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ANALYSIS OF PREDNISONE, PREDNISOLONE AND THEIR 20β-HYDROXYLATED METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic technique primarily developed for use on samples from kidney perfusion studies is presented for simultaneous determination of prednisone, prednisolone and their 20β-hydroxylated metabolites. The technique employs 6β-hydroxycortisol as the internal standard. Samples are extracted with ethyl acetate, washed with sodium hydroxide and water and injected onto a silica gel column with UV detection at 254 nm. Inter- and intraday variability of the assay was determined at two concentrations of each steroid and was less than 10%. Assay steroid recovery ranged from 54.1% for prednisone to 63.2% for 20β-hydroxyprednisone. Sensitivity is 4—10 ng/ml for the steroids measured. The chromatographic conditions may be modified to permit quantitation of these steroids from plasma samples. This method may alternatively be used for quantitation of 6β-hydroxycortisol, an endogenous indicator of enzyme induction. A perfusate concentration—time profile is presented from a kidney perfusion study using prednisolone.

INTRODUCTION

Perfusion studies examining the renal disposition of prednisone and prednisolone have demonstrated extensive metabolism of these compounds by the rat kidney [1, 2]. In a similar experiment exploying cortisol, a high degree of metabolism to cortisone as well as production of the 20β-hydroxylated derivatives of cortisol and cortisone was demonstrated using radioactive cortisol and thin-layer chromatography [3]. Given the similarities in structure between the prednisone–prednisolone and cortisone—cortisol steroid pairs, the 20β-hydroxy derivatives of prednisone and prednisolone may be major metabolites resulting from the renal biotransformation of these substrates by the kidney

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[3]. To quantitatively examine the renal metabolism of prednisone and prednisolone in the isolated perfused kidney, a sensitive and specific assay for measurement of these steroids was needed. This report describes a high-performance liquid chromatographic (HPLC) technique for determinations of prednisone, prednisolone, and their 20β-hydroxylated metabolites in perfusate and urine samples. Modification of the chromatographic conditions to permit quantitation of these compounds in plasma is also described.

EXPERIMENTAL

Materials

The HPLC system utilized in this procedure consisted of a Waters Model 6000A solvent delivery system, U6K universal loop injector and a Model 440 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.). The UV absorbance of all steroids was measured at 254 nm. A Zorbax™ Sil (DuPont Labs., Wilmington, DE, U.S.A.) column (25 cm × 4.6 mm I.D., 5—6 μm particle size) equipped with a 70 × 6 mm stainless-steel Whatman precolumn was used to separate the compounds. The precolumn was packed with HC-Pellosil (37—53 μm particles) (Whatman, Clifton, NJ, U.S.A.). The ethyl acetate used in the extraction procedure and the heptane and methylene chloride used in the mobile phase were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The pure ethyl alcohol (U.S.A.) employed in the mobile phase was obtained from U.S. Industrial Chemicals (New York, NY, U.S.A.). The glacial acetic acid, also used in the mobile phase was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Prednisone and prednisolone were purchased from Sigma (St. Louis, MO, U.S.A.). The 1,4-pregnadiene-17α,20β,21-triol-3,11-dione (20β-hydroxyprednisone) was a gift from Schering Corporation (Bloomfield, NJ, U.S.A.). The 1,4-pregnadiene-11β,17α,20β,21-tetrol-3-one (20β-hydroxyprednisolone) was a gift from the Steroid Reference Collection (Westfield College, London, Great Britain) and 6β-hydroxycortisol was a gift from Lederle Laboratories (American Cyanamide, Pearl River, NY, U.S.A.).

Standard preparation

Stock solutions of prednisone, prednisolone, 20β-hydroxyprednisone and 20β-hydroxyprednisolone prepared in an acetonitrile—methanol mixture (1:1) were added to perfusate and water to produce standard concentrations ranging from 50—1500 ng/ml.

Extraction procedure

Samples of perfusate or urine (1 ml) were added to glass culture tubes. The internal standard, 6β-hydroxycortisol (ca. 600 ng) was added to each of the samples. Each tube was vortexed to mix the internal standard and sample. To this mixture, 20 ml of ethyl acetate was added. The tubes were shaken for 20 min. The aqueous layer was removed and the organic phase was washed with 1 ml of 0.1 *N* sodium hydroxide and centrifuged. After removal of the aqueous layer the organic phase was washed with 1 ml of distilled, deionized water. After centrifugation and aspiration of the aqueous phase, 1 g of anhydrous sodium sulfate was added to dry the organic phase, which was sub-

sequently evaporated to dryness at 45°C under a continuous nitrogen stream.

Chromatography

Approximately 200 μl of mobile phase was used to reconstitute the residues just prior to injection. The mobile phase consisted of a heptane—methylene chloride—ethanol—acetic acid (60.8:29.5:8.1:1.6) mixture and was pumped through the column at a solvent speed of 3 ml/min.

Steroid recovery

The assay recovery of each steroid was assessed at 100 ng/ml and 1000 ng/ml in the following manner. Five perfusate samples (1 ml) containing each steroid were extracted and injected. Five injections of the same amount of steroid (e.g. 100 or 1000 ng) in mobile phase were directly injected. The peak heights of the steroids in both sets of samples were measured. The assay recovery of each steroid was computed using the following equation:

$$\% \text{ Recovery} = \frac{\text{Peak height extract}}{\text{Mean peak height direct injection}} \times 100$$

Perfusion

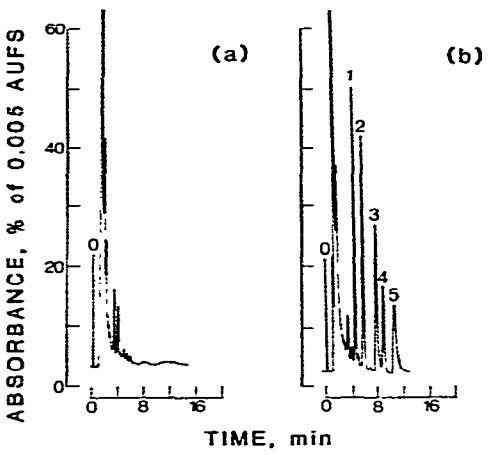
A kidney perfusion study was performed to assess if the 20β-hydroxy metabolites of prednisone and prednisolone were major renal metabolites of these steroids. A rabbit kidney was perfused with prednisolone at an initial perfusate concentration of approximately 900 ng/ml for a period of 90 min. Perfusate samples were obtained at selected times during the experiment and were assayed for prednisone, prednisolone, 20β-hydroxyprednisone and 20β-hydroxyprednisolone.

RESULTS

The chromatograms resulting from the injection of blank perfusate as well as perfusate spiked with prednisone, prednisolone, 20β-hydroxyprednisone, 6β-hydroxycortisol and 20β-hydroxyprednisolone are presented in Fig. 1. Fig. 1b illustrates the response to steroid concentrations of approximately 250 ng/ml and to 625 ng of internal standard.

The steroid recoveries at two concentrations and sensitivity limits for each of the steroids are presented in Table I. The mean assay recovery ranged from 54.1% for prednisone to 63.2% for 20β-hydroxyprednisone; the approximate sensitivity limits of the assay range from 4—10 ng/ml (Table I). A typical calibration plot resulting from the injection of perfusate standards containing the steroids at selected concentrations in the 0—1500 ng/ml range is illustrated in Fig. 2. Detector response was linear in this range for each of the steroids with the greatest response observed for prednisone judging from the slopes of the calibration plot.

The intraday and interday variabilities of the assay for each of the steroids are presented for low and high concentrations in Table II. Both variability studies resulted in coefficients of variation of less than 10% for each steroid examined.



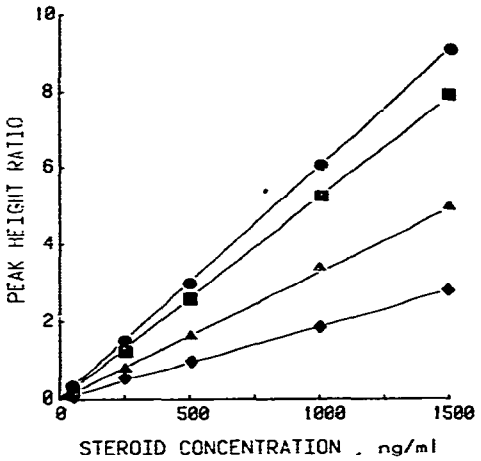


Fig. 1. Chromatograms of (a) blank perfusate extract; (b) perfusate extract spiked with 250 ng of (1) prednisone, (2) prednisolone, (3) 20β-hydroxyprednisone and (5) 20β-hydroxyprednisolone. 6β-Hydroxycortisol (4), 625 ng, is the internal standard. The symbol (o) designates the injection point.

Fig. 2. Calibration curve for the determination of prednisone (●), prednisolone (■), 20β-hydroxyprednisone (▲) and 20β-hydroxyprednisolone (◆) in a 1-ml perfusate extract.

TABLE I

STEROID RECOVERIES AT TWO CONCENTRATIONS AND SENSITIVITY LIMITS OF THE ASSAY

Steroid	Recovery (%)			Sensitivity limit* (ng/ml)
	100 ng/ml**	1000 ng/ml**	Mean	
Prednisone	50.8 (5.0)	57.4 (9.2)	54.1	10
Prednisolone	53.0 (5.0)	70.3 (5.8)	61.6	10
20β-Hydroxyprednisone	62.1 (5.2)	64.4 (4.6)	63.2	4.0
6β-Hydroxycortisol	54.7 (2.6)	63.5 (4.2)	59.1	—
20β-Hydroxyprednisolone	58.9 (4.7)	56.1 (6.5)	57.5	5.0

*Based on a signal-to-noise ratio of 2.5.
**Mean (± S.D.).

Although the assay was primarily developed for use on perfusate and urine samples obtained from kidney perfusion experiments, the assay may be adapted for use on plasma samples through alteration of the mobile phase. Fig. 3 presents chromatograms resulting from the injection of blank plasma (Fig. 3a) and plasma spiked with each steroid (Fig. 3b) to a concentration of approximately 250 ng/ml. The mobile phase employed during injection of the plasma extracts was heptane—methylene chloride—ethanol—acetic acid (71.4:20.4:6.9:1.3) at a solvent speed of 2 ml/min. This modification of the chromatographic conditions results in good separation of all steroids from each other and from interferences present in the plasma samples. The interference between prednisone and prednisolone in Fig. 3b is probably endogenous

TABLE II

INTRADAY AND INTERDAY COEFFICIENTS OF VARIABILITY

All variability statistics are based on ten measurements.

Steroid	Intraday variability coefficient of variation (%)		Interday variability coefficient of variation (%)	
	50 ng/ml	1000 ng/ml	50 ng/ml	1000 ng/ml
Prednisone	8.6	5.7	8.1	7.8
Prednisolone	5.0	3.1	4.4	4.4
20β-Hydroxy-prednisone	6.0	5.9	8.4	4.4
20β-Hydroxy-prednisolone	9.8	6.2	8.2	6.4

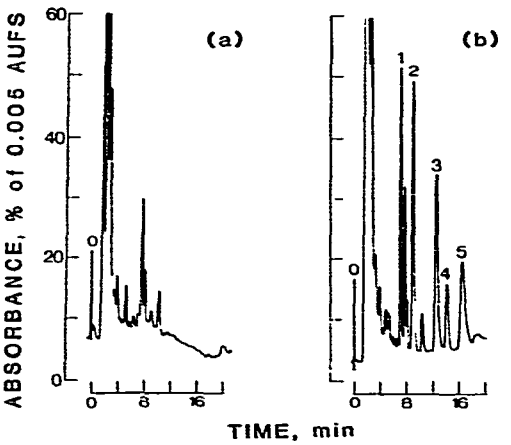


Fig. 3. Chromatograms of (a) blank plasma extract; (b) plasma extract spiked with 250 ng of (1) prednisone, (2) prednisolone, (3) 20β-hydroxyprednisone and (5) 20β-hydroxyprednisolone. 6β-Hydroxycortisol (4), 625 ng, is the internal standard. The symbol (○) designates the injection point.

cortisol which is eluted between prednisone and prednisolone employing this solvent system. Since 6β-hydroxycortisol is an endogenous by-product of cortisol metabolism, it is not suitable for use as an internal standard for samples procured from in vivo experimentation. An alternative internal standard for these biological samples is triamcinolone which possesses a retention time under these chromatographic conditions of 15.8 min.

The results of the kidney perfusion study are presented in Fig. 4. Metabolism of prednisolone to prednisone by the kidney occurred and was accompanied by further metabolism of these compounds to their 20β-hydroxylated metabolites. Peak concentrations of 20β-hydroxyprednisone and 20β-hydroxyprednisolone during the perfusion were 127 and 118 ng/ml, respectively.

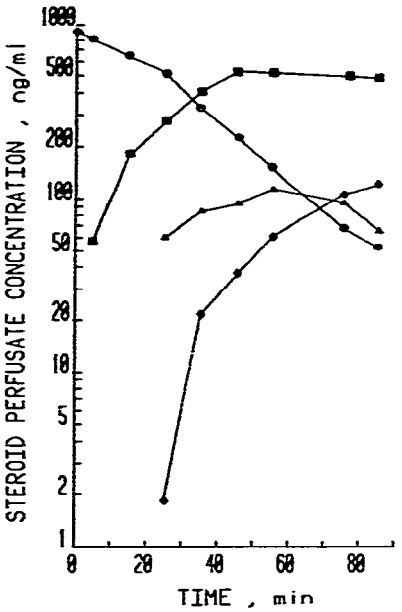


Fig. 4. Perfusate concentrations of prednisolone (●), prednisone (■), 20β-hydroxyprednisolone (▲) and 20β-hydroxyprednisone (◆) as a function of time during perfusion of a rabbit kidney with prednisolone.

DISCUSSION

This HPLC technique provides a sensitive and reproducible means of determining concentrations of prednisone and prednisolone as well as their 20β-hydroxylated derivatives in perfusate and urine. The method will also detect the glucocorticoids and their metabolites listed in Table III. Furthermore, this technique may be modified to permit plasma determinations of these steroids, allowing simultaneous examination of the disposition of prednisone and prednisolone as well as the formation and disposition of these metabolites in man.

TABLE III

RETENTION TIMES OF SELECTED CORTICOSTEROIDS AND THEIR METABOLITES

Corticosteroid	Retention time (min)
Methylprednisone	4.05
17α, 20α, 21-Trihydroxy-1,4-pregnadiene-3,11-dione	4.17
Cortisone	4.17
Dexamethasone	4.17
Cortisol	4.25
Methylprednisolone	5.92
Triamcinolone	7.75
6α-Hydroxycortisol	8.55

The analytical capability for detecting these compounds will enable precise quantitation of the metabolic disposition of prednisone and prednisolone in perfused organ systems, which is the initial step in understanding the non-linear pharmacokinetics of these compounds in vivo [4]. The results of the renal perfusion study (Fig. 4) confirm the metabolism of prednisolone to prednisone and the formation of the 20β-hydroxylated derivatives of both of these compounds by the kidney. The pharmacokinetics of these compounds will be reported more fully in subsequent reports.

There are several HPLC methods available for steroid analysis [5—8]. None of these methods quantitate the parent compounds and the steroid metabolites with the sensitivity and rapidity of the proposed procedure.

An alternative use for this technique arises from its ability to detect 6β-hydroxycortisol, an endogenous metabolite of cortisol. The excretion rate of this compound has been used to reflect enzyme induction or inhibition in man [9]. Through the use of either prednisone, prednisolone or their 20β-hydroxylated metabolites as an internal standard, 6β-hydroxycortisol determinations are possible. Thus this technique can be used to evaluate drug metabolizing enzyme activity in man.

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