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Simultaneous analysis of prednisone, prednisolone and their major hydroxylated metabolites in urine by high-performance liquid chromatography

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ABSTRACT

A high-performance liquid chromatographic technique for the simultaneous determination of prednisone, prednisolone and their major hydroxylated metabolites, *viz.*, 20β-hydroxyprednisone, 6β-, 20α- and 20β-hydroxyprednisolone, in human urine is presented. The retention times were 6.5, 11.4, 18.1, 24.2, 31.6 and 35.3 min, respectively. The technique employs betamethasone as the internal standard. Samples are extracted with ethyl acetate using a diatomaceous earth extraction column, and the extract was dried and injected onto a silica gel column with ultraviolet detection at 254 nm. The calibration curve is linear within the studied range 50–1500 ng/ml for prednisolone and 50–750 ng/ml for the other steroids. The intra-day and inter-day coefficients of variation are less than 10% for prednisone and prednisolone but higher for the metabolites. The assay was used to study the excretion rate profile of each of these steroids in the urine of a normal male subject receiving a 49.3-mg intravenous dose of prednisolone. The results indicate that prednisone, 6β-, 20α- and 20β-hydroxyprednisolone may be the major unconjugated metabolites of prednisolone while 20β-hydroxyprednisone may be a minor metabolite.

INTRODUCTION

Prednisone and prenisolone are widely used synthetic corticosteroids. Prednisone is both a prodrug and a metabolite of the active drug prednisolone. Although the metabolites of these steroids (see Fig. 1) have been identified [1–3], their excretion pattern in the urine is not established perhaps due to the lack of a suitable high-performance liquid chromatographic (HPLC) assay. Frey and Frey [4] described a method for the simultaneous measurement of prednisone, prednisolone and 6β-hydroxyprednisolone in human urine. Using that method they were able to investigate the influence of drug interactions and disease states on the formation of 6β-hydroxyprednisolone [5]. However, at present there is no HPLC method available for the simultaneous determination of prednisone, prednisolone, 6β-hydroxyprednisolone and the 20-hydroxylated metabolites of prednisone and prednisolone.

Recently Teng and Benet [6] have also described an HPLC assay for simulta-

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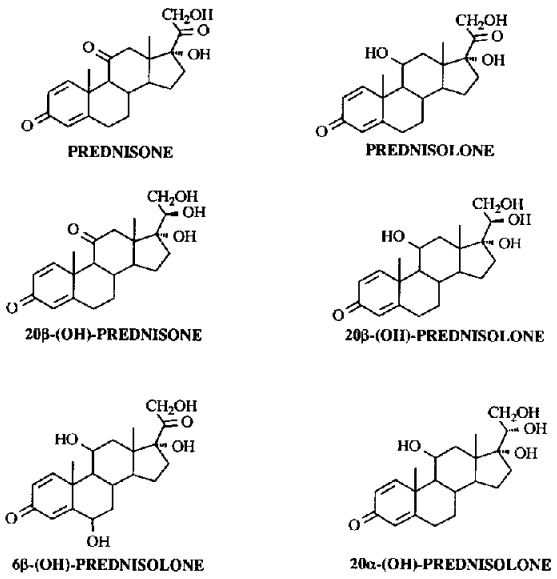

Fig. 1. Structures of prednisone, prednisolone and their major hydroxylated metabolites.

neous determination of prednisone, prednisolone and 6β-hydroxyprednisolone in human urine. We have modified their method to include three other hydroxylated metabolites of prednisone and prednisolone, *viz.*, 20β-hydroxyprednisone, 20α-hydroxyprednisolone and 20β-hydroxyprednisolone. This provides the most comprehensive HPLC assay for the determination of the metabolites of prednisone and prednisolone in human urine to date.

EXPERIMENTAL

Apparatus

The HPLC system used in this method consisted of a Waters Model 6000A solvent delivery system, an automated injector (Waters WISP Model 712) and a Waters Model 441 UV fixed-wavelength (254 nm) absorbance detector (Waters Assoc., Milford, MA, U.S.A.). Chromatographic separation was achieved with a Zorbax SIL (DuPont, Wilmington, DE, U.S.A.) column (250 mm × 4.6 mm I.D., 5–6 μm particle size) equipped with a 70 mm × 6 mm stainless-steel Whatman guard column packed with HC-Pellocil (37–53 μm particles) (Whatman, Clifton, NJ, U.S.A.). Peak heights were recorded on a Hewlett-Packard Model 3392A integrating recorder (Avondale, PA, U.S.A.).

Chemicals and reagents

The ethyl acetate used in the extraction procedure and the methanol and methylene chloride used in the mobile phase were of HPLC grade and obtained from

Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The glacial acetic acid used in the mobile phase was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). The anhydrous sodium sulfate used in extraction was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Prednisone, prednisolone, betamethasone, 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one, 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one, tetrahydrocortisone, 3α,11β-dihydroxyetiocholan-17-one and 3α-hydroxyetiocholane-11,17-dione were purchased from Sigma (St. Louis, MO, U.S.A.). 6β-Hydroxyprednisolone and 20α-hydroxyprednisolone were purchased from Steraloids (Wilton, NH, U.S.A.). 20β-Hydroxyprednisone and 20β-hydroxyprednisolone were gifts from Dr. D.F. Johnson (National Institutes of Health, Bethesda, MD, U.S.A.).

Extraction procedure

The internal standard used was betamethasone (500 ng/ml of sample). The rest of the extraction procedure was the same as described by Teng and Benet [6]. Briefly, 0.1–1.0 ml of urine samples, diluted if necessary to a total 1-ml volume with water, was mixed with the internal standard and poured into an inert, high-surface-area diatomaceous earth extraction column (Chem Elut, Analytichem International, Harbor City, CA, U.S.A.). After 5 min, the columns were flushed twice with 6 ml of ethyl acetate. The eluate was collected and washed twice with 1 ml of 0.2 *M* sodium hydroxide after which the organic layer was separated and dried with 1 g of anhydrous sodium sulfate. The solvent was evaporated to dryness at 30°C under a gentle stream of nitrogen.

Chromatography

Approximately 250 μl of mobile phase were used to reconstitute the residue, and a 50-μl aliquot was injected onto the column. The mobile phase consisted of methylene chloride–glacial acetic acid–methanol (91.3:7.5:1.2, v/v/v) and was pumped at a flow-rate of 2.0 ml/min.

Steroid recovery

The assay recovery of each steroid was assessed at three different concentrations in the following manner. Standards were made in urine and extracted as described. Betamethasone (500 ng) was added to each sample after extraction. After reconstitution with mobile phase (250 μl), 50–120 μl were injected onto the column. The ratio of the peak heights of each steroid relative to betamethasone was calculated. Next, the same amounts of steroids were added to 500 ng of betamethasone, reconstituted with the mobile phase and injected directly. The peak-height ratio was calculated for each steroid as before. The procedure was repeated twice ($n = 3$) and recovery was calculated using the following equation:

$$\text{recovery} = \frac{\text{mean peak-height ratio, extracted drug}}{\text{mean peak-height ratio, direct injection}} \times 100\%$$

Human studies

The assay was used to assess the excretion rate and urinary recovery of prednisolone and its major hydroxylated metabolites. An intravenous dose of prednisolone sodium phosphate (Hydeltrasol, Merck Sharp and Dohme, West Point, PA, U.S.A.) equivalent to 49.3 mg of prednisolone was administered to a healthy male subject as part of a clinical study. Urine was collected prior to prednisolone administration and at selected intervals for a period of 32 h after dosing. The volume of urine obtained in each interval was recorded, and a 10 ml aliquot was withdrawn from each interval collection and stored at −20°C until analysis.

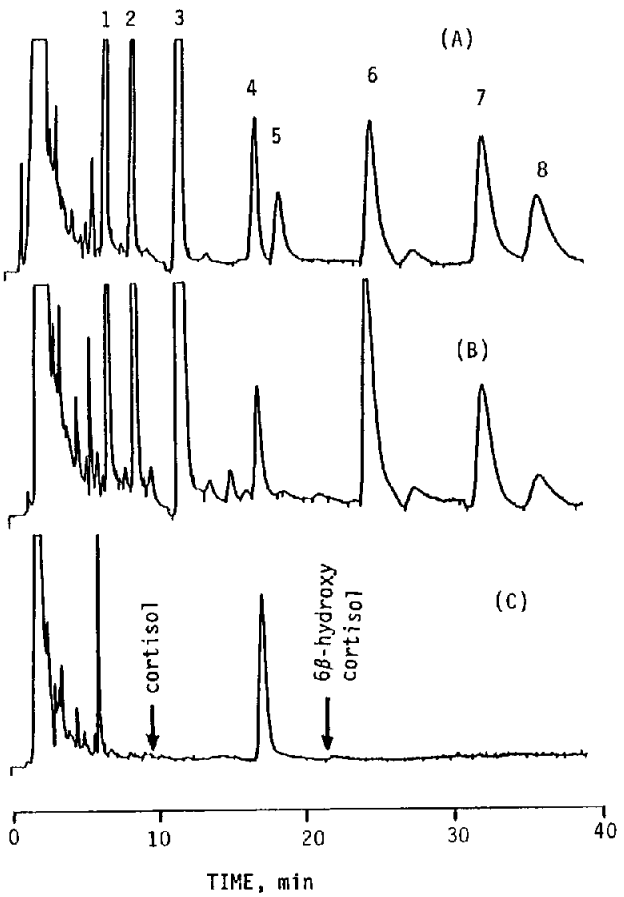

Fig. 2. Representative chromatograms obtained with (A) standards (prednisolone concentration 1500 ng/ml, others are about 750 ng/ml) made in diluted (1:3) blank urine and (B) diluted (1:6) urine sample obtained 0–2 h after a 49.3-mg intravenous dose of prednisolone to a normal male subject. A chromatogram of (C) blank extracted urine is shown for comparison with arrows indicating expected positions of endogenous cortisol and 6β-hydroxycortisol. Peaks: 1 = prednisone; 2 = internal standard; 3 = prednisolone; 4 = unknown endogenous substance; 5 = 20β-hydroxyprednisone; 6 = 6β-hydroxyprednisolone; 7 = 20α-hydroxyprednisolone; 8 = 20β-hydroxyprednisolone.

RESULTS

The HPLC conditions described allow baseline resolution of prednisone, prednisolone, the four metabolites and the internal standard (Fig. 2). The assay exhibited linearity over the tested concentration ranges of 50–1500 ng/ml for prednisolone and 50–750 ng/ml for the other steroids.

The lower limits for routine analysis in urine are 90 ng/ml for 20α-hydroxyprednisolone and 20β-hydroxyprednisolone and about 50 ng/ml for the other compounds.

The intra-day and inter-day variabilities were determined for all the compounds (Table I) except 20α-hydroxyprednisolone as this compound was not available at the time of validation of the assay procedure. For the hydroxy metabolites the coefficients of variation ranged from 10.1 to 30.2% for the lower concentration ranges, but were lower (3.6–11.1%) for the medium and high end of the standard curve. For prednisone and prednisolone the accuracy and precision were good over the entire range of the standard curves.

The extraction recovery of prednisolone was about 75% whereas for the other steroids, including prednisone, the recoveries averaged about 65% (Table II). These values seemed to be independent of the concentration of steroid in the urine.

TABLE I

INTRA-DAY AND INTER-DAY ASSAY VARIABILITIES

Steroid	Actual concentration (ng/ml)	Intra-day		Inter-day	
		Mean ($n = 5$) measured concentration (ng/ml)	C.V. (%)	Mean ($n = 7$) measured concentration (ng/ml)	C.V. (%)
Prednisone	103.6	106.1	4.6	105.4	2.9
	310.8	307.6	1.9	309.8	2.8
	621.6	642.3	2.4	624.5	4.7
Prednisolone	208.0	218.0	2.2	212.5	3.2
	624.0	615.9	2.8	622.9	2.2
	1248.0	1288.1	3.0	1280.7	5.7
20β-Hydroxy-	64.8	99.6	30.2	76.1	25.5
prednisone	194.4	197.9	10.9	195.1	11.1
	388.8	398.2	8.8	403.2	6.4
6β-Hydroxy-	90.8	96.7	23.0	93.2	10.1
prednisolone	272.4	265.0	5.1	268.4	6.6
	544.8	571.0	5.0	566.2	7.5
20β-Hydroxy-	108.0	108.4	12.8	101.3	29.7
prednisolone	324.0	304.2	6.7	318.0	3.6
	648.0	675.8	6.6	683.7	8.0

TABLE II

EXTRACTION RECOVERIES OF STEROIDS

Steroid	Concentration (ng/ml)	Recovery (mean, $n = 3$) (%)	Mean (%)
Prednisone	103.6	69.1	67
	310.8	65.0	
	621.6	66.0	
Prednisolone	208.0	73.5	76
	624.0	75.4	
	1248.0	78.6	
20β-Hydroxy-prednisone	64.8	62.6	69
	194.4	70.9	
	388.8	72.5	
6β-Hydroxy-prednisolone	90.8	64.4	65
	272.4	63.8	
	544.8	65.7	
20α-Hydroxy-prednisolone	98.4	64.7	66
	295.2	63.6	
	590.4	70.3	
20β-Hydroxy-prednisolone	108.0	62.8	65
	324.0	58.2	
	648.0	75.0	

The results of the human studies are summarized in Table III which shows the percentage of the prednisolone dose recovered in the urine unchanged or as metabolites, and in Fig. 3 which shows the excretion rate as a function of time. The results indicate that prednisone, 6β-, 20α- and 20β-hydroxyprednisolone may be major metabolites while 20β-hydroxyprednisone may be a minor metabolite of prednisolone. We have found similar results in another (unpublished) study in-

TABLE III

PERCENTAGE OF DOSE RECOVERED IN THE URINE FOLLOWING A 49.3-mg INTRAVENOUS DOSE OF PREDNISOLONE TO A NORMAL MALE SUBJECT

Steroid	Recovery (%)
Prednisone	3.3
Prednisolone	16.2
20β-Hydroxyprednisone	0.04
6β-Hydroxyprednisolone	4.7
20α-Hydroxyprednisolone	3.5
20β-Hydroxyprednisolone	5.5
Total	33.2

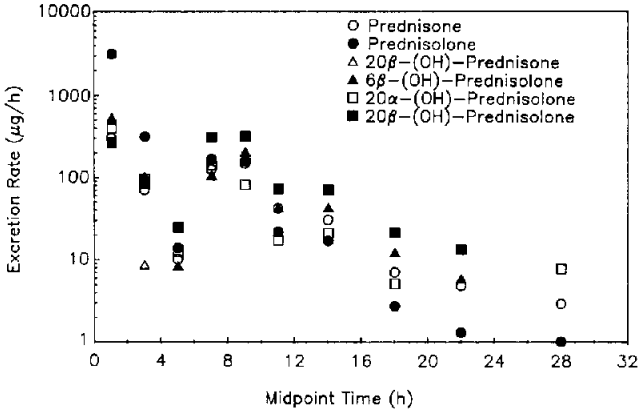

Fig. 3. Semilog plot of urinary excretion rate of prednisolone and its major metabolites as a function of time in a normal male subject after an intravenous dose of 49.3 mg prednisolone.

volving twelve, healthy, male volunteers receiving either an intravenous dose of 0.8 mg/kg prednisolone phosphate or an oral dose of 0.8 mg/kg prednisone.

Fig. 3 shows the decline of the excretion rate of prednisolone and its metabolites on a semilog plot. If the 5-h time points (corresponding to 4–6 h interval) are excluded from the picture, the remaining points show a smooth decline with time. During the 4–6 h interval the total urine volume was only 43 ml and it is possible that some urine was lost in collection.

DISCUSSION

The proposed normal-phase HPLC assay provides a simple and sensitive means of determining concentrations of prednisone, prednisolone and their major hydroxylated metabolites in the urine. The assay is also free from interference from endogenous cortisol and 6β-hydroxycortisol when they are present in high enough concentrations to be detected. The extraction recoveries were similar for the hydroxylated metabolites and prednisone but slightly higher for prednisolone.

Previous methods for determining the 20-hydroxy metabolites involved extraction, preliminary purification using thin-layer chromatography followed by derivatization and quantification by gas chromatography [2,3]. Rocci and Jusko [7] described an HPLC assay for the 20β-hydroxylated metabolites of prednisone and prednisolone in rabbit kidney perfusate. However, we could not apply their assay to human urine because of interference from an endogenous substance (peak 4 in Fig. 2).

At least six other metabolites of prednisone and prednisolone have been identified in the urine [8]. All of these have saturated ring systems (Fig. 4) and a mixture of five of these metabolites (one of them, 17α, 21-dihydroxy-5β-pregn-1-

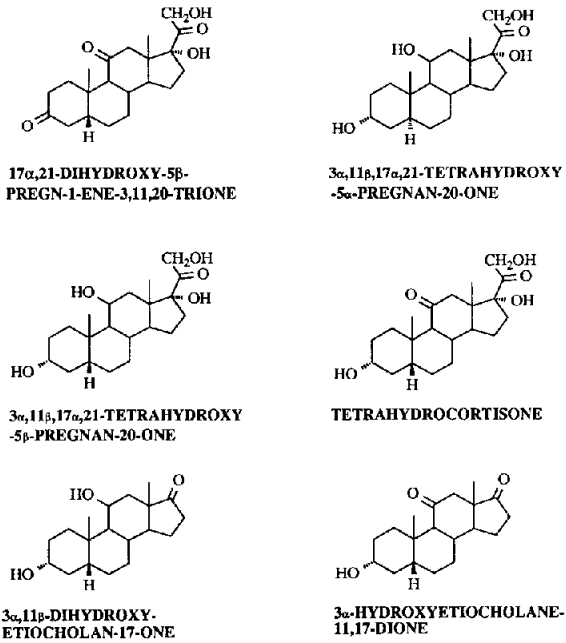

Fig. 4. Metabolites of prednisone and prednisolone with saturated rings.

ene-3,11,20-trione, was not available to us) failed to show any peaks in our system. This was not unexpected as ring saturation would decrease the absorptivity of these metabolites in the UV range.

The limitations of our method are long retention times and high variability at low concentration ranges of the metabolites. If 20β-hydroxyprednisone, which seems to be a minor metabolite, is eliminated from the assay, it may be possible to shorten the retention times and improve sensitivity and precision. For routine clinical studies, however, the sensitivity of our assay may be adequate. We have been able to measure all the metabolites after a 16.4-mg intravenous dose of prednisolone during a 24-h urine collection (unpublished work). By proper dilution it should be possible to measure the metabolites after lower doses (about 10 mg) of prednisone or prednisolone.

In conclusion, the proposed assay allows the most comprehensive examination to date of the metabolites of prednisone and prednisolone in the urine using HPLC.

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