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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR METHYLPREDNISOLONE AND ITS SOLUBLE PRODRUG ESTERS IN DOG PLASMA

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SUMMARY

A high-performance liquid chromatographic method with ultraviolet detection (λ_{max} = 243 nm) has been developed for the simultaneous determination of methylprednisolone (MP) and its water-soluble prodrug esters methylprednisolone hemisuccinate (MPS) and N,N,N′-triethylethylenediamine amide of 6α-methylprednisolone-21-hemisuberate hydrochloride (TMPS) in dog plasma. A reversed-phase liquid chromatographic separation was performed on a Microsorb C_8 (3 μm) column equipped with a C_8 5-μm guard column. The mobile phase composition was water—acetonitrile—methanol—dimethyloctylamine—acetic acid (65.5:34:0.4:0.04:0.04). The methyl ester of phenethylcarbamate was employed as an internal standard. The chromatographic responses were linear up to 25 μg/ml for MP, 70 μg/ml for MPS, and 95 μg/ml for TMPS. The sensitivity of the assay by ultraviolet detection is approximately 4, 8, and 12 ng/ml of plasma for MP, MPS and TMPS, respectively. The assay variability in terms of 95% confidence limit for each steroid is < 4.5%. Plasma concentration—time curves are reported for MP, MPS, and TMPS after intravenous administration of MPS and TMPS equivalent to 3, 10 and 30 mg MP per kg body weight of dog. The assay methodology is simple, selective and reproducible for the quantitative determination of MP, MPS and TMPS in dog plasma.

INTRODUCTION

The hydrochloride salt of N,N,N′-triethylethylenediamine amide of methylprednisolone-21-hemisuberate (TMPS) and the sodium salt of methylprednisolone hemisuccinate (MPS) are water-soluble prodrugs of methylprednisolone (MP). After intravenous administration of these prodrugs, they hydrolyze and revert to the parent drug methylprednisolone (Fig. 1).

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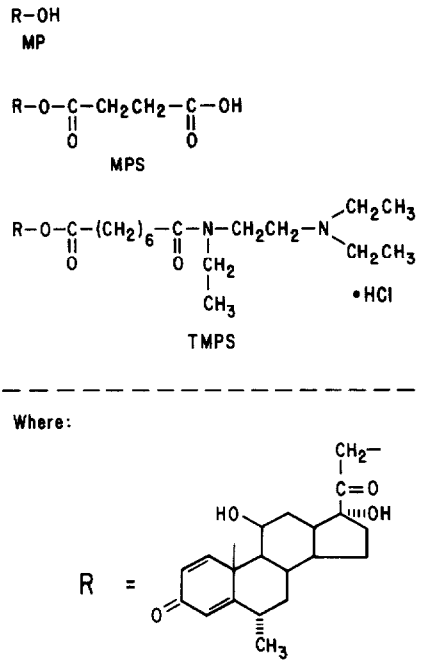

Fig. 1. Structures of MP, MPS and TMPS.

Methylprednisolone is an important steroid for the treatment of various diseases. This steroid has particular utility in therapy of acute bronchospasm (status asthmaticus) when it is administered as its water-soluble sodium succinate ester [1—4]. Due to instability of MPS in solution, TMPS was synthesized as a new water-soluble prodrug for the possible replacement of MPS.

In order to perform a bioequivalency comparison between MPS and TMPS, it was essential to develop a sensitive analytical method for the measurements of MP, MPS and TMPS in biological fluids. Although several analytical techniques have been reported for the measurement of MP and its soluble prodrug MPS, many of them lack the desired extraction efficiency and assay sensitivity and do not have the ability to simultaneously analyze MP, MPS and TMPS from biological fluids [5—12]. Ebling et al. [13] described a sensitive assay for cortisol, MP and MPS. In this procedure, the concentration of MPS was measured indirectly as the difference between MP concentration from hydrolyzed and unhydrolyzed samples. The procedure is more elaborate because it involves many steps as well as double sample preparation and analysis.

This paper reports a simple, rapid method using reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet detection for the simultaneous determination of MP, MPS and TMPS in dog plasma.

EXPERIMENTAL

Reagent and materials

Methylprednisolone (MP) and methylprednisolone hemisuccinate (MPS)

were UpJohn (Kalamazoo, MI, U.S.A.) control reference standards with purity value of 99.4 and 99.3%, respectively. The hydrochloride salt of N,N,N′-triethylethylenediamine amide of methylprednisolone-21-hemisuberate (TMPS) and the methyl ester of phenethylcarbamate (internal standard) were prepared by Upjohn. Dimethyloctylamine was obtained from Ames Labs. (Milford, CT, U.S.A.). The analytical-grade glacial acetic acid was purchased from Mallinckrodt (Paris, KY, U.S.A.). The acetonitrile and methanol were UV grade, purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water used was sterile water for irrigation, (U.S.P.).

Chromatographic equipment and conditions

An LDC Constametric III pump and an LDC spectromonitor III variable-wavelength UV detector were used in conjunction with a Rheodyne Model 7125 low-dead-volume injector. The column was a Microsorb, 3-μm, 100 × 4.6 mm I.D. C_8 column. A 30-mm Brownlee Spherisorb guard column packed with 5-μm RP-8 was attached to the analytical column. Data were recorded on a Linear dual-pen strip chart recorder.

Mobile phase

The mobile phase composition for the determination of MP, MPS and TMPS was water–acetonitrile–methanol–dimethyloctylamine–acetic acid (65.5:34:0.4:0.04:0.04). The solution was prepared by mixing exact volumes of the above components, followed by degassing the liquid under vacuum.

Chromatographic operation conditions

The ultraviolet detector was fixed at 243 nm. The sensitivity of the detector was 0.02 a.u.f.s. (absorbance units full scale) for MP, MPS and TMPS. The flow-rate was held constant at 1.0 ml/min. The column pressure was 147 bar (2200 p.s.i.).

Dog study protocol

A single beagle dog was fasted overnight before and at least 4 h after dosing. The dog was weighed just before dosing and the concentration of the injection solution adjusted to give doses of TMPS or MPS in MP equivalents of 3 mg/kg, 10 mg/kg and 30 mg/kg. The injection volume was a constant 10 ml using sterile saline as the vehicle given over a 1-min period. Blood samples were drawn into pre-heparinized glass syringes and transferred to an ice-chilled 5-ml centrifuge tube. The samples were immediately centrifuged at 1000 *g* at 4°C for 5 min. A 200-μl aliquot of plasma was added to an appropriately labelled 15-ml centrifuge tube containing 20 μl of 25% acetic acid solution and 2 ml acetonitrile. The contents were mixed and immediately frozen at −70°C for future assay.

Assay procedure

Preparation of stock solutions. A stock solution containing ca. 4 μg/ml MP, 12 μg/ml MPS and 20 μg/ml TMPS was prepared in acetonitrile. An internal standard solution of ca. 84 μg/ml was prepared in the mobile phase.

Preparation of plasma standards. Aliquots of stock solution for an eight-

point standard curve were pipetted into 15-ml centrifuge tubes and the solvent was evaporated to dryness under nitrogen. Pre-dose dog plasma (200 μl), 25% aqueous acetic acid (20 μl), and acetonitrile (2 ml) were added to each of the above tubes and mixed well by vortexing for about 5 sec. The plasma protein was fully floculated by keeping at room temperature for approximately 10 min and then centrifuged at 2500 *g* for 10 min. After decanting all of the clear liquid into fresh centrifuge tubes, the solvent was evaporated to dryness under nitrogen. The residue was reconstituted with 100 μl of internal standard solution, centrifuged at 2600 *g* for 5 min, and then 20-μl aliquots of the clear solution were injected into the chromatograph.

Preparation of plasma samples. Dog plasma samples (200 μl) were pipetted into labelled 15-ml centrifuge tubes which contained 20 μl of 25% aqueous acetic acid and 2.0 ml of acetonitrile. After vortex mixing of the samples, they were allowed to stand at room temperature for 10 min to precipitate plasma proteins. Subsequent steps involving centrifugation, evaporation of supernatant and reconstitution with the mobile phase were carried out as described for the standard plasma samples.

Calculations

Calibration curves of MP, MPS and TMPS were constructed by plotting their peak-height ratios versus concentration of MP, MPS and TMPS. Concentrations of MP, MPS and TMPS in plasma samples were calculated from the peak-height ratios using the appropriate slope and intercept obtained by linear regression of the calibration data.

RESULTS AND DISCUSSION

Fig. 2A shows a typical HPLC profile of blank dog plasma spiked with internal standard and Fig. 2B shows a chromatogram of plasma containing 0.686 μg/ml MP, 1.80 μg/ml MPS, 2.41 μg/ml TMPS and internal standard. The assay provides complete resolution of MP, MPS, TMPS and internal standard from endogenous hydrocortisone and other components. Each steroid of interest is completely resolved. The retention times of MP, MPS, TMPS and internal standard are 4.8, 9.0, 18.0 and 6.8 min, respectively. If desired, hydrocortisone is measurable at a retention time of 3.2 min as confirmed by a chromatogram using a hydrocortisone reference standard.

Table I shows the extraction efficiencies at six different concentrations of MP, MPS and TMPS from dog plasma. The average extraction efficiency was 99 ± 3% for MP, 96 ± 4% for MPS and 103 ± 6% for TMPS. The values were calculated based on known concentrations of unextracted MP, MPS and TMPS samples prepared in mobile phase compared to the same concentrations extracted from plasma samples. Published assay procedures [5—11, 13] reported incomplete extraction of some steroids compared with the virtually 100% extraction of all of the steroids tested using this methodology.

Linearity of response and sensitivity

Linear regression analysis of the concentration data indicated no significant deviations from linearity for MP up to 25 μg/ml, for MPS up to 70 μg/ml, and

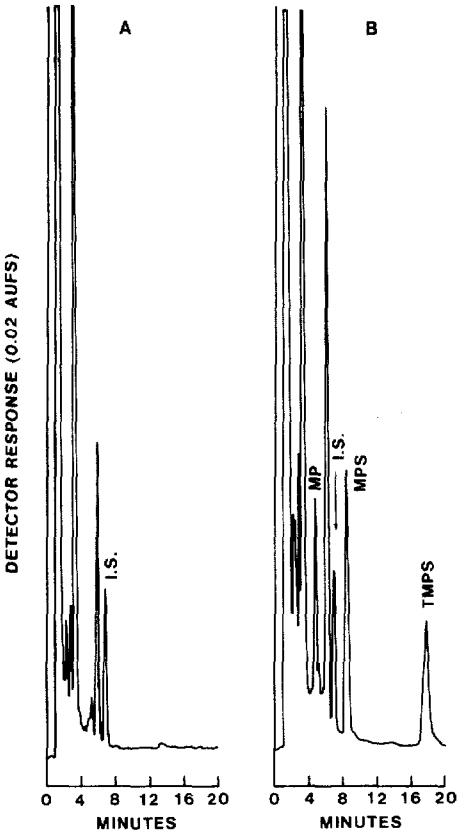

Fig. 2. Chromatograms of dog plasma extract of (A) blank plasma containing internal standard (I.S.), and (B) blank plasma spiked with MP, MPS, and TMPS.

for TMPS up to 95 μg/ml in dog plasma. The slope, intercept and correlation coefficient values were determined by the regression analysis of the peak-height ratio (peak height of MP, MPS or TMPS over peak height of internal standard) as a function of MP, MPS or TMPS concentrations. Correlation coefficients for standard curves prepared for MP, MPS and TMPS were better than 0.999 over an eight-week period at six different times. The assay sensitivity of this method was determined to be 4, 8 and 12 ng/ml for MP, MPS and TMPS, respectively, when using sample volumes of 0.2 ml of dog plasma. The above sensitivities were calculated based on minimum measurable peak heights and a 30-μl sample injection out of a final volume of 50 μl.

Assay precision and accuracy

The assay precision and accuracy were established by assaying samples containing known concentrations of MP, MPS and TMPS in 0.2 ml dog plasma. Samples were prepared on six different days at concentrations within the standard curve range and were treated as unknowns for the HPLC analysis. Table II shows the accuracy and precision data for the recovery of MP, MPS and TMPS from dog plasma. The average inter-day recovery for MP, MPS and TMPS was 99.6 ± 1.9%, 101.1 ± 3.7% and 101.7 ± 4.5%, respectively. Assay

TABLE I

EXTRACTION EFFICIENCY OF MP, MPS AND TMPS FROM DOG PLASMA COMPARED TO UNEXTRACTED STANDARD IN MOBILE PHASE

Compound	Concentration added (μg/ml)	Concentration found (μg/ml)	Percentage recovery
MP	0.14	0.14	100.0
	1.37	1.35	98.5
	2.74	2.60	94.9
	5.49	5.33	97.1
	10.98	11.03	100.0
	27.44	27.84	101.5
Mean ± 95% confidence limit			98.7 ± 3.5
MPS	0.36	0.32	88.9
	3.62	3.76	103.9
	7.23	7.13	98.6
	14.46	13.65	94.4
	28.93	29.13	100.7
	72.72	68.89	94.7
Mean ± 95% confidence limit			96.9 ± 4.5
TMPS	0.49	0.55	112.2
	4.86	5.06	104.1
	9.72	10.14	104.3
	19.44	18.38	94.5
	38.88	40.97	105.4
	97.20	97.38	100.2
Mean ± 95% confidence limit			103.4 ± 6.2

TABLE II

ACCURACY AND PRECISION FOR THE RECOVERY OF MP, MPS AND TMPS FROM DOG PLASMA (n = 6)

Compound	Concentration added (μg/ml)	Concentration found (mean ± 95% C.L.*) (μg/ml)	Percentage recovery (mean ± 95% C.L.)
MP	1.37	1.36 ± 0.03	99.6 ± 1.9
	5.49	5.46 ± 0.11	99.5 ± 2.1
MPS	3.62	3.66 ± 0.13	101.1 ± 3.7
	14.46	14.84 ± 0.56	102.6 ± 3.9
TMPS	4.86	4.94 ± 0.22	101.7 ± 4.5
	19.44	19.31 ± 0.83	99.3 ± 4.2

*C.L. = Confidence limit.

variability is given in terms of the 95% confidence interval and in terms of the coefficient of variation (Table III).

Applicability of the methodology

The utility of the analytical method for pharmacokinetic studies was demon-

TABLE III

COMPARISON OF THE ASSAY VARIANCE AND COEFFICIENT OF VARIATION FOR MP, MPS AND TMPS IN DOG PLASMA

Compound	Mean concentration found (μg/ml)	Assay variance, σ^2	Coefficient of variation (%)
MP	0.069	$1.333 \cdot 10^{-6}$	1.6
	1.365	$6.123 \cdot 10^{-4}$	1.8
	5.462	$1.214 \cdot 10^{-2}$	2.0
MPS	0.185	$1.20 \cdot 10^{-5}$	1.9
	3.658	$1.562 \cdot 10^{-2}$	3.4
	14.84	$2.922 \cdot 10^{-1}$	3.6
TMPS	0.245	$1.21 \cdot 10^{-4}$	4.5
	4.942	$4.342 \cdot 10^{-2}$	4.2
	19.31	$6.202 \cdot 10^{-1}$	4.1

strated by monitoring plasma levels of MP, MPS and TMPS in the dog. Plasma samples were analyzed after intravenous administration of MPS and TMPS at doses equivalent to 3, 10 and 30 mg MP per kg body weight. Fig. 3 shows a typical plasma concentration—time curve for MP levels after intravenous administration of MPS and TMPS at doses equivalent to 30 mg MP per kg body weight in the dog. Fig. 4 shows typical MPS or TMPS levels after intravenous administration of MPS and TMPS at doses equivalent to 10 mg/kg body weight in the dog.

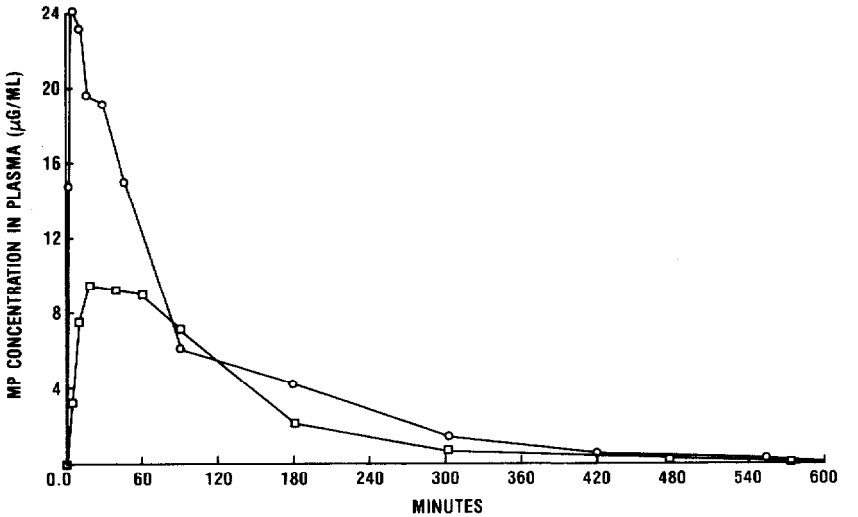

Fig. 3. MP levels in dog after intravenous administration of MPS (□) or TMPS (○) at doses equivalent to 30 mg MP per kg body weight.

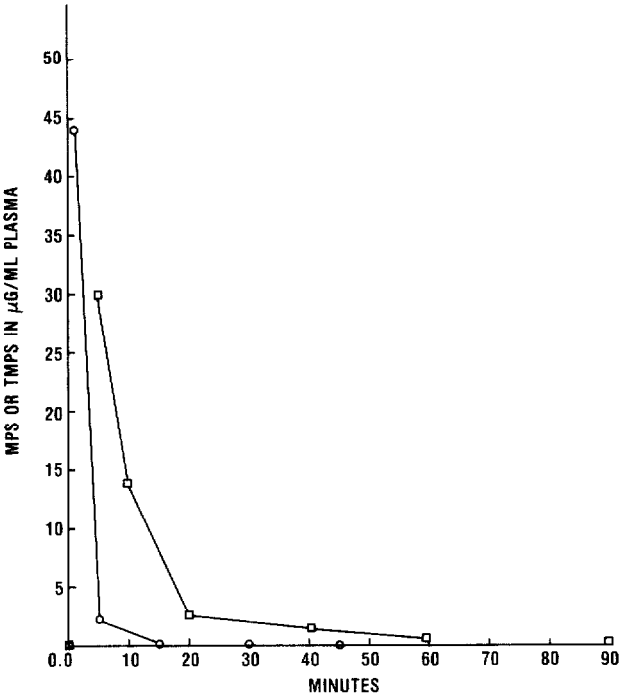

Fig. 4. MPS (□) and TMPS (○) levels in dog after intravenous administration of MPS or TMPS at doses equivalent to 10 mg MP per kg body weight.

Analytical error behavior

The data in Table II can be used to estimate the effect of sample concentration on the precision of the estimation. This information is important for at least two reasons. The confidence that might be required in the assay result may require replication of the assay. A knowledge of the error behavior allows the number of replicates needed to be calculated. If the assay procedre is to be used for pharmacokinetic studies, then a knowledge of the error behavior is important in determining the weighting factors to be used for any curve fitting.

The criteria for determining error behavior have been given in the literature. The two cases treated are constant error variance as a function of concentration and constant coefficient of variation as a function of concentration. Constant variance means that all measurements are equally precise and no weighting factors are needed. Constant coefficient of variation means that measurement of different concentrations have different variances and therefore need different weighting factors. The number of replicates needed at a particular concentration level to achieve needed pharmacokinetic precision can be calculated from a knowledge of the assay variance at that particular concentration. A comparison of the variance and coefficient of variation for this assay is shown in Table III. It is obvious that the variance in the assay is not constant and is a non-linear function of sample concentration. Therefore a weighting factor, such as the reciprocal of the variance, shoudl be used for pharmacokinetic curve fitting.

CONCLUSION

Advantages of this assay methodology over others are complete recovery of all the assay components and simultaneous detection of both MPS and TMPS from the same prepared sample. The assay methodology is simple, sensitive, selective and reproducible for the quantitative determination of MP, MPS and TMPS.

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