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ANALYSIS OF METHYLPREDNISOLONE, METHYLPREDNISONE AND CORTICOSTERONE FOR ASSESSMENT OF METHYLPREDNISOLONE DISPOSITION IN THE RAT

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SUMMARY

A sensitive, specific and precise high-performance liquid chromatographic assay for the simultaneous determination of methylprednisolone, methylprednisone and corticosterone using betamethasone as the internal standard is reported. Rat serum (0.5 ml) is extracted with methylene chloride, washed with sodium hydroxide, then water and the extract is injected onto a microparticulate silica gel column with ultraviolet detection at 254 nm. Calculated limits of quantitation are less than 10 ng/ml and the intra-day coefficient of variation is less than 5% for each steroid. This assay has been applied to preliminary studies of methylprednisolone disposition in the rat. The plasma concentration–time profile for each steroid was determined following intravenous administration of methylprednisolone (10 mg/kg). Peak serum methylprednisone concentrations of ca. 250 ng/ml occurred within 5 min of methylprednisolone administration and the average area under the curve ratio (methylprednisolone/methylprednisone) was 9.3 These findings demonstrate that methylprednisone is a metabolite of methylprednisolone in the rat and suggest that the metabolic back-conversion of methylprednisone to methylprednisolone may be less than in other species.

INTRODUCTION

Recent studies have characterized the interaction of prednisolone or dexamethasone with hepatic cytosolic "steroid receptors" and have examined the time course of glucocorticoid action in relation to steroid and receptor concentrations in the rat [1]. Steroid disposition studies are complicated due to the reversible metabolism of compounds such as prednisolone and cortisol to their inactive 11-keto steroid forms, prednisone and cortisone [2]. Likewise, methylprednisolone is oxidized to its 11-keto metabolite, methylprednisone, in the rabbit and man [3]. To examine the fundamental clearance processes operative in such a reversible system it is necessary to measure serum concentrations of both the parent

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drug and metabolite after administration of each compound [2, 4]. A normal-phase high-performance liquid chromatographic (HPLC) method was developed for the analysis of methylprednisolone, methylprednisone and corticosterone. The assay is suitable for disposition studies of methylprednisolone–methylprednisone interconversion in the rat. Previous HPLC techniques lack the required specificity or sensitivity for such studies [5–12]. A reversed-phase HPLC procedure for determination of methylprednisone and methylprednisolone was reported, but the present method requires smaller sample volumes and also avoided the use of dry ice–acetone during the extraction procedure [3]. In addition to measuring serum concentrations of methylprednisolone and methylprednisone, the present method was also used for the analysis for corticosterone, the major endogenous glucocorticoid present in rat serum.

EXPERIMENTAL

Materials

The HPLC system consisted of a Model M45 solvent delivery system and a Model 441 UV fixed-wavelength (254 nm) absorbance detector (Waters Assoc., Milford, MA, U.S.A.). Chromatographic separation was obtained with a Zorbax SIL (Dupont, Wilmington, DE, U.S.A.) column (25 cm×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.). Injections were made with a Model 7125 universal loop injector (100-μl volume) (Rheodyne, Berkeley, CA, U.S.A.). Peak heights were recorded on a Hewlett-Packard Model 3390A integrating recorder (Avondale, PA, U.S.A.).

The methylene chloride and heptane used for sample extraction and in preparation of the mobile phase were purchased from American Burdick and Jackson (Muskegon, MI, U.S.A.). Glacial acetic acid obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and absolute ethanol (U.S.P.) purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY, U.S.A.) were also used in the mobile phase preparation.

Methylprednisone was a gift of Upjohn (Kalamazoo, MI, U.S.A.). Methylprednisolone, corticosterone, betamethasone and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). Pharmaceutical-grade decolorizing carbon (Norit-A) was purchased from Amend Drug and Chemical Co. (Irvington, NJ, U.S.A.). Anhydrous sodium sulfate, sodium hydroxide and sodium dihydrogenphosphate (monohydrate) were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Dipotassium hydrogenphosphate was purchased from J.T. Baker.

Preparation of standards

Albumin-based standards were prepared by dissolving 1.392 g dipotassium hydrogenphosphate, 0.276 g sodium dihydrogenphosphate and 8.77 g sodium chloride in a volume of water to yield 1 l. After adjusting the pH to 7.4, approximately 180 ml were transferred to a 250-ml glass beaker. Bovine serum albumin (9.0 g) was added and allowed to dissolve. The solution was then brought to a final volume of 200 ml with phosphate buffer. To the phosphate-buffered serum albumin

solution were added standards of corticosterone, methylprednisolone and methylprednisone in acetonitrile–methanol (1:1) to provide concentrations of 25–850 ng/ml.

Serum-based standards were prepared from pooled rat serum which had been "stripped" of endogenous glucocorticoids using activated charcoal. To 15 ml pooled rat serum was added Norit-A (0.04 g/ml) with constant stirring at room temperature for 2 h. The suspension was then centrifuged (13 400 *g*) for 30 min at 6–8 °C and the serum layer was decanted and then filtered through a 5-μm Millipore filter and then through a 0.45-μm Millipore filter to remove carbon particles. Serum standards were then prepared from stock solutions of each steroid as described above.

Extraction procedure

To 25 mm × 150 mm screw-capped centrifuge tubes were added 50 μl of a methanol–acetonitrile solution (1:1) containing ca. 200 ng betamethasone per 0.05 ml and the solutions were taken to dryness under a gentle stream of nitrogen. To each tube was added 0.5 ml albumin-based standard or 0.2–0.5 ml rat serum and the solutions were brought to a final volume of 1.0 ml with the addition of phosphate-buffered serum albumin. The solutions were then vortex-mixed and methylene chloride (15 ml) was added. The mixture was then shaken on a reciprocating shaker for 20 min and centrifuged. The methylene chloride phase was then washed with 1 ml of 0.1 *M* sodium hydroxide followed by 1 ml water and the aqueous phase was discarded.

Chromatography

The residue obtained by evaporation of the solvent under nitrogen was reconstituted with 200 μl mobile phase and vortex-mixed just prior to injection onto the column. The mobile phase was prepared by mixing 600 ml methylene chloride, 350 ml heptane, 10 ml glacial acetic acid and 32 ml ethanol. The solution was then passed through a GVWP Millipore filter to degas and remove particulates. The mobile phase flow-rate was maintained at 2.0 ml/min.

Steroid recovery

To screw-capped centrifuge tubes containing ca. 200 ng betamethasone internal standard was added 0.5 ml "stripped" rat serum or 0.5 ml phosphate-buffered serum albumin containing ca. 50–550 ng/ml corticosterone, methylprednisone and methylprednisolone. The solutions were brought to a final volume of 1.0 ml with phosphate-buffered serum albumin and extracted as described above except that all volumes were transferred during the extraction procedure using volumetric pipettes. A 10-ml volume of the final extract was then transferred to a 20-ml glass scintillation vial containing 50 μl of a prednisone (external standard) stock solution (9.2 μg/ml in acetonitrile–methanol). The solution was taken to dryness, 200 μl mobile phase were added and then an aliquot was injected onto the HPLC column. For comparison, solutions containing each steroid were mixed with 50 μl external standard stock solution and were taken to dryness. The residues were mixed with mobile phase and were then injected onto the HPLC col-

umn. The peak heights of the steroids in each set of samples were measured. The assay recovery of each steroid was computed using the following equation:

$$\text{recovery} = 100\% \times (\text{peak-height ratio}_{\text{extracted}}) / (\text{peak-height ratio}_{\text{direct injection}})$$

The sample to external standard peak-height ratio for extracted drug was corrected by a factor of 1.5 to account for incomplete volume transfer during the extraction procedure.

Precision studies

The within-day ($n=10$) and between-day ($n=7$) reproducibility of the assay was determined in bovine serum albumin-based standards containing ca. 60 ng/ml or ca. 600 ng/ml of each steroid.

Animal studies

The disposition of methylprednisolone was studied in two male rats following a single 10 mg/kg intravenous dose of methylprednisolone. The dosage form was prepared by placing 25 mg methylprednisolone into a 5-ml volumetric flask and then adding 3 ml polyethylene glycol 400 (Sigma), 0.6 ml ethanol and 1 ml distilled water with gentle heating (40°C) until solution occurred. The solution was then brought to a final volume of 5 ml with distilled water. A rapid intravenous injection was administered using a jugular vein cannula which was then rinsed with 0.4 ml saline. Blood samples (0.5–1.0 ml) were obtained prior to and at least 3 h after methylprednisolone administration through the same cannula. The volume of blood withdrawn at each sampling time was measured and the blood volume was replaced with donor rat blood preserved with citrate–dextrose anticoagulant solution [13].

RESULTS

Chromatograms obtained from the analysis of an albumin-based standard and a standard prepared from "stripped" rat serum (0.5 ml) containing 57 ng/ml corticosterone, 49 ng/ml methylprednisone and 52 ng/ml methylprednisolone are presented in Fig. 1A and B. The extraction of each steroid from rat serum was similar to that from bovine serum albumin solution. For comparison, a chromatogram obtained from the analysis of "stripped" rat serum is provided (Fig. 1C).

The extraction recovery of each steroid from bovine serum albumin or pooled rat serum did not vary with steroid concentration (Table I). To further examine any possible differences in extraction, analytical standards for each steroid were prepared in bovine serum albumin and pooled rat serum. The albumin-based standards were then used to construct a standard curve for the quantitation of each steroid in rat serum. Measured concentrations were within 10% of the corresponding nominal value at serum concentrations of each steroid ranging from 50 to 870 ng/ml.

The relationship between assay response and steroid concentration was linear over the 25–800 ng/ml concentration range. The assay response (peak-height ratio) variance increased with steroid concentration and therefore the analysis

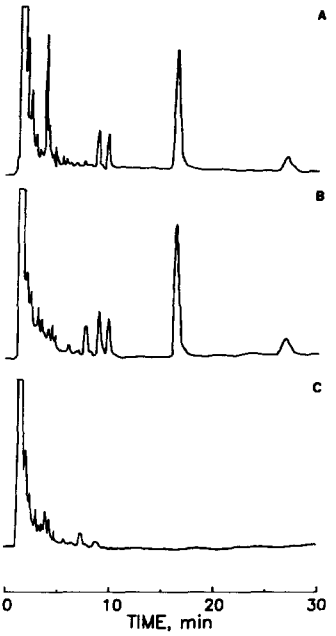

Fig. 1. Chromatograms obtained from the analysis of 0.5 ml bovine serum albumin (A) and charcoal-treated rat serum (B) containing 56 ng/ml corticosterone, 49 ng/ml methylprednisone and 56 ng/ml methylprednisolone. A chromatogram obtained from the analysis of 0.5 ml blank charcoal-treated rat serum is shown for comparison (C). The retention times for corticosterone, methylprednisone, betamethasone and methylprednisolone were ca. 8.5, 9.5, 16.0 and 26.2 min.

TABLE I

STEROID EXTRACTION RECOVERIES FROM BOVINE ALBUMIN AND RAT SERUM

Steroid	Nominal concentration (ng/ml)	Recovery (mean ± S.D., $n=3$) (%)	
		4.5% Bovine albumin	Rat serum
Corticosterone	56.6	94.9 ± 5.1	95.9 ± 7.4
	565.5	94.1 ± 3.3	93.5 ± 3.4
Methylprednisone	49.4	90.2 ± 5.3	86.6 ± 4.4
	493.5	85.1 ± 4.1	85.9 ± 2.5
Methylprednisolone	51.5	74.6 ± 3.7	73.6 ± 2.8
	515.0	74.8 ± 4.6	76.5 ± 4.5
Betamethasone ($n=6$)	403.2	69.4 ± 3.8	71.2 ± 3.7

of each standard curve was performed using a weighted linear regression technique with the data weighted according to the square of the observed assay response [14].

Assay sensitivity criteria were calculated by the procedure of Oppenheimer et al. [14]. The determination limit (concentration which would be expected to be

detected with a coefficient of variation of 10%) was less than 10 ng/ml for each steroid.

The between-day and within-day coefficient of variation (C.V.) of the assay ranged from 1.4 to 9.6% at steroid concentrations of ca. 60 and ca. 600 ng/ml (Table II).

The serum concentrations of each steroid measured after intravenous administration of 10 mg/kg methylprednisolone are shown in Fig. 2. Peak serum concentrations of methylprednisone were nearly one order of magnitude lower than the corresponding methylprednisolone concentration. The conversion of methylprednisolone to methylprednisone was rapid in the rat. The decline in serum corticosterone concentration was discontinuous, and the serum corticosterone concentration decreased to less than 10% of the initial value within 4 h of methylprednisolone administration. The pertinent pharmacokinetic parameters for

TABLE II

INTRA-DAY AND INTER-DAY ASSAY PRECISION

Steroid	Nominal concentration (ng/ml)	Intra-day (mean, $n=10$)		Inter-day (mean, $n=7$)	
		Measured concentration (ng/ml)	C.V. (%)	Measured concentration (ng/ml)	C.V. (%)
Corticosterone	67.9	71.3	4.2	71.1	5.1
	678.6	705.8	2.9	685.6	2.8
Methylprednisone	59.2	62.0	3.5	59.7	9.6
	592.2	607.5	2.8	594.9	3.2
Methylprednisolone	61.8	63.7	3.0	64.2	3.5
	618.0	636.8	1.4	620.1	1.9

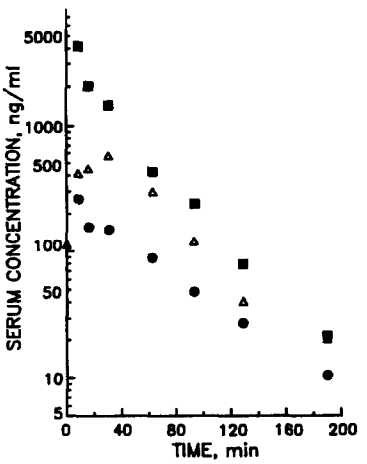

Fig. 2. Serum concentrations of methylprednisolone (■), methylprednisone (●) and corticosterone (△) measured in the rat following intravenous administration of 10 mg/kg methylprednisolone.

TABLE III

PHARMACOKINETIC PARAMETERS FOR METHYLPREDNISOLONE/METHYLPREDNISONE DISPOSITION IN THE RAT

Total clearance, steady-state distribution volume (V_{ss}), central distribution volume (V_c), mean residence time (MRT), half-life and area under the serum concentration–time curve (AUC) for methylprednisolone or methylprednisone were calculated as described previously [17].

	Methylprednisolone				
	Total clearance (ml/min/kg)	V_{ss} (l/kg)	V_c (l/kg)	MRT (min)	Half-life (min)
Rat 1	82.5	2.52	1.83	31	29
Rat 2	68.1	2.72	1.18	40	39

	Methylprednisone (MP)				
	Dose/AUC (l/h/kg)	MRT (min)	Half-life (min)	AUC ratio MPn/MP	Half-life ratio MP/MPn
Rat 1	47.4	54	42	9.57	1.45
Rat 2	37.5	85	52	9.17	1.33

methylprednisone and methylprednisolone are listed in Table III. These data demonstrate that methylprednisolone is metabolized to methylprednisone in the rat, but the calculated pharmacokinetic parameters may be confounded by back-conversion of methylprednisone to the parent compound as demonstrated in other animal species [4]. Additional studies of methylprednisone disposition are planned to confirm this hypothesis.

DISCUSSION

The proposed normal-phase HPLC assay is suitable for pharmacokinetic studies of methylprednisolone interconversion in the rat. Recent animal studies demonstrate that methylprednisolone is metabolized to the 11-keto steroid methylprednisone and then back-converted to the parent compound [3]. This confounds any traditional pharmacokinetic analysis based on serum concentrations of the parent compound [2, 4]. A more meaningful characterization of the interconversion system entails analysis of both the parent compound (methylprednisolone) and the reversible metabolite (methylprednisone).

Several HPLC assays for the analysis of methylprednisolone and related esters have been reported [3–12]. A reversed-phase HPLC assay was reported for the analysis of methylprednisolone and methylprednisone, but the proposed normal-phase assay offers the advantages of increased sensitivity and less tedious extraction procedure [3]. Moreover, the present assay was validated for the simultaneous analysis of corticosterone, the major endogenous glucocorticoid in rat serum.

The mobile phase composition for the present method was modified from a normal-phase assay for methylprednisolone developed in this laboratory [7]. Substitution of heptane for hexane and a slight decrease in ethanol content re-

sulted in adequate resolution of corticosterone and methylprednisone with no interference from endogenous compounds.

Other investigators have used a logarithmic parabolic equation to describe the relationship between assay response and steroid concentration to improve assay precision at low steroid concentrations [6]. We used a weighted linear regression technique which resulted in improved assay precision at low steroid concentrations and also permitted calculation of assay sensitivity criteria [14].

The extraction recovery of each steroid from bovine serum albumin solution was identical to that from rat serum. Standard curve specimens are prepared in 4.5% bovine serum albumin to avoid the use of charcoal-stripped plasma and the filtration of charcoal-treated plasma in the preparation of analytical standards.

Serum corticosterone concentrations increased slightly in both rats shortly following methylprednisolone administration (Fig. 2). These observations are consistent with the episodic nature of corticosterone secretion. Prominent periodicities in the time series of corticosterone secretion have been observed in the rat [15].

The pharmacokinetic parameters for methylprednisolone and methylprednisone disposition are similar to those reported for a structurally similar steroid, prednisolone, in the rat [16]. Although serum methylprednisone concentrations were ten-fold lower than corresponding methylprednisolone concentrations, the specificity and sensitivity of the proposed HPLC method were adequate to determine the serum concentration–time profile of each steroid.

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