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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE 21-[8-[METHYL-(2-SULFOETHYL)AMINO]-8-OXOCTANOATE] SODIUM SALT IN HUMAN PLASMA

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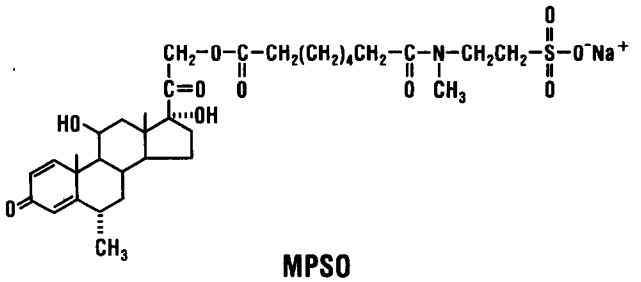
SUMMARY

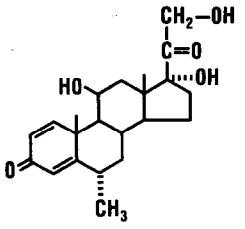
A high-performance liquid chromatographic assay method with ultraviolet detection at 243 nm has been developed for the quantitative determination of methylprednisolone (MP) and methylprednisolone 21-[8-[methyl-(2-sulfoethyl)amino]-8-oxoctanoate] sodium salt (MPSO) in human plasma. The method is simple, rapid and sensitive to detect MP and MPSO in human plasma following administration of therapeutic doses of MPSO. The assay procedure involved stabilization of plasma samples by addition of disodium ethylenediaminetetraacetic acid and ion-pair extractions of MPSO with tetraethylammonium chloride. After extracting both drugs and internal standard into chloroform, the extract was evaporated to dryness under nitrogen. The resulting residue was reconstituted in 200–500 μl of mobile phase and chromatographed on a C_{18} IBM reversed-phase column (5 μm). The mobile phase was a mixture of water–actetonitrile–isopropanol (71:19.9:10) containing 50 μl of 0.1 *M* hydrochloric acid and 0.497 g tetraethylammonium chloride. Propyl *p*-hydroxybenzoate was used as an internal standard. The chromatographic responses were linear up to about 200 μg/ml for MP and 80 μg/ml for MPSO in human plasma. The assay detection limit was approximately 7 ng/ml for MP and 25 ng/ml for MPSO in human plasma. Statistical analysis indicated an average recovery of 102.0 ± 4.71% for MP and 75.2 ± 2.88% for MPSO. Human plasma levels are reported for MP and MPSO following single-dose intravenous administration of 100-mg equivalents of MPSO.

INTRODUCTION

Methylprednisolone 21-[8-[methyl-(2-sulfoethyl)amino]-8-oxoctanoate] sodium salt (MPSO) is a new water-soluble prodrug of methylprednisolone (MP) that is being evaluated as a second-generation replacement for methylprednisolone sodium succinate (MPS). After intravenous administration of prodrug MPSO, it hydrolyzes and converts to the parent drug MP (Fig. 1).



MPSO



MP

Fig. 1. Structures of MP and MPSO.

MP is an important steroid for the treatment of various diseases [1–4]. This steroid has particular utility in the therapy of acute bronchospasm (status asthmaticus) when it is administered as the water-soluble prodrug of MP. Due to instability of MPS in solution, MPSO was synthesized as a new water-soluble prodrug for the possible replacement of MPS. MPSO has an estimated room temperature stability of at least two years [5] which should allow marketing of a ready-to-inject solution, as well as multidose vials.

In order to perform a bioequivalency comparison between MPS and MPSO, it was essential to develop a sensitive analytical method for the measurement of MP and MPSO in biological fluids. Although several analytical techniques have been reported for the measurement of MP and its soluble prodrug MPS, they apparently lack the desired assay sensitivity and do not have the ability to simultaneously analyze MP and its soluble prodrugs from biological fluids [6–12]. Ebling et al. [13] described a sensitive assay for cortisol, MP and MPS in which the concentration of MPS was measured indirectly as the difference between the MP concentrations in hydrolyzed and unhydrolyzed samples. Since this procedure involves double sample preparation and analysis time and several extra assay steps it is a rather time-consuming and laborious assay procedure. A simple and sensitive assay method was developed by us for the simultaneous determination of MP and its soluble prodrug esters in dog plasma [14]; but this method was found unsuitable for the quantitation of MPSO due to the long retention time of this compound.

This paper describes a simple, sensitive and rapid ion-pair extraction method for the simultaneous determination of MP and MPSO in human plasma utilizing

a high-performance liquid chromatographic (HPLC) method with ultraviolet detection at 243 nm.

EXPERIMENTAL

Reagents and materials

MP and MPSO were Upjohn (Kalamazoo, MI, U.S.A.) control reference standards with purity of 99.4 and 96.6%, respectively. Propyl *p*-hydroxybenzoate, 99% pure (internal standard) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and analytical-grade hydrochloric acid was purchased from Mallinckrodt (Paris, KY, U.S.A.). Tetraethylammonium chloride (TEACl) was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Chloroform, acetonitrile and isopropanol were UV grade, purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water used in this assay was Sterile Waters for Irrigation, USP (American McGaw, Irvine, CA, U.S.A.).

Chromatographic equipment and conditions

An LDC Constametric III pump and an LDC Spectromonitor III variable-wavelength ultraviolet detector or Kratos Spectroflow 783 programmable absorbance detector were used in conjunction with a Rheodyne Model 7125 low-dead-volume injector or Waters WISP 710B autosampler. The column was a C_{18}, 5 μm, 250×4.5 mm I.D. IBM column fitted with an RP-8, Spheri-5, 3-cm guard column from Brownlee Labs. Data were recorded on a Linear dual-pen strip chart recorder.

The mobile phase composition for the determination of MP and MPSO was water–acetonitrile–isopropanol (71:19.9:10) containing 50 μl of 0.1 *M* hydrochloric acid and 0.497 g TEACl. The solution was prepared by adding exact volumes of the above components, followed by degassing under vacuum.

The ultraviolet detector was set at 243 nm and the sensitivity of the detector was set at 0.01 a.u.f.s. (absorbance units full scale) for MP and MPSO. The flow-rate was held constant at 1.5 ml/min. The column pressure was 160 bar.

Human study protocol and sample handling

A single-dose intravenous tolerance study of MPSO (MP equivalent doses: 10, 40, 100, 250, 500, 1000, 1500, 2000 and 3000 mg) in humans was performed in normal male volunteers, aged between 18 and 55 years. Volunteers were smokers and non-smokers with body weight within 15% of ideal body weight. The subjects were not permitted to use any other drug for fourteen days prior to entering the study. The evening prior to administration of the drug, all subjects received a light snack. On the medication day, they were fasted until noon and then received a low fat meal. The drugs were infused at the rate of 100 mg MP equivalent per min, except for the 10-, 40-, and 100-mg doses, which were given over 1 min.

A 10-ml sample of blood was drawn into a chilled 10-ml ethylenediaminetetraacetic acid (EDTA) (equivalent to 1.5 mg EDTA per ml blood) vacutainer at the following recorded time intervals: predose, 1, 2, 5, 10, 20, 45, 90, 180, 360, 480 and 720 min. Plasma was separated from the blood as quickly as possible and was

frozen in dry ice. The frozen samples were stored at −70°C as soon as possible for future analysis.

Preparation of stock solutions

A stock solution containing 20 μg/ml MP and 40 μg/ml MPSO was prepared in acetonitrile. An internal standard solution of 7 μg/ml was prepared in acetonitrile. Stability data for both of these stock solutions showed that they are stable in acetonitrile for at least three months at 4°C. A 1.0 *M* TEACl solution and 0.1 *M* hydrochloric acid solution were prepared in sterile water.

Preparation of plasma standards

A 100-μl aliquot of 15 mg/ml Na_2EDTA was pipetted into a series of 15-ml centrifuge tubes and evaporated to dryness under nitrogen. Aliquots of stock solution and 100 μl of stock internal standard were added to each tube and the contents were evaporated to dryness under nitrogen. Then 1-ml aliquots of blank human plasma plus 300 μl of 1.0 *M* TEACl solution were added to each centrifuge tube and mixed well by vortexing for 5 s. Immediately, 5 ml of chloroform were added and mixed for about 2 s. Each sample was processed to the first chloroform extraction stage before the next plasma sample was mixed with TEACl. Samples were immediately extracted by placing them in a horizontal shaker for approximately 15 min, then they were centrifuged at 2500 *g* for 5 min. The chloroform layer was transferred to a clean labelled centrifuge tube and the aqueous layer was immediately re-extracted with 5 ml chloroform as before. The combined chloroform extracts were evaporated to dryness with nitrogen at room temperature. The dried extracts were reconstituted with 200–500 μl of mobile phase by vortexing for at least 8–10 s and then 50–100 μl were injected into the HPLC system for analysis.

Preparation of unknown plasma samples

Unknown plasma samples were prepared by pipetting 100 μl of stock internal standard solution into a series of centrifuge tubes and evaporating to dryness under nitrogen. Then 1-ml-aliquots of plasma and 300 μl of 1.0 *M* TEACl solution were added to each centrifuge tube and vortexed for 5 s. Samples were immediately extracted with 5 ml of chloroform and subsequent centrifugation, evaporation and reconstitution steps were carried out as described for the plasma standards. The above procedure is suitable following doses of MPSO below 0.5-g equivalents of MP. Samples obtained following doses of MPSO equal to 0.5–1 g equivalents of MP were assayed by diluting 0.2 ml quenched plasma sample with 0.8 ml blank human plasma (harvested from EDTA-treated blood). Similarly, 0.05-ml quenched plasma samples following MPSO dose of 1.5, 2 and 3 g equivalents of MP were diluted with 0.95 ml of blank human plasma. The diluted samples were processed the same as the undiluted samples as described above.

Calculations

The calculation of MP and MPSO in plasma samples was accomplished by using the appropriate slopes obtained by linear regression analysis of peak-height

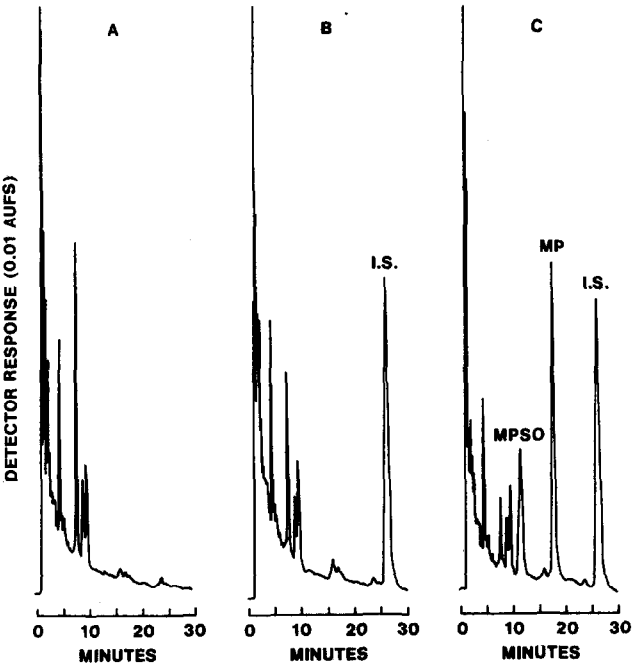


Fig. 2. Chromatograms of human plasma: (A) blank plasma; (B) plasma containing internal standard (I.S.); (C) plasma containing I.S., MP and MPSO.

ratios versus concentration data. In all cases, there were no statistically significant intercepts. To calculate concentrations of MP and MPSO in diluted plasma samples for MPSO doses of 500 mg and above, the calculated concentrations were multiplied by the appropriate dilution factor.

RESULTS AND DISCUSSION

In the development of this assay methodology several problems had to be resolved. First, it was necessary to find conditions that can provide efficient extraction of MP, MPSO and internal standard from human plasma with minimum interference from plasma endogenous components. Second, proper chromatographic conditions had to be established to obtain baseline separation for all three components from endogenous hydrocortisone. Third, due to instability of MPSO in biological fluids, appropriate test conditions had to be found under which MPSO in plasma samples would remain relatively stable during storage and analysis.

Several reversed-phase chromatographic columns were tested for the selection of the one which would yield optimum resolution of drug-related material from coextracted endogenous components. The columns tested were Rainin C_8, 10 cm, 3 μm; C_{18}, 10 cm, 3 μm; Supelcosil LC-8, 25 cm, 5 μm; IBM C_8, 25 cm, 5 μm; C_{18}, 25 cm, 5 μm. A number of mobile phase compositions consisting of water–acetonitrile–tetrabutylammonium chloride or water–acetonitrile–

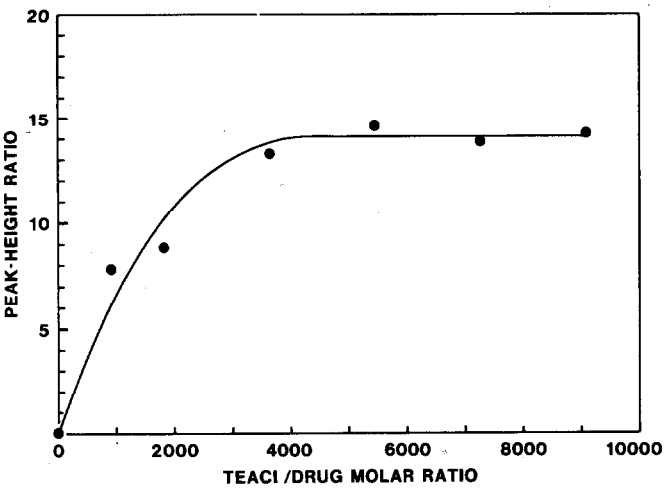


Fig. 3. Effect of TEACl on MPSO extraction.

isopropanol containing 0.1 *M* hydrochloric acid and TEACl were tested. Among all these, a 25-cm, C_{18}, 5-μm IBM column and a mobile phase composed of water–acetonitrile–isopropanol (71:19.9:10) containing 50 μl of 0.1 *M* hydrochloric acid and 0.497 g TEACl provided optimum resolution of MP, MPSO and internal standard from endogenous plasma components. After screening several steroids and a large number of other compounds for use as an internal standard, propyl *p*-hydroxybenzoate was chosen as the internal standard. It met the requirements of stability and quantitative recovery from biological fluids.

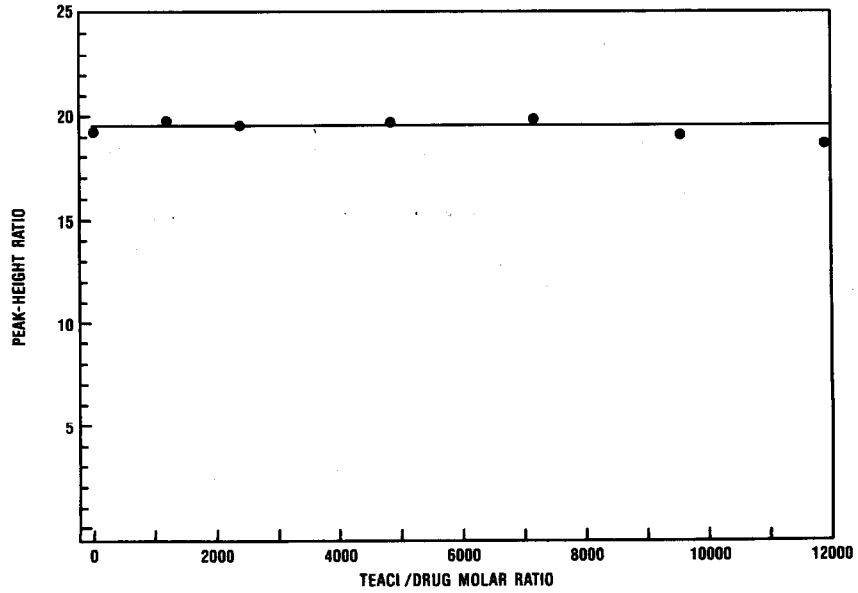


Fig. 4. Effect of TEACl on MP extraction.

TABLE I

INTER-DAY ACCURACY AND PRECISION OF MP AND MPSO FROM HUMAN PLASMA

Compound	Label concentration (μg/ml)	Recovery (mean ± S.D., $n=3$) (%)
MP	0.785	98.4 ± 4.68
	3.14	103.1 ± 7.07
	6.28	101.9 ± 7.02
MPSO	1.85	97.3 ± 6.75
	7.40	100.1 ± 9.13
	13.20	105.2 ± 6.67

TABLE II

STABILITY OF MPSO IN PLASMA CONTAINING 1.5 mg/ml Na_2EDTA (STORED AT −70°C)

Date assayed	Storage time	Plasma concentration (μg/ml)
November 20th, 1985	Fresh sample	38.66
December 5th, 1985	After two weeks	38.51
January 21st, 1986	After eight weeks and thawed twice	38.17
January 27th, 1986	After nine weeks	38.76

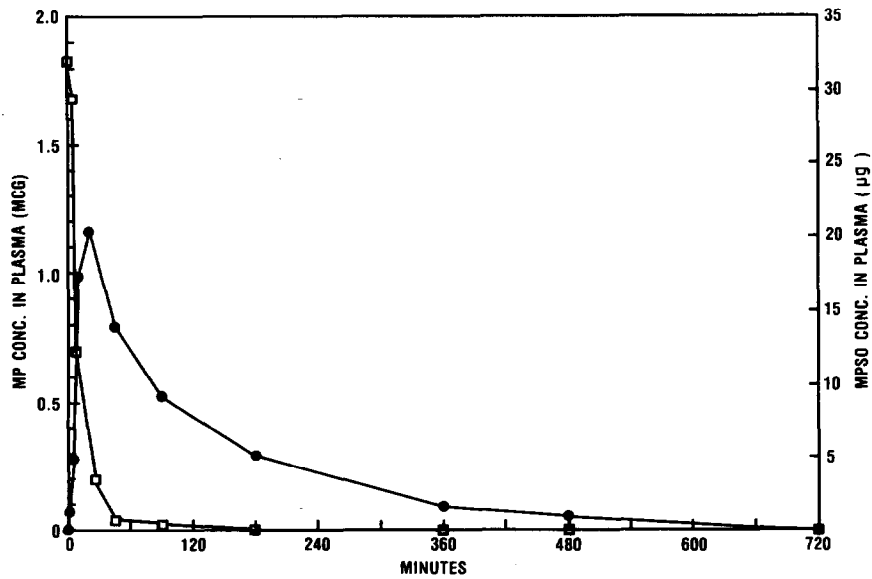


Fig. 5. MP (●) and MPSO (□) concentrations in human plasma following a 100-mg MP equivalent intravenous dose of MPSO.

TABLE III

PHARMACOKINETIC PARAMETERS FOR MP IN HUMAN PLASMA AFTER VARIOUS INTRAVENOUS DOSES OF MPSO IN NORMAL MALE VOLUNTEEES

C_{max} = maximum MP plasma concentration; t_{max} = time of maximum MP plasma concentration; K_{elim} = terminal rate constant for MP elimination; $t_{\frac{1}{2}elim}$ = half-life of MP associated with terminal rate constant; $AUC_{0-270\ min}$ = area under the MP plasma concentration-time curve from 0 to 270 min.

Subject No.	Dose★ (mg)	C_{max} (μg/ml)	t_{max} (min)	K_{elim} (min^{-1})	$t_{\frac{1}{2}\ elim}$ (min)	$AUC_{0-270\ min}$ ($\mu g/ml\ min^{-1}$)
2	10	0.120	46	0.006	108	16.92
5	10	0.116	46	0.0064	108	22.74
1	40	0.378	21	0.0064	108	64.34
9	100	1.162	21	0.0061	114	157.2
31	1000	19.56	20	0.0045	152	1969.06
32	1000	10.70	20	0.0044	156	2098.53

★MPSO equivalent of MP.

Several methods were tried for complete extraction of MP and MPSO from plasma. These trials involved precipitation of plasma proteins with reagents such as perchloric acid, methanol, acetonitrile and acetone. However, these attempts were unsuccessful in isolating MP and MPSO from interfering plasma endogenous components. Subsequent work led to employing an ion-pairing reagent TEACl in the extraction which provided excellent resolution of MP, MPSO and the internal standard from plasma with minimum background interferences. Prior to selection of chloroform as an extraction solvent, several solvents were examined to achieve optimum recoveries of MP, MPSO and internal standard from human plasma. A single chloroform extraction resulted in 80 and 52% recovery of MP and MPSO, respectively. A second 5-ml chloroform extraction provided total recoveries of 102 and 75% for MP and MPSO, respectively, from plasma. Stability of MPSO in biological fluids was maintained by using Na_2EDTA as a plasma stabilizer and storing the samples at $-70°C$. After resolving the above problems, assay conditions described in the Experimental section provided optimum resolution for the simultaneous quantitative analysis of MP and MPSO from human plasma.

Fig. 2A shows typical HPLC profile of blank human plasma. Fig. 2B shows plasma containing internal standard. Fig. 2C shows plasma containing MP, MPSO and internal standard. The asay provides complete resolution of MP, MPSO and internal standard from endogenous hydrocortisone and other components. The retention times of MPSO, MP and internal standard are 12, 18 and 26.5 min, respectively. If desired, hydrocortisone is also measurable at a retention time of 11.5 min as confirmed by a chromatogram using a hydrocortisone reference standard.

Fig. 3 shows the effect of varying the amounts of ion-pairing agent on the extent of formation of the TEACl ion pair with MPSO. A molar ratio of drug to TEACl of 1:5400 gave optimum ion-pair formation of MPSO in human plasma. Fig. 4 shows that use of TEACl has no effect on the extraction of MP. The data points in Figs. 3 and 4 represent single measurements with no replicates.

Extraction efficiency

Blank plasma specimens (1 ml) were spiked with known concentrations of MP from 0.78 to 15.69 μl/ml and MPSO from 1.85 to 37.0 μg/ml. These samples were extracted according to the procedure described for the plasma standards. The absolute extraction efficiencies of MP and MPSO from plasma were calculated by comparing the absorbance of known concentrations of MP and MPSO reference standards in mobile phase. The results demonstrated an average (±S.D.) extraction efficiency of 102.0±4.7% for MP over a concentration range of 0.78–15.69 μg/ml. The average (±S.D.) extraction efficiency of MPSO was 75.2±2.9% over a concentration range of 1.85–37.01 μg/ml.

Linearity of response and detection

Linear regression analysis of the concentration data indicated no significant deviations from linearity for MP upto approximately 200 μg/ml and for MPSO up to 80 μg/ml. The slope, intercept and correlation coefficient values were determined by regression analysis of the peak-height ratio (peak height MP or MPSO over peak height of internal standard) as a function of MP or MPSO concentrations. Correlation coefficients for standard curves prepared for MP and MPSO were better than 0.999 over an eight-week period at five different times. The assay detection limits for MP and MPSO in human plasma were approximately 7 ng/ml and approximately 25 ng/ml, respectively, when 1.0 ml of human plasma was analyzed. The detection limits for both compounds were calculated based on a signal-to-noise ratio of 3:1 and a 100-μl injection out of a 200-μl final reconstituted volume.

Assay precision and accuracy

The assay precision and accuracy were established by assaying samples containing known concentrations of MP and MPSO in 1.0 ml blank human plasma. Samples were prepared on three different days at various levels (within the standard curve range) and were treated as unknowns in the HPLC analysis. Table I shows the inter-day precision and accuracy results of MP and MPSO. The average inter-day recoveries for MP ranged from about 98 to 103%, with an S.D. of about 4 to 7% and for MPSO 97 to 105% with an S.D. of about 6 to 9%, respectively.

Stability of MPSO in human plasma

The stability of MPSO was first determined at room temperature and at −70°C storage without adding Na_2EDTA. Although, MPSO is relatively more stable upon storage at −70°C, it degrades rapidly during the process of equilibrating the sample to room temperature and analysis. Thus, studies were conducted to define the stability of MPSO in human plasma and to determine the proper handling and storage procedure. Results in Table II show that plasma samples containing MPSO are stable for at least two months when they contain 1.5 mg Na_2EDTA per ml plasma and are stored at −70°C. The EDTA may operate by interfering with carboxy esterases thought to be present in the plasma.

Applicability of the methodology

The utility of the analytical method for bioavailability and pharmacokinetic studies was demonstrated by monitoring plasma profiles of MP and MPSO in humans. Plasma samples were analyzed after intravenous administration of MPSO at doses from 10 to 3000 mg MP equivalents. Fig. 5 shows typical plasma concentration–time curves for MP and MPSO after intravenous administration of an MPSO dose equivalent to 1.31 mg MP per kg body weight (approximately 100 mg). The plasma levels of MP and MPSO were used to estimate pharmacokinetic parameters using graphic techniques. Table III shows pharmacokinetic parameters for MP after various intravenous doses of MPSO in normal male volunteers.

CONCLUSION

The assay methodology is simple, sensitive, specific and reproducible for simultaneous quantitative determination of MP and MPSO from human plasma and provides complete recovery of parent drug MP.

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