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CORTICOSTEROID ANALYSIS IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, specific, and reproducible high-performance liquid chromatographic assay for the simultaneous determination of prednisone, prednisolone and cortisol in biological fluids was developed with dexamethasone as the internal standard. Samples are extracted with methylene chloride, washed with sodium hydroxide and then water, and chromatographed on a microparticulate silica gel column with UV detection at 254 nm. Sensitivity was greater than 15 ng for all four steroids. Specificity was supported by use of dual wavelength UV detection and/or radioimmunoassay. The assay has been applied in pharmacokinetic studies and a typical plasma concentration—time profile for the three steroids is presented for one subject who received 50 mg of prednisone.

INTRODUCTION

Bioavailability and pharmacokinetic studies of synthetic glucocorticoids require efficient, sensitive, specific and reproducible analytical techniques. Most studies have employed radioimmunoassay (RIA) techniques to assess the bioavailability of prednisone or prednisolone formulations [1—3]. The RIA technique for these drugs, however, suffers from poor specificity [4] and poor reproducibility [1]. Only recently have high-performance liquid chromatography (HPLC) techniques been developed for glucocorticoid determinations in plasma [5—8]. However, these methods are either not efficient or not specific.

This report describes an HPLC method for the simultaneous assay of prednisone, dexamethasone, cortisol and prednisolone in plasma, urine, or saliva.

EXPERIMENTAL

Materials

The HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system with a U6K universal loop injector and a Model 440 UV absorbance detector with dual wavelength capability (Waters Assoc., Milford, Mass., U.S.A.). Wavelenghts of 254 and 280 nm were used. A 0.25 m × 4.6 mm I.D. stainless-steel column packed with 5—6 μm Zorbax™ SIL particles (DuPont, Wilmington, Del., U.S.A.) was attached to a 6 × 70 mm stainless-steel Whatman precolumn packed with HC Pellosil® (Whatman, Clifton, N.J., U.S.A.). Solvents for the mobile phase were purchased from Burdick & Jackson Labs., Muskegon, Mich., U.S.A. Analytical standards of prednisone, prednisolone, cortisol and dexamethasone were purchased from Sigma, St. Louis, Mo., U.S.A. Beclomethasone, 17α,20β,21-trihydroxy-1,4-pregnadiene-3,11-dione and 17α,20α,21-trihydroxy-1,4-pregnadiene-3,11-dione were gifts from Schering, Kenilworth, N.J., U.S.A. Methylprednisolone was a gift from Upjohn, Kalamazoo, Mich., U.S.A. and 6β-hydroxycortisol was a gift from Lederle Labs. (American Cyanamide), Pearl River, N.Y., U.S.A.

Prednisolone-6,7-^{3}H(N) with a specific activity of 45.4 Ci/mmole and cortisol-1,2-^{3}H(N) with a specific activity of 125 mCi/mg were obtained from New England Nuclear (Boston, Mass., U.S.A.). Pharmaceutical grade decolorizing carbon was obtained from the Amend Drug and Chemical Co., Irvington, N.J., U.S.A.

Preparation of standards. Four grams of decolorizing carbon were added to 100 ml of human plasma and stirred for 2 h at room temperature. Charcoal was then removed from the plasma by centrifugation overnight at 24,000 *g*. To this cortisol-stripped plasma, standards of dexamethasone, prednisone, prednisolone and cortisol in acetonitrile—methanol (1:1) were added to provide concentrations of 50—500 ng/ml.

Extraction procedure. Samples of saliva, plasma, or urine (1 ml) were added to 10 ml of methylene chloride. The internal standard, dexamethasone (125 ng) was then added and the glass culture tubes were shaken for 20 min. The tubes were centrifuged and the aqueous layer and creamy interface aspirated. The organic phase was then washed with 1 ml of 0.1 *N* sodium hydroxide and subsequently with 1 ml of water. After aspirating the aqueous phase, 1 g of anhydrous sodium sulfate was added to dry the organic phase. The latter was evaporated at 45° under a nitrogen gas stream.

Chromatography. The residue was reconstituted with approximately 200 μl of mobile phase for injection. The mobile phase was methanol—methylene chloride (3:97) at a column flow-rate of 2 ml/min. Concentrations of the individual steroids in plasma, urine, or saliva were determined from the slope of the plot of the peak height ratio of steroid: dexamethasone against standard concentrations of steroids.

Extraction recoveries. Trace quantities of tritiated cortisol or prednisolone were added to serum containing 50 or 500 ng/ml concentrations of dexamethasone, prednisone, prednisolone, and cortisol to determine the extraction

efficiency of the steroids. Four replicates of each concentration were extracted together with four samples of charcoal-stripped plasma. The washed organic phase was evaporated to dryness in a liquid scintillation counting vial and 10 ml of Aquasol® (New England Nuclear) were added. Samples were counted in a Packard Tri-Carb Model 3255 liquid scintillation counter (Packard, Downers Grove, Ill., U.S.A.) with a counting error of less than 1%. The sample channels ratio method of quench correction was employed to determine counting efficiency. The extraction efficiency was then calculated from dpm's obtained from samples and dpm's obtained from vials containing spike concentrations of ^{3}H-prednisolone or ^{3}H-cortisol.

RESULTS

A chromatogram of an extract of cortisol-stripped plasma spiked with prednisone, dexamethasone, cortisol and prednisolone is shown in Fig. 1. The

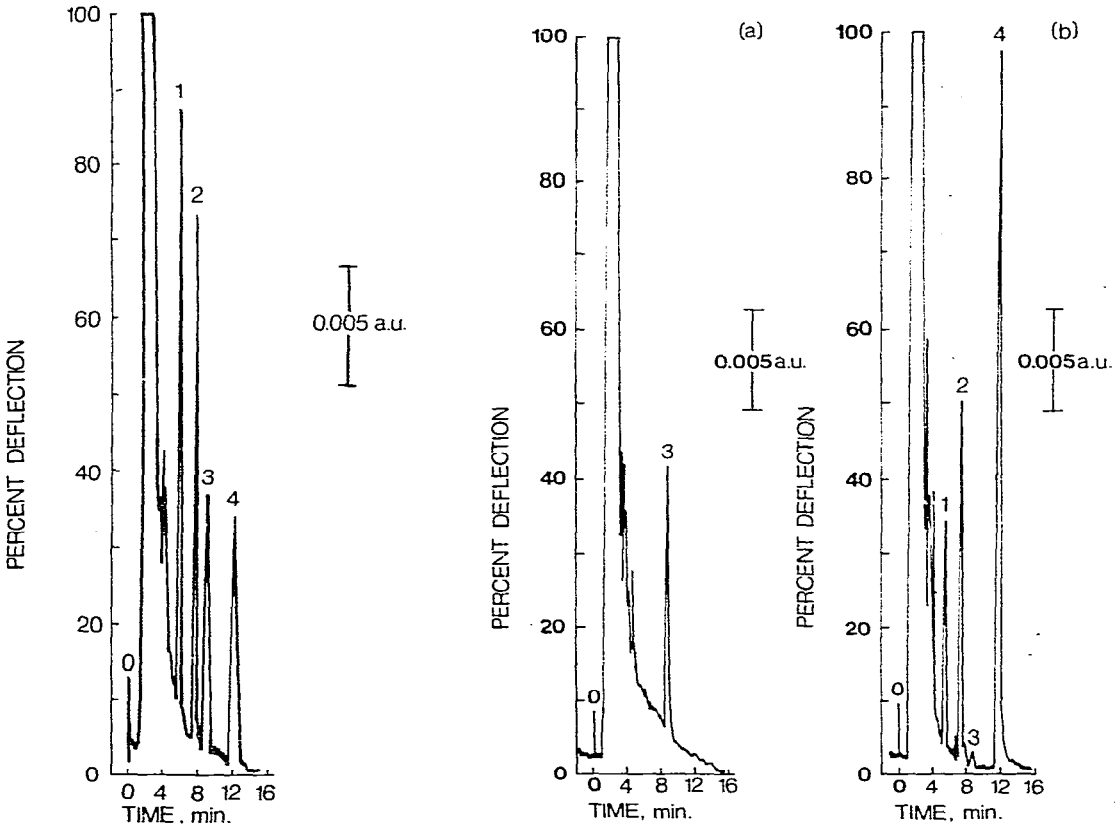


Fig. 1. Chromatogram of an extract of 1.0 ml of human plasma spiked with 100 ng of: (1) prednisone; (3) cortisol; (4) prednisolone. Dexamethasone, 125 ng (2) is the internal standard. The symbol (0) designates the injection point.

Fig. 2. Chromatograms of extracts of plasma: (a) taken prior to a dose of prednisone; (b) taken 3 h after a single oral dose of 50 mg prednisone. See Fig. 1 for peak assignments.

chromatogram illustrates the response to steroid concentrations of 100 ng per ml plasma and to 125 ng of dexamethasone, which was used as the internal standard. In Fig. 2a, the chromatogram of a plasma sample taken before the administration of a 50-mg tablet of prednisone is shown without the internal standard. Fig. 2b illustrates the response to steroid concentrations 3 h after the dose of prednisone, and represents concentrations of 56 ng/ml for prednisone, 10 ng/ml for cortisol, and 426 ng/ml for prednisolone.

Steroid concentrations in urine and saliva were also determined with this method after an oral dose of 50 mg of prednisone. Chromatograms of saliva and urine concentrations of steroids are shown in Fig. 3. In Fig. 3b, a chromatogram of 1.0 ml of urine taken from a 0—3 h urinary collection interval is monitored at wavelengths of 254 and 280 nm. A constant ratio of peak height of a steroid at the two wavelengths served as an added measure of specificity of the assay. The 280 to 254 nm ratios for the four steroids were: prednisone, 0.07; dexamethasone, 0.08; cortisol, 0.03; prednisolone, 0.09. While monitoring at dual wavelengths improves assay specificity, the 254 nm wavelength yields nearly optimum absorbance of several corticosteroids as demonstrated in Fig. 4. This wavelength is common, essentially, to all HPLC instruments which contain a mercury lamp and increases the general applicability of the assay technique.

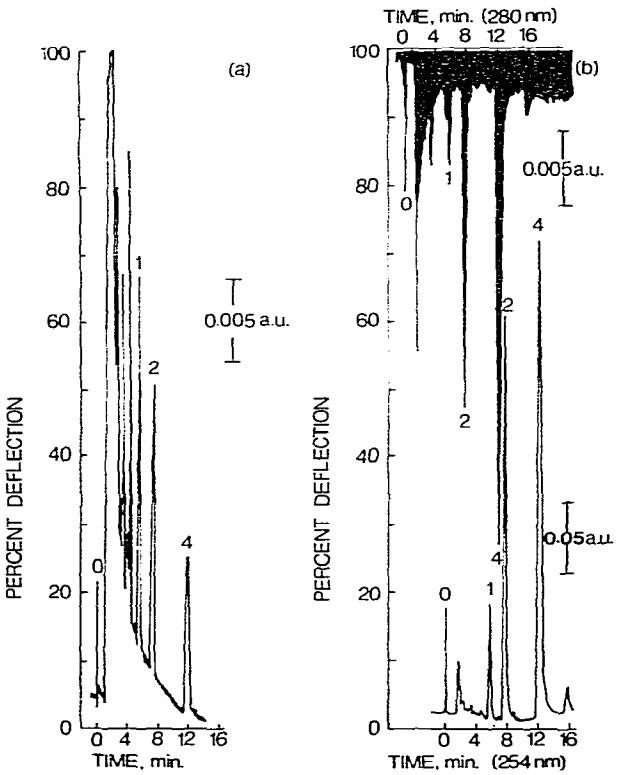


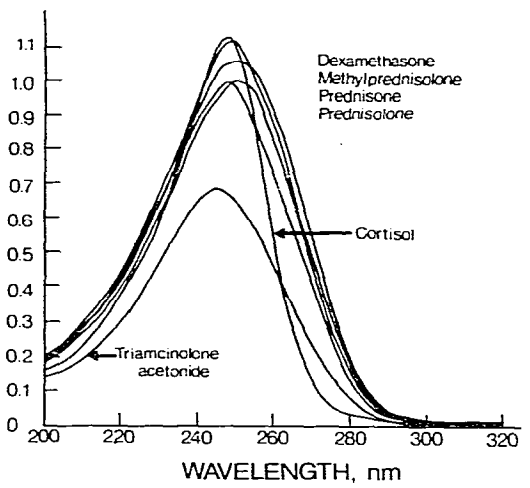
Fig. 3. Chromatograms of: (a) an extract of 3.0 ml of saliva collected 3 h after a 50-mg oral dose of prednisone; (b) an extract of human urine taken after a 50-mg oral dose of prednisone and monitored at wavelengths of 254 and 280 nm. See Fig. 1 for peak assignments.

The recovery of cortisol and prednisolone from plasma was approximately 83% (Table I) and was independent of concentration. The sensitivity limit of the assay for prednisone, cortisol and prednisolone was about 5 ng/ml when a signal-to-noise ratio of 2.5 or greater was used as a criterion for a significant response.

The response of the HPLC system was linear over the 0 to 500 ng/ml steroid concentration range, and as demonstrated in Fig. 5, a greater response was seen with prednisone than with cortisol or prednisolone.

The precision of the assay was determined with successive samplings of pooled patient plasma. Sixteen successive samplings gave coefficients of variation of 12, 5, and 8% for prednisone, prednisolone, and cortisol, respectively, as demonstrated in Fig. 6.

The specificity of the assay was determined by comparing retention times of standards to those of samples, by comparing the peak height ratio of a



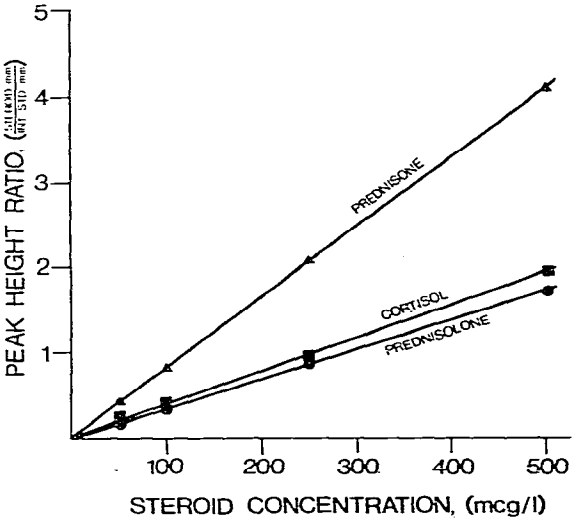


Fig. 4. UV absorbance spectra of several corticosteroids at concentrations of 25 μg/ml in acetonitrile—ethanol (1:1).

Fig. 5. Calibration curve for the determination of prednisone, prednisolone and cortisol in a 1.0-ml plasma extract.

TABLE I

EXTRACTION EFFICIENCY OF ^{3}H-PREDNISOLONE OR ^{3}H-CORTISOL FROM PLASMA CONTAINING DEXAMETHASONE, CORTISOL, PREDNISONE AND PREDNISOLONE

Steroid concentration (ng/ml)	Recovery (%)		(%)
	^{3}H-Prednisolone	^{3}H-Cortisol	
50	83.0 ± 2.68*	82.8 ± 2.6	
500	83.9 ± 2.6	81.4 ± 3.7	

*Mean ± S.D. of 4 determinations.

steroid at two wavelengths (254 and 280 nm) to the ratio of the standards, and also by correlating the observed concentrations of a steroid assayed by HPLC with concentrations determined by an RIA technique. For the latter, concentrations of prednisolone following a single intravenous dose of 40 mg of prednisolone sodium succinate were determined by this HPLC method and are compared with concentrations determined by RIA in Fig. 7. Nearly perfect

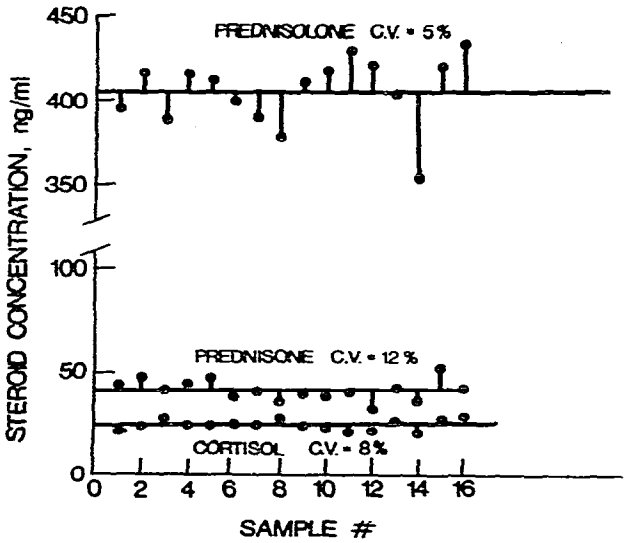


Fig. 6. Reproducibility of the HPLC assay method determined by 16 successive assays. The mean (horizontal bar) and coefficient of variation (C.V.) are as indicated.

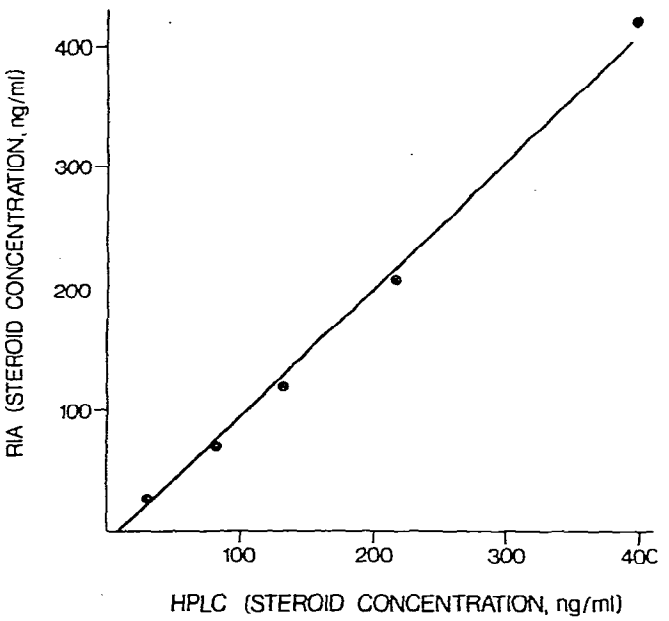


Fig. 7. Correlation between plasma prednisolone concentrations measured by HPLC and RIA ($r = 0.985$).

TABLE II

RETENTION TIMES OF SELECTED GLUCOCORTICOIDS AND THEIR METABOLITES

Steroid	Retention time (min)
Cortisone	4.2
Triamcinolone acetonide	4.3
Prednisone	5.4
Beclomethasone	6.5
Dexamethasone	7.1
Cortisol	8.4
Methylprednisolone	10.5
Prednisolone	11.6
17α,20α,21-Trihydroxy-1,4-pregnadiene-3,11-dione	15.5
17α,20β,21-Trihydroxy-1,4-pregnadiene-3,11-dione	18.0
6-β-Hydroxycortisol	18.4

agreement is seen. An additional check for specificity involved examination of other glucocorticoids and/or metabolites of prednisone. These materials had retention times as listed in Table II. The assay method clearly separates this series of closely related compounds. Many other conjugated metabolites of these steroids probably do not warrant concern as the organic extraction technique precludes the lipid-insoluble biotransformation products.

DISCUSSION

The simultaneous determination of prednisone, cortisol and prednisolone by this HPLC method proves to be efficient, precise, sensitive and selective. The procedure facilitates the characterization of prednisone and its pharmacologically active metabolite, prednisolone. Further, it allows examination of the effect these steroids have on circulating cortisol concentrations. The data in Fig. 8 were obtained in a healthy male volunteer who received a 50-mg prednisone dose in a bioavailability trial. These and other pharmacokinetic data confirm the expected disposition characteristics of these corticosteroids and the effect on cortisol secretion. The pharmacokinetics of prednisone and prednisolone will be reported more fully in subsequent reports.

Recent pharmacokinetic and bioavailability studies of prednisone have employed RIA techniques. However, poor precision with such RIA methods, as suggested by a coefficient of variation of 12—25% over the therapeutic concentration range [1], and a significant cross-reactivity with endogenous steroids or metabolites [4] complicate these results. In addition, endogenous interferences confound the assay performance to such an extent that standards must be constituted in each patient's zero hour plasma sample [1].

HPLC has been used only recently for steroid analysis in plasma [5—8], but such methods are not as efficient, specific or as versatile as the proposed procedure. Our method has proved specific and reliable. Over 2000 samples

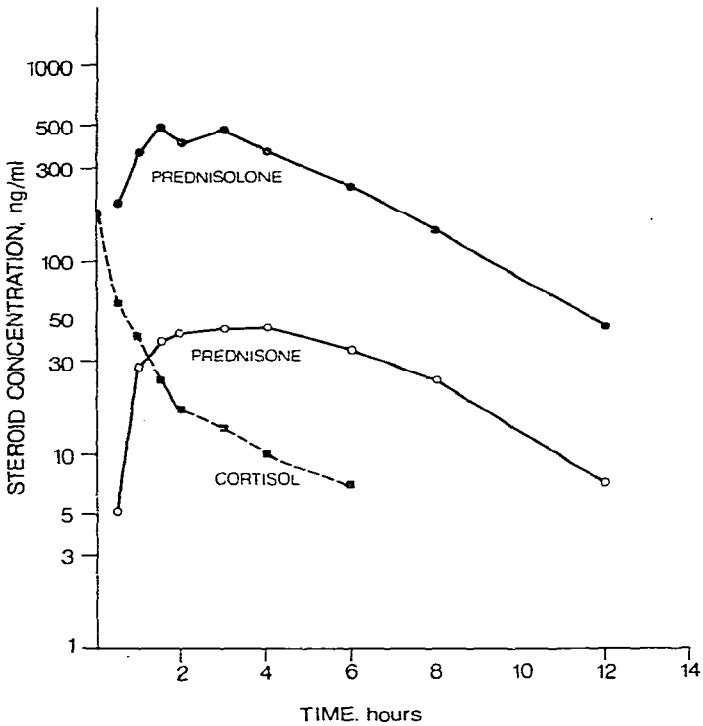


Fig. 8. Steroid plasma concentration—time profile of a human volunteer given a 50-mg oral dose of prednisone.

have been analyzed by this method in studies of the disposition kinetics of prednisone, cortisol, dexamethasone, prednisolone and methylprednisolone.

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