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ANALYSIS OF PREDNISOLONE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, specific high-performance liquid chromatographic procedure for the determination of prednisolone in plasma is described. The organic solvent extract from plasma is chromatographed on a silica gel column using a mobile phase of 0.2% glacial acetic acid, 6% ethanol, 30% methylene chloride in *n*-hexane on a high-performance liquid chromatograph fitted with an ultraviolet detector (254 nm). Quantitation of plasma samples containing 25 ng/ml prednisolone is reported. Metabolites and endogenous hydrocortisone do not interfere with prednisolone. The determination of prednisolone concentrations in plasma following administration of a 10-mg single oral dose to a human subject is described.

INTRODUCTION

At present, the analytical procedures for the determination of prednisolone in plasma samples (obtained during bioavailability studies) are based on either radio-immunological or competitive protein binding principles [1, 2]. Qualitative [3] and quantitative [4] high-performance liquid chromatographic (HPLC) procedures for the determination of corticosteroids have been reported. The procedure reported by Trefz et al. [4] for the determination of hydrocortisone in plasma uses prednisolone as the internal standard. Although no attempt is made to do so, the method appears to be sensitive enough to allow also the quantitation of prednisolone. However, the long retention time and comparatively complex extraction procedure are not attractive for use in a bioavailability trial involving several thousand samples.

This report describes an HPLC method that is sufficiently sensitive and specific for the determination of plasma samples containing 25 ng/ml of prednisolone.

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EXPERIMENTAL

Materials

Prednisolone (U.S.P. reference) was used for the preparation of standard solutions. 20β-Dihydrocortisone (Sigma, St. Louis, Mo., U.S.A.) was used as the internal standard. Solvents used for the mobile phase were glacial acetic acid (Mallinckrodt Canada, Montreal, Canada), methylene chloride (Spectrograde, Caledon Labs., Georgetown, Canada) and "UV"-grade *n*-hexane (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.).

Anhydrous diethyl ether was freshly distilled prior to use. Prednisolone [6,7-^{3}H (nominal)] with a specific activity of 40 Ci/mmole (New England Nuclear, Montreal, Canada) was used in the extraction study.

Chromatographic procedure

A constant-volume high-performance liquid chromatograph (Waters Assoc., Milford, Mass., U.S.A.) containing a Model 6000A pump, U6K injector and Model 440 detector at 254 nm was used at an attenuation of 0.005 absorbance units full scale (a.u.f.s.). The column (250 × 3.2 mm I.D., 316 stainless steel) was packed with nominal 5-μm silica gel (LiChrosorb SI 60, Brinkman, Westbury, N.Y., U.S.A., manufactured by E. Merck, Darmstadt, G.F.R.) using a balanced density slurry packing procedure similar to that described by Majors [5]. The mobile phase, consisting of 0.2% glacial acetic acid, 6% ethanol, 30% methylene chloride in *n*-hexane (v/v), was prepared fresh daily. A flow-rate of 120 ml/h (2000 p.s.i.) was used.

Preparation of standard solutions

Prednisolone, accurately weighed, was dissolved in redistilled ethanol in a 1.0-ml volumetric flask. An aliquot of this solution was diluted with ethanol to produce a final solution of the desired concentration. Spiked plasma solutions (25—200 ng/ml) were prepared by addition of various volumes of standard prednisolone solution using a 10-μl syringe (Hamilton, Reno, Nev., U.S.A.).

The internal standard 20β-dihydrocortisone was prepared by a similar procedure.

General procedure

A 2.0-ml aliquot of plasma sample was added to a 15-ml Corex tube (Ingram & Bell, Don Mills, Canada) along with 2 μl (1 mg/ml) of internal standard solution and 6.0 ml of ether—methylene chloride (60:40). Parafilm was used to seal the tube which was then shaken for 15 min in an Evapo-Mix Constant shaker (Buchler, Fort Lee, N.J., U.S.A.) and centrifuged (10 min) at 7000 *g* (Sorvall Model RC2-B). A 5.0-ml aliquot of the organic phase was transferred to a 15-ml Corex tube containing 1.0 ml of 0.1 *N* aqueous hydrochloric acid, the tube shaken, and centrifuged as described previously. A 4.0-ml aliquot of the organic phase was transferred to a 5-ml conical tube and evaporated under nitrogen in a constant-temperature (55°) bath. The residue was immediately reconstituted with 200 μl of mobile phase and stored −20° prior to analysis. A 100-μl aliquot of the sample solution was chromatographed.

Calculations

The concentration of prednisolone in the plasma sample was determined from the following expression:

$$C_p = \frac{R}{m}$$

where C_p = concentration of the drug in plasma (ng/ml);
R = peak height ratio (drug/internal standard);
m = slope of the calibration curve.

Radioactive recovery experiment

[^{3}H]Prednisolone (62,395 dpm) was added to 2.0 ml of plasma containing either 25 ng/ml or 100 ng/ml of prednisolone and was extracted using the procedure described. The organic extract was transferred into a scintillation vial and evaporated to dryness. Ten milliliters of cocktail (BBS-3; Beckman, Fullerton, Calif., U.S.A.) were added and the radioactivity was determined by a Beckman L.S. 150 scintillation counter equipped with an automatic quench correction devise.

RESULTS AND DISCUSSION

The reproducibility and efficiency of the extraction procedure was determined using [^{3}H]prednisolone. Results, summarized in Table I, show that the extraction efficiency and reproducibility are comparable at plasma concentrations of 25 ng/ml and 100 ng/ml.

Fig. 1 shows the chromatogram obtained following the injection of a mixture of prednisolone, prednisone, dexamethasone, hydrocortisone and the internal standard. Note that prednisolone is well separated from its metabolite prednisone and endogenous hydrocortisone. A second metabolite of prednisolone, 20β-dihydroprednisolone [6—10] has a retention time greater than 20 min.

Fig. 2a shows the chromatogram obtained from the analysis of a spiked plasma sample containing 50 ng/ml of prednisolone using the system described above. This represents an injection of 16 ng of prednisolone. Note that in order to achieve this sensitivity, an attenuation of 0.005 a.u.f.s. is required. The peak to peak noise level at this attenuation was less than 1% of full-scale deflection.

Fig. 2b shows a chromatogram from pooled blank plasma obtained from 4 subjects. No interfering compounds were extracted from plasma using the described procedure.

TABLE I

EXTRACTION RECOVERIES OF [^{3}H]PREDNISOLONE FROM PLASMA

Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%) (n = 4)
25	76.1	1.6
100	74.6	1.5

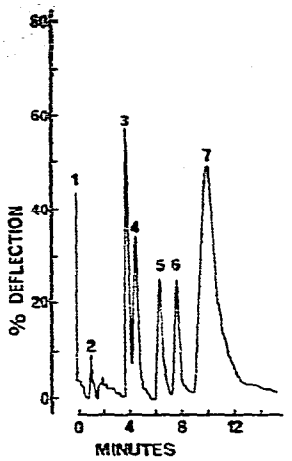

Fig. 1. High-performance liquid chromatogram showing separation of injected standards (qualitative). 1 = Injection; 2 = solvent front; 3 = prednisone; 4 = dexamethasone; 5 = hydrocortisone; 6 = prednisolone; 7 = internal standard.

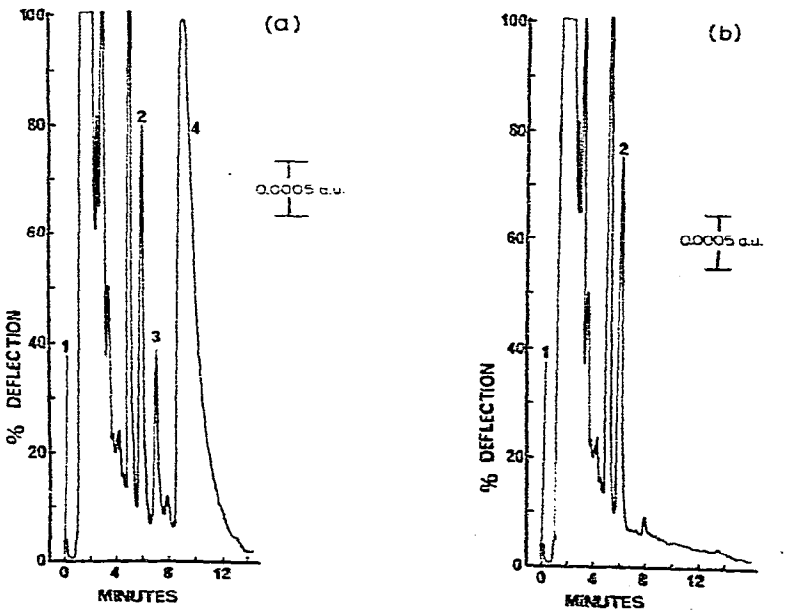

Fig. 2. High performance liquid chromatograms of (a) extracted human plasma spiked with prednisolone (50 ng/ml) and (b) human blank plasma extract. 1 = Injection; 2 = hydrocortisone; 3 = prednisolone; 4 = internal standard.

It must be emphasized that the resolution of prednisolone from endogenous substances is critically dependent on the percentage of ethanol used. The optimal percentage of ethanol necessary for a particular column was determined empirically. The mobile phase was always freshly prepared. Another problem encountered was that prednisolone extracted from plasma decomposed when stored overnight in the dried state. This problem was resolved by storing the extract in the mobile phase at −20°. Under this condition, the samples could be stored for several days without significant decomposition.

A calibration curve was obtained by plotting the peak height ratio (prednisolone/internal standard) versus the concentration of prednisolone in spiked plasma. The plot is linear and passes through the origin ($y = mx$) over the concentration range of 25 ng/ml to 200 ng/ml. The slope value is 0.0085 and its upper and lower 95% confidence limits are 0.0087 and 0.0083, respectively. The regression coefficient (R^2) is 0.999.

Three additional complete calibration curves, were constructed and the slopes were within the 95% confidence limit of the one reported. The slopes were also determined several times from the means of 4 spiked plasma standards (100 ng/ml) and these were also within the confidence limits reported.

Table II lists the results obtained from the analysis of a number of spiked plasma samples (25 ng/ml). These data reflect the accuracy and precision of the method. The mean recovery and the coefficient of variation were 98.6% and 3.9%, respectively.

The plasma prednisolone profile of a human subject (male, 90 kg) following

TABLE II

ESTIMATION OF PREDNISOLONE (25 ng/ml) IN PLASMA

Sample number	Theoretical (ng/ml)	Estimated (ng/ml)	Recovery (%)*
1	25	24.7	98.8
2	25	24.5	98.0
3	25	25.9	103.6
4	25	23.5	94.0
Mean			98.6
Coefficient of variation			± 3.9

*Recovery (%) = (estimated/theoretical) × 100%

TABLE III

PLASMA PREDNISOLONE CONCENTRATIONS IN A HUMAN SUBJECT FOLLOWING ORAL ADMINISTRATION OF A 10-mg DOSE

Time (h)	Prednisolone (ng/ml)
1	300
2	250
3	190
5	116
7	69

oral ingestion of two 5-mg prednisolone tablets (Delta Cortef; Upjohn, Toronto, Canada) is summarized in Table III. Blood was collected in heparinized 10-ml Vacutainer (Becton-Dickinson, Toronto, Canada) and the plasma, separated by centrifugation, was transferred to a 10-ml glass tube and stored at −20° prior to use.

The plasma drug concentration decayed in a mono-exponential manner with time. The $t_{1/2}$ was 3 h which is in reasonable agreement with the mean $t_{1/2}$ of 2.5 h as reported by Sullivan et al. [11].

In summary, the HPLC method is a sensitive and specific procedure for the determination of prednisolone in plasma following single dose (10 mg) administration in humans. The method has been used for assessing the specificity and accuracy of a radio-immunological assay (R.I.A.) for prednisolone and can be used in laboratories which are not equipped to carry out radio-immunological assays.

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