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Note

Determination of prednisolone in plasma by high-performance liquid chromatography

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Recent clinical interest in the bioavailability of prednisolone (11β,17α,21-trihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione) in paediatric applications indicates the need for a specific and sensitive assay. Unfortunately established radioimmunoassay techniques suffer from poor reproducibility [1] and poor specificity [2]. However, the introduction of high-performance liquid chromatography (HPLC) has led to the development of specific methods for the measurement of corticosteroids in biological fluids [3—8]. This paper describes an HPLC method which is both specific and sufficiently sensitive to allow the precise determination of prednisolone in small sample volumes usually available in paediatric practice. A mobile phase consisting of dichloromethane—ethanol—water—glacial acetic acid (500:30:30:1% total volume on separation, v/v) is used in conjunction with a 10-μm porous silica column. Operating at a flow-rate of 2 ml/min and employing ultraviolet (UV) detection at 254 nm permits the simultaneous determination of prednisolone, prednisone (17α,21-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,11,20-trione) and cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione) concentrations in plasma.

EXPERIMENTAL

Chemicals

Ethanol (absolute alcohol AR grade) was obtained from James Burrough, London, Great Britain. Dichloromethane (general purpose reagent grade) and diethyl ether, glacial acetic acid, hydrochloric acid and sodium hydroxide ("Analar" quality) were all purchased from BDH, Poole, Great Britain. Prednisone, dexamethasone, cortisol and prednisolone were supplied by Sigma

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(London), Poole, Great Britain. [6,7(n)-^{3}H]prednisolone with a specific activity of 41 Ci/mmol was provided by the Radiochemical Centre, Amersham, Great Britain. Bovine serum albumin (30% solution) was acquired from Armour Pharmaceutical, Eastbourne, Great Britain.

General procedure

A 1-ml volume of heparinised plasma containing 150 ng of dexamethasone (9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) as the internal standard, 1 ml of 0.1 *N* NaOH and 10 ml of ether—dichloromethane (60:40, v/v) were added to a 15-ml glass stoppered, round bottomed centrifuge tube and shaken for 10 min. After separation, achieved by centrifuging for 5 min at 1000 rpm (approx. 300 *g*), the organic phase was removed and washed by shaking with 1 ml of 0.1 *N* HCl for 5 min. Following centrifugation, as before, the organic layer was transferred to a 15-ml conical tube and evaporated to dryness under nitrogen at 55°C. The residue was reconstituted in 100 μl of the mobile phase and a 50-μl aliquot was injected onto the column.

Preparation of mobile phase

A 30-ml volume of water (double glass distilled), an equal volume of ethanol and 500 ml of dichloromethane were mixed for 1 h in a stoppered conical flask using a magnetic stirrer. Following separation in a glass separating funnel, the lower organic-rich layer was removed, and its volume determined. After the addition of glacial acetic acid (equivalent to 1% of the measured volume) and final mixing, the solvent was filtered through a 0.5-μm Millipore filter (type FH) using a Waters Assoc. Solvent Clarification Kit attached to a vacuum pump (approx. 15 inches of Hg).

Chromatography

The high-performance liquid chromatograph consisted of a Waters Assoc. Model 6000A constant volume pump, a U6K universal loop injector and a Model 440 UV detector. Absorbance was measured at 254 nm with an attenuation of 0.005 absorbance units full scale (a.u.f.s.). A standard 30 cm × 3.9 mm I.D. stainless steel μPorasil column (10 μm porous silica) was used in conjunction with a guard column packed with μBondapak C_{18}/Corasil. All of the chromatography equipment and columns were supplied by Waters Assoc., Hartford, Great Britain. The mobile phase, which was prepared fresh daily, was used at a flow rate of 2 ml/min (approximately 68 bars).

Preparation of steroid standards for calibration

Stock solutions of prednisolone and dexamethasone (100 μg/ml in ethanol) were diluted with ethanol to give standard solutions of 1 μg/ml and 10 μg/ml, respectively. From these, calibration standards were prepared in 1 ml of plasma or 3% bovine serum albumin so that each contained 25, 50, 100 or 150 ng of prednisolone and 150 ng of dexamethasone as the internal standard.

Extraction recovery experiment

Tritiated prednisolone (449,762 dpm) was added to 1-ml aliquots of plasma

TABLE I

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

Prednisolone conc. (ng/ml)	[^{3}H]Prednisolone added (dpm)*	[^{3}H]Prednisolone recovered (dpm)**	[^{3}H]Prednisolone recovered corrected for volume losses (dpm)**	Percentage recovery**	Coefficient of variation** (%)
0	449 672.2	352 294.04	409 163.22	90.99	2.41
25	449 672.2	350 262.00	407 276.78	90.57	2.74
100	449 672.2	348 312.06	408 877.96	90.93	1.91

*Mean, $n = 4$.
**Mean, $n = 5$.

containing 0, 25 and 100 ng of prednisolone plus 150 ng of dexamethasone and extracted using the procedure described. The organic extract was transferred to a scintillation vial and evaporated to dryness. Ten millilitres of Lumagel scintillation cocktail (LKB-Wallac, Turku, Finland) were added and the samples counted in an LKB-Wallac Rack Beta Model 1215 liquid scintillation counter equipped with automatic quench calibration. Using the external standards ratio method of quench correction it was possible to determine counting efficiency and express all results as disintegrations per min.

RESULTS

It is readily apparent from Table I that the extraction efficiency (approximately 91%) and reproducibility (coefficient of variation < 2.74) are independent of prednisolone concentration. Calibration curves were obtained by comparing the peak height ratio (prednisolone/internal standard) with the actual concentration of prednisolone in spiked aliquots of plasma or 3% bovine serum albumin. A linear relationship exists over the concentration range 25—150 ng/ml, with the line passing through the origin. Slope values are 0.0052 and 0.0055 for 3% bovine serum albumin and plasma, respectively, and the correlation coefficient (r) is 0.999 in both cases.

The effects of sample storage on reproducibility of results were examined and the findings are listed in Table II. Analyses were carried out in duplicate, at weekly intervals, on ten replicate samples containing either 20 or 100 ng/ml prednisolone in 3% bovine serum albumin. These had been stored at —20°C for up to four weeks maximum. The mean recovery and coefficient of variation were 103.9% and 8.04% and 101.6% and 6.08% for prednisolone concentrations of 20 and 100 ng/ml, respectively. Clearly the method is valid and storage for one month at —20°C has no adverse effects.

TABLE II

DETERMINATION OF PREDNISOLONE IN 3% BOVINE SERUM ALBUMIN

Samples stored at —20°C and assayed in duplicate at weekly intervals over a period of four weeks.

Prednisolone added (ng/ml)	Prednisolone estimated* (ng/ml)	Recovery (%)	Coefficient of variation (%)
20	20.78	103.9	8.04
100	101.6	101.6	6.08

*Mean, $n = 10$.

The separation of prednisolone from other steroids was also examined. A representative chromatogram obtained following the injection of a mixture of prednisone, dexamethasone, cortisol and prednisolone is illustrated in Fig. 1. It can be seen that complete separation of these steroids is achieved by this method.

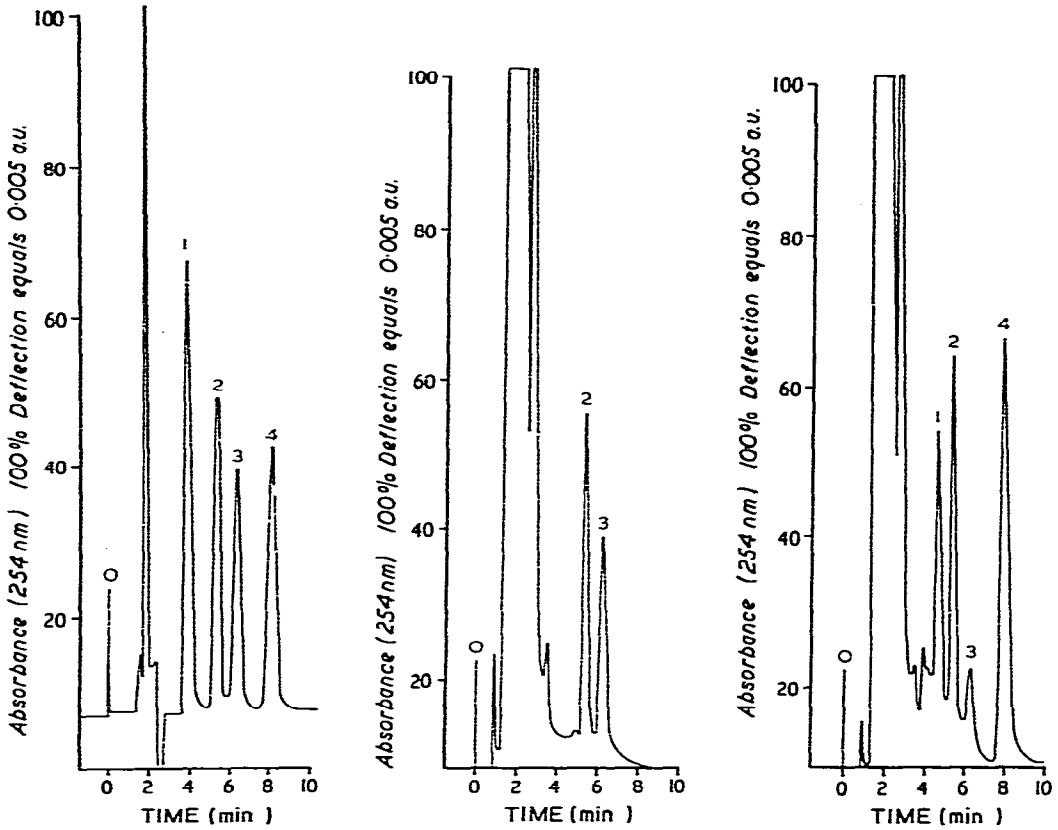

Fig. 1. Chromatogram showing separation of a standard steroid mixture. Peaks: 0 = injection, 1 = prednisone, 2 = dexamethasone, 3 = cortisol, 4 = prednisolone.

Fig. 2. Chromatogram showing an extracted human plasma blank obtained immediately before oral administration of 10 mg of prednisolone. Peaks: 0 = injection, 2 = dexamethasone, 3 = cortisol.

Fig. 3. Chromatogram of extracted human plasma obtained from the same volunteer control as in Fig. 2, 1 h after ingesting 10 mg of prednisolone. Peaks: 0 = injection, 1 = prednisone, 2 = dexamethasone, 3 = cortisol, 4 = prednisolone.

In Fig. 2, the chromatogram of an extracted plasma sample, taken before the administration of a 10-mg tablet of prednisolone, is shown. Cortisol and the internal standard peaks are present and it is readily apparent that no interfering compounds were extracted from the plasma.

Fig. 3 illustrates the response of the same patient 1 h after the 10-mg dose of prednisolone. The cortisol peak is considerably suppressed as the prednisolone level approaches its maximum. A peak corresponding to prednisone may also be observed.

The application of the method in clinical practice is shown by the dose—response curve of a human female (74 kg) following the ingestion of 10 mg of prednisolone (Table III).

TABLE III

PLASMA PREDNISOLONE CONCENTRATIONS FOLLOWING A 10-mg DOSE ADMINISTERED ORALLY TO A VOLUNTEER FEMALE CONTROL (74 kg)

Time (h)	Prednisolone (ng/ml)
0.5	115
1.0	232
1.5	279
3.0	225
5.0	114.5
7.0	61
10.0	24
15.0	9

DISCUSSION

The separation and subsequent estimation of steroids in biological fluids by HPLC has been achieved using both silica and reversed-phase columns [3—8]. Separations involving the use of silica packings may be facilitated either by an adsorption mechanism [3, 6, 7] or by liquid—liquid partition, such as the method of Trefz et al. [8] utilizing a solvent system based on that of Hesse and Hövermann [9]. The method described in this paper evolved from attempts to reduce the affinity of the silica packing material for the steroids and so shorten the retention times. This was achieved by employing a water-saturated mobile phase to deactivate the silica [10]. The further addition of 1% glacial acetic acid served to improve resolution and sharpen peak shapes. There are, however, obvious similarities between our method and that of Trefz et al. [8], who proposed that the separation mechanism probably involves liquid/liquid partition.

The determination of prednisolone by this method proves to be efficient, precise, sensitive and selective, offering the advantage of both faster analysis time and greater sensitivity compared to other HPLC techniques [3—8]. Furthermore, the sensitivity limit of the assay (approximately 10 ng/ml) could be enhanced by reducing the amount of internal standard added and injecting larger sample volumes, thus permitting the accurate measurement of concentrations as low as 5 ng/ml.

An additional advantage is the ability to measure simultaneously prednisone, cortisol and prednisolone. Indeed, the appearance of a prednisone peak can be observed in patient samples as the prednisolone level approaches its maximum (see Fig. 3) and disappears as the prednisolone level falls. This confirms the observations of Scott et al. [5] and provides further evidence of the metabolism of prednisolone to prednisone [11]. The decay and eventual disappearance of the cortisol peak may also be observed, indicating that suppression of endogenous corticosteroid production by the adrenal cortex occurs under the influence of increasing plasma prednisolone levels [12].

The chromatograms illustrated in Figs. 2 and 3 are those obtained from the plasma of an adult female volunteer and are representative of the many samples

analysed. Similar peak patterns are present in the extracted plasma from paediatric patients and there appear to be no interfering peaks peculiar to paediatric plasma. In fact, in nearly two years we have not encountered any interferences in either adult or paediatric samples.

The comparison of calibration standards revealed that it is permissible to replace authentic plasma with 3% bovine serum albumin to provide calibration data and this is the practice we have adopted.

The application of this method to the measurement of steroid concentrations in saliva, urine and cerebrospinal fluid is currently under investigation and these findings will be reported later.

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