance liquid chromatographic (HPLC) techniques have been developed for prednisolone determination in plasma [3—5]. Furthermore, these methods allow the simultaneous determination of prednisone and prednisolone in plasma. The method of Rose et al. [4, 6] has also been shown to be suitable for measuring prednisolone and prednisone in urine. No method is currently available for routine measurement of 6β-hydroxyprednisolone. This report describes an HPLC method for the simultaneous assay of prednisolone, prednisone and 6β-hydroxyprednisolone in urine, with [^{3}H]prednisolone and 6β-[^{3}H]hydroxycortisol as the internal standards (Fig. 1).

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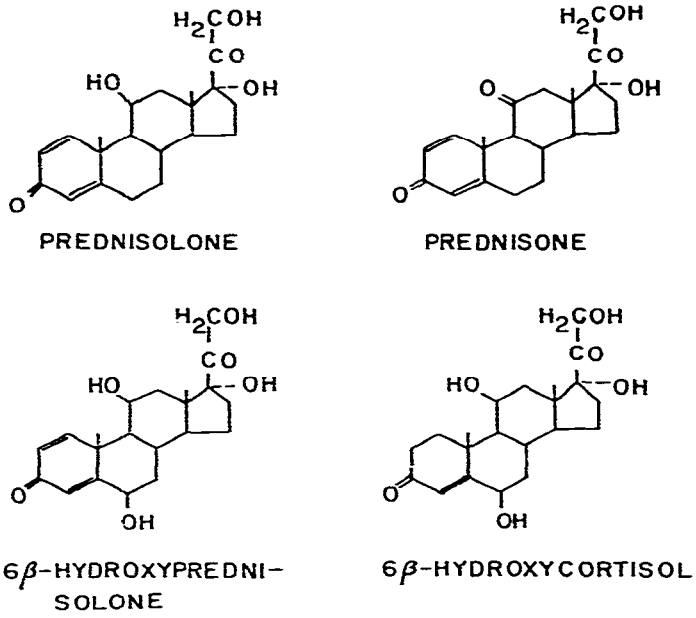

Fig. 1. Structure of prednisolone, prednisone, 6β-hydroxycortisol and 6β-hydroxyprednisolone.

MATERIALS AND METHODS

Reagents and standards

Prednisone and prednisolone were purchased from Sigma (St. Louis, MO, U.S.A.); 6β-hydroxyprednisolone and 6β-hydroxycortisol were obtained from Steraloids (Wilton, NH, U.S.A.). Radioactive [6,7(*n*)-^{3}H] prednisolone (specific activity 41 Ci/mmol) and 6β-hydroxy-[1,2-^{3}H] cortisol (specific activity 43 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Great Britain). Reagents used were of analytical grade, solvents of HPLC grade (Fluka, Buchs, Switzerland). Prednisone and prednisolone were initially dissolved in methanol—glacial acetic acid (99.9:0.1, v/v) (10 μg/ml); 6β-hydroxycortisol and 6β-hydroxyprednisolone were dissolved in ethyl acetate (10 μg/ml). Similarly, the internal standards [^{3}H] prednisolone and 6β-[^{3}H] hydroxycortisol were prepared; the final solution used contained 100,000 dpm per 100 μl of each of the two steroids. To prepare standard curves, the steroids were added to normal human urine.

The absorption maximum of prednisone, prednisolone and 6β-hydroxyprednisolone was determined by UV spectrophotometry (Fig. 2).

All glassware was cleaned with acetone, decontaminated with Deconex 11 (Borer Chemie, Solothurn, Switzerland), ultrasonicated for 15 min, washed with distilled water and dried at 80° C.

Extraction procedure

Saturate 0.5—3 ml of urine containing 100,000 dpm of [^{3}H] prednisolone and 100,000 dpm of 6β-[^{3}H] hydroxycortisol as internal standards with sodium

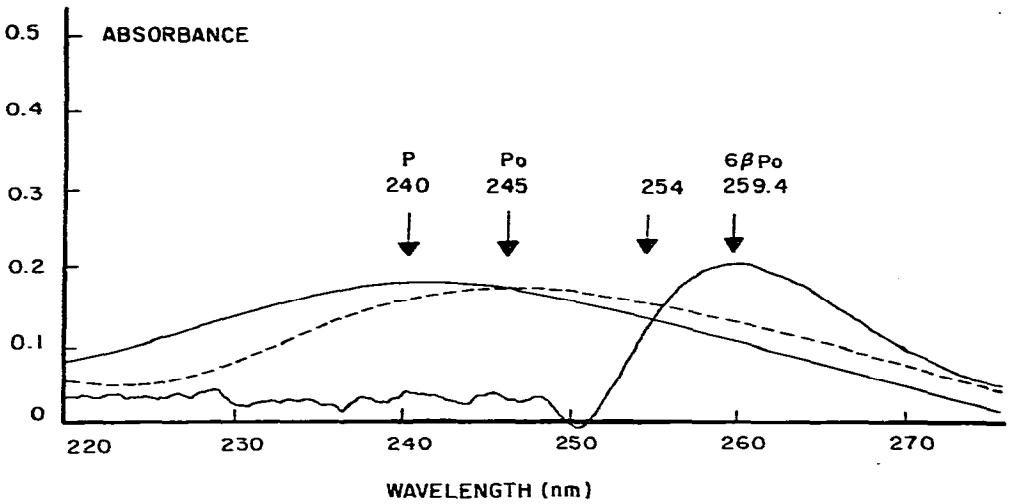

Fig. 2. UV absorbance spectra of prednisone (P), prednisolone (Po) and 6β-hydroxyprednisolone (6βPo). Prednisolone and prednisone were dissolved in methanol—glacial acetic acid (99.9:0.1, v/v) (10 μg/ml) and 6β-hydroxyprednisolone was dissolved in ethyl acetate (10 μg/ml). The arrows indicate the absorbance maxima. The UV detector used for the HPLC assay measures the absorbance at 254 nm.

sulfate for 10 min at 37°C. Extract with 9 ml of ethyl acetate using a mechanical shaker. Transfer the organic phase into a 16 × 160 mm glass tube and wash twice with 0.25 mol/l sodium hydroxide saturated with sodium sulphate. Remove the organic layer and evaporate it to dryness under a stream of nitrogen gas. Dissolve the residue in 3 ml of ethanol, mix for 20 sec, transfer into a new glass tube and evaporate to dryness. Dissolve the residue in 150 μl of ethyl acetate. For clinical samples inject 150 μl onto the column. Use a mobile phase consisting of hexane (55.9%), diethyl ether (31%), tetrahydrofuran (6.5%), ethanol (2.3%) and glacial acetic acid (0.3%). The observed pressure on the column is 186 MPa at a flow-rate of 1.8 ml/min. Set the UV detector at 0.004 A full scale and the double-pen recorder at 50 mV and 500 mV.

Apparatus

The HPLC pump was a Model 6000 A from Waters Assoc. (Milford, MA, U.S.A.), the UV detector an LC-15 from Perkin-Elmer (Norwalk, CT, U.S.A.) and the radioactivity detector an LB 504 with glass scintillator cell (0.5 ml) from Laboratorium Prof. Dr. Berthold (Wildbad, G.F.R.). The linear double-pen recorder was from Tegal Scientific (Martinez, CA, U.S.A.). The HPLC column, 250 × 3.2 mm, silica (SI-60), 5 μm average particle diameter was from Altex, (Berkeley, CA, U.S.A.). The UV spectrophotometer was an Aminco DW-2_a^{TM} (Division of Travenol Laboratories Inc., Silver Spring, MD, U.S.A.).

RESULTS

The chromatogram of pure prednisone, prednisolone, 6β-hydroxycortisol

and 6β-hydroxyprednisolone assessed by the UV detector at 254 nm and the radioactive peaks of the internal standards [^{3}H]prednisolone and 6β-[^{3}H]-hydroxycortisol assessed by the radioactivity monitor LB 504 are given in Fig. 3. The retention times for prednisone, prednisolone, 6β-hydroxycortisol and 6β-hydroxyprednisolone were 11, 14, 17, and 21 min, respectively. The ratio of the peak heights (cm) obtained by UV absorbance and radioactivity monitoring (counts) was used for internal standardization. 6β-[^{3}H]Hydroxycortisol was used as an internal standard for 6β-hydroxyprednisolone, and [^{3}H] prednisolone as an internal standard for prednisone and prednisolone. The analytical recovery for 6β-hydroxyprednisolone, (n = 12) and for [^{3}H]prednisolone (n = 12) was 70 ± 2% ($\bar{X}$ ± S.D.) and 85 ± 3%, respectively. The standard curves for all three steroids exhibit good linearity ($r^2 > 0.96$) from 200 to 7500 ng.

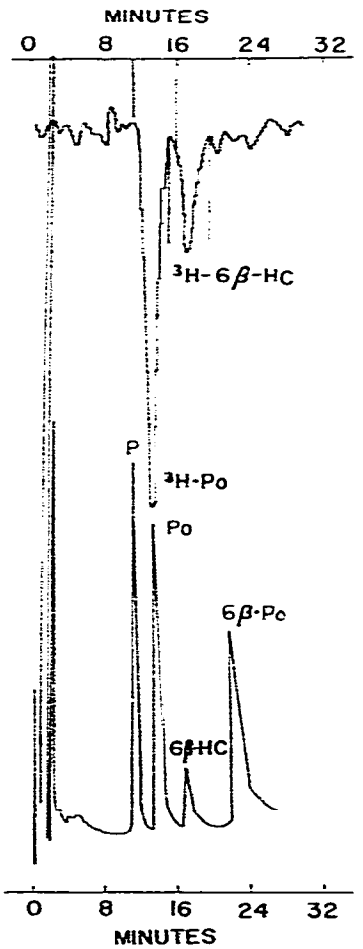

Fig. 3. The lower trace delineates the UV absorbance of injected standards of prednisone (P), prednisolone (Po), 6β-hydroxycortisol (6β-HC) and of 6β-hydroxyprednisolone (6β-Po). The upper trace depicts the radioactivity measurements of the internal standards [^{3}H]prednisolone (^{3}H-Po) and 6β-[^{3}H]hydroxycortisol (^{3}H-6β-HC). In this trace the negative mark indicates the start, and the positive mark indicates the end, of the integration of a peak.

Chromatograms of extracts of a blank urine and of urine containing 6β-hydroxyprednisolone, prednisolone and prednisone from the same subject are depicted in Fig. 4a and b. The blank urine shows no interference with the three steroids. The chromatogram in Fig. 4b was obtained by extracting 1 ml of urine after a dose of 50 mg of prednisolone was given. For routine analysis of 1 ml of urine, 200—7500 ng of each of the three steroids can be measured.

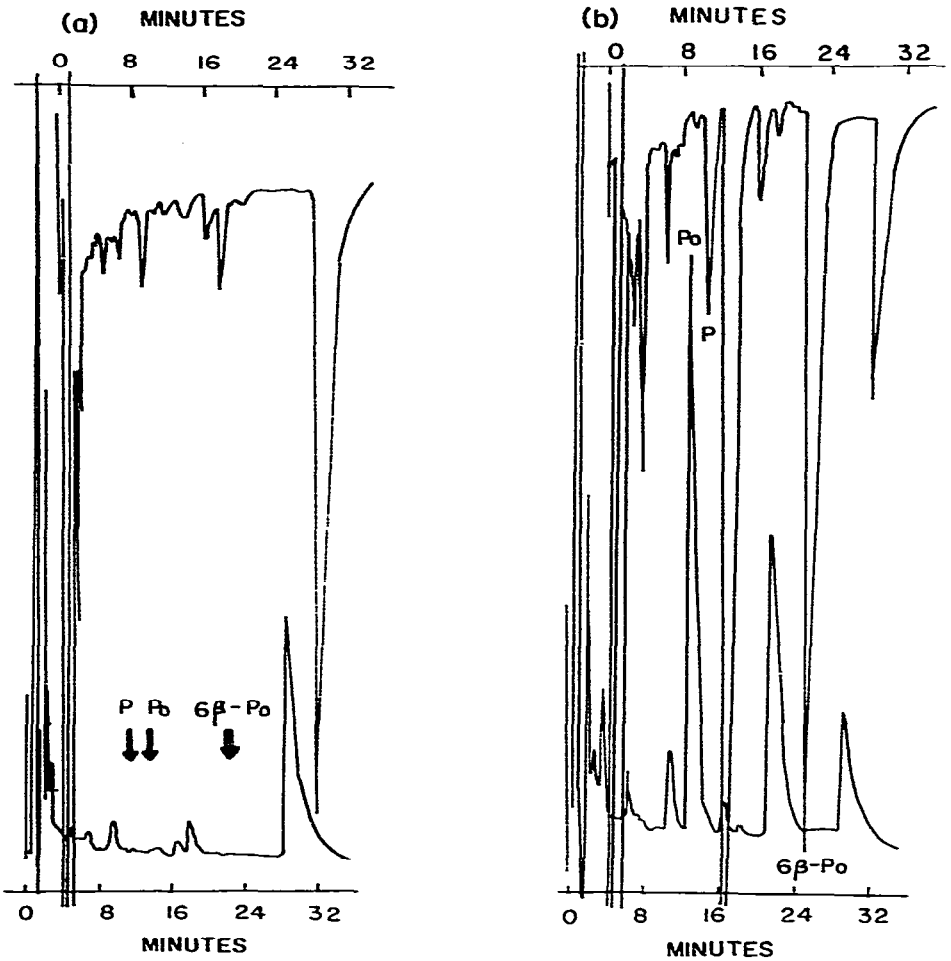

Fig. 4. (a) Dual-pen recording of chromatogram for blank human urine extract. The attenuation of the lower pen is 2.5 times that for the upper pen recording. P = prednisone; Po = prednisolone; 6β-Po = 6β-hydroxyprednisolone. (b) Dual-pen recording of chromatogram for urine sample from the same subject, treated with prednisolone.

The intra-day and inter-day variabilities for twelve measurements for each steroid are given in Table I. The urine used for these measurements was obtained from a patient on chronic prednisone treatment (0.7 mg/kg per day). To determine the stability of the three steroids, urine from a patient with a nephrotic syndrome treated with prednisone was stored at 4°C for four months; 0.5 ml of that urine was extracted and analyzed repeatedly (Fig. 5).

TABLE I

INTRA- AND INTER-DAY VARIABILITY OF THE METHOD

	C.V. (%)	
	Intra-day variability (n = 12)	Inter-day variability (n = 12)
6β-Hydroxyprednisolone	10.3	11.1
Prednisolone	5.5	4.5
Prednisone	10.9	11.6

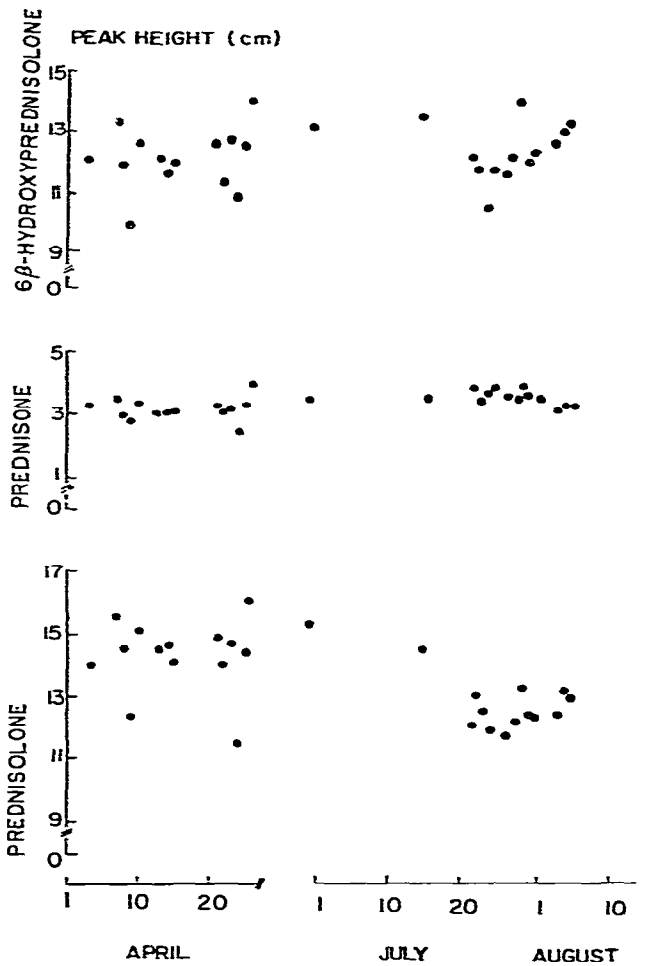

Fig. 5. Stability of prednisone, prednisolone and 6β-hydroxyprednisolone when urine of a patient treated with prednisone is stored at 4°C. On the y-axis the peak height of each of the three steroids measured by the UV detector is given.

Prednisone and 6β-hydroxyprednisolone are stable, while the UV absorbance attributable to prednisolone decreases by about 15% over a time period of four months.

Potential interference by 33 drugs commonly used was investigated by chromatographing extracts of urine obtained from patients treated with these drugs (Table II).

TABLE II

POTENTIAL INTERFERENCE* OF DRUGS WITH PREDNISONE, PREDNISOLONE AND 6β-HYDROXYPREDNISOLONE

Drug	Prednisone	Prednisolone	6β-Hydroxyprednisolone
Allopurinol	—	—	—
Amiodarone HCl	—	—	—
Atenolol	—	—	—
Calcitriol	—	—	—
Captopril	—	—	—
Chlorpromazine	—	—	—
Cimetidine	—	+	—
Clomethiazole	—	—	—
Clonidine HCl	—	—	—
Digoxine	—	—	—
Dihydralazine	—	—	—
Disulfiram	—	—	+
Ethinylestradiol—Linestrenol	—	—	—
Etofylline	—	—	—
Flucloxacilline	+	+	—
Furosemide	—	—	—
Heparin	—	—	—
Indapamide	—	—	—
Isosorbide dinitrate	—	—	—
Lynestrenol—Ethinylestradiol	—	—	—
Medigoxin	—	—	—
L-α-Methyldopa	—	—	—
Nifedipine	—	—	—
Norgestrel—Ethinylestradiol	—	—	—
Phenazone	—	—	—
Phenobarbital	—	—	—
Phenprocoumon	—	—	—
Phenytoin	—	—	—
Propranolol HCl	—	—	—
Pyridoxine	—	—	—
Sulfinpyrazone	—	+	—
Theophylline	—	—	—
Triameterene	—	—	—

*—, no interference; + interference.

Normal volunteers were given intravenous prednisolone and the amount of prednisolone, prednisone and 6β-hydroxyprednisolone in the urine was determined (Table III). For comparison, prednisolone and prednisone were measured by the method detailed in this paper and by our HPLC assay published previously [3]. Both methods provide similar results (Table III).

TABLE III

URINARY EXCRETION OF PREDNISOLONE, PREDNISONE AND 6β-HYDROXYPREDNISOLONE FOLLOWING INTRAVENOUS DOSING OF PREDNISOLONE IN FIVE NORMAL SUBJECTS

Subject	Dose (mg)	Prednisolone (mg per 24 h)	Prednisone (mg per 24 h)	6β-Hydroxyprednisolone (mg per 24 h)
1	52.5	15.5 (17.4)*	1.26 (1.68)	1.96
2	55.7	19.0 (17.6)	1.58 (1.25)	1.95
3	45.9	9.4 (9.0)	0.76 (0.83)	2.71
4	52.5	17.4 (19.6)	1.17 (1.28)	3.80
5	57.4	12.3 (13.8)	1.56 (1.63)	3.80

*Values in parentheses are the amounts obtained using the method of Frey et al. [3].

DISCUSSION

With the assay described we can simultaneously measure prednisone, prednisolone and 6β-hydroxyprednisolone in urine. The three steroids are extracted with ethyl acetate. Ethyl acetate was chosen for two reasons: (1) Frantz et al. [7] used ethyl acetate for the extraction of 6β-hydroxycortisol, a compound with close structural similarities to 6β-hydroxyprednisolone (Fig. 1), and (2) Morrison et al. [8] extracted prednisone and prednisolone with ethyl acetate. In our procedure the analytical recovery of 6β-[^{3}H]hydroxycortisol and [^{3}H]-prednisolone is higher than 70%. The analytical recovery of prednisone and 6β-hydroxyprednisolone was not determined accurately beause no labelled compound was available. However, comparison of the peak heights of these two steroids following direct injection with the peak heights following the extraction procedure indicates an analytical recovery of a similar magnitude.

The solvent system we previously described for measuring prednisone and prednisolone in plasma [3] allows the separation of urinary prednisone, prednisolone and 6β-hydroxyprednisolone on a silica column; however, the retention time for 6β-hydroxyprednisolone is about 1 h and no suitable internal standard was found. Roots et al. [9] determined 6β-hydroxycortisol by a normal-phase chromatographic procedure using a solvent system consisting of hexane (470 ml), methylene chloride (410 ml), ethanol (112 ml), and water (15 ml); likewise, these authors found no internal standard [9]. We tried more than 30 modifications of different solvent systems for separating the three steroids and presumptive internal standards. The solvent system described in the Methods section allows the separation of prednisone, prednisolone and 6β-hydroxyprednisolone; however, no internal standard was found. Therefore, we decided to add commercially available [^{3}H]prednisolone and 6β-[^{3}H]hydroxycortisol for internal standardization of the structurally related prednisone, prednisolone and 6β-hydroxyprednisolone. The radioactivity was monitored by a recently improved radioactivity detector coupled with the HPLC system. The solvent system described in our assay procedure does not contain the most commonly used constituent for glucocorticoid extraction and separation,

namely methylene chloride [3—5, 10]. This has the advantage that the radioactivity of tritium can be detected with the usually available monitors coupled with HPLC requiring scintillator fluid for detection. The latter detectors cannot be used for measuring radioactivity of tritium-labelled compounds, since the chlorinated solvents quench the radioactivity. Note that the recently developed high-efficiency glass scintillator cells allow the detection of tritium radioactivity also when chlorinated solvents are used.

There are several hints that our chromatographic peaks are attributable to prednisone, prednisolone and 6β-hydroxyprednisolone. Changing the retention times by changing the composition of the solvent system did not uncover additional peaks and/or induce differences in retention times between the pure compound injected directly on the column and the corresponding compound assessed in urine following glucocorticoid dosing. Prednisone and prednisolone amounts measured in urine by two different extraction procedures and separated by two different assay systems were of similar magnitude (Table III). For the clinical studies in progress, we are aware of potential chromatographic interference due to commonly used drugs (Table II); there is the possibility of using our assay system previously published [3] in the case of interference with prednisone and prednisolone. Possible interferences due to commonly used drugs were not reported for the method published by Rose and Jusko [4].

The renal excretion of prednisone measured in five healthy volunteers is within the range reported by Rose et al. [6]. The fraction of the dose of prednisolone excreted by our volunteers is higher than the fraction of the dose excreted by the volunteers of Rose et al. This is probably due to the higher dose of prednisolone given to our volunteers [6]. The increase in renal clearance of prednisolone with increasing dose of prednisolone administered, may best be explained by the non-linear binding of prednisolone to plasma proteins [11]. No data are available from the literature for comparison of the amount of 6β-hydroxyprednisolone excreted in urine, because no practical assay is available. The influence of disease states on the excretion of prednisone, prednisolone and 6β-hydroxyprednisolone, and the relevance of abnormal excretion of these steroids for prednisone dosing, will be reported in subsequent papers.

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