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SIMULTANEOUS DETERMINATION OF METHYLPREDNISOLONE AND METHYLPREDNISOLONE 21-[8-[METHYL-(2-SULFOETHYL)AMINO]-8-OXOOCTANOATE] SODIUM SALT IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

A reversed-phase high-performance liquid chromatographic assay 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-oxooctanoate] sodium salt (MPSO) in human urine following therapeutic doses in humans The assay procedure involves stabilization of urine samples by addition of disodium ethylenediaminetetraacetic acid (Na_2EDTA) and ion-pair extractions of MPSO using tetraethylammonium chloride (TEACl) as the counter ion 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 an IBM C_{18} reversed-phase column (5 μm) The mobile phase was a mixture of water–acetonitrile–isopropanol (71 2 18 8 10 0, v/v) containing 75 μl of 0 1 *M* hydrochloric acid and 0 450 g of TEACl per liter Propyl *p*-hydroxybenzoate was used as an internal standard The extraction efficiencies of MP and MPSO were greater than 90% using the ion-pairing agent TEACl The chromatographic responses were linear up to about 200 μg/ml for MP and 80 μg/ml for MPSO and had sufficient precision and accuracy to provide quantitative data from human urine The assay detection limit was about 8 ng/ml for MP and 25 ng/ml for MPSO in human urine Stability studies in urine indicated that without Na_2EDTA stabilization and at room temperature, rapid degradation of MPSO occurred in urine Addition of EDTA to the urine specimen and storage at $-70\,^{\circ}C$ increased the stability of MPSO, and little or no degradation was observed in urine stored for more than 60 days The method has been used in the simultaneous determination of MP and MPSO in urine specimens obtained from a single-dose tolerance study of MPSO in normal male volunteers

INTRODUCTION

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

MP has particular utility in therapy of acute bronchospasm (status asthmaticus) when it is administered intravenously as a water-soluble prodrug [1–4]. MPSO was synthesized as a possible replacement for the currently marketed prodrug, MPS, which has poor aqueous stability MPSO has an estimated aqueous stability at room temperature (25°C) of at least two years [5], which should allow marketing of a ready-to-inject solution as a multi-dose vial

To perform a bioequivalency comparison of 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 for pharmacokinetic studies in humans Rohdewald et al [6] developed an assay for the simultaneous determination of MP, MPS and endogenous hydrocortisone in plasma Previously, we developed a simple and sensitive assay method for the simultaneous determination of MP and its soluble prodrug esters in dog plasma [7], but this method proved unsuitable for human urine due to an interfering peak near MP Ebling

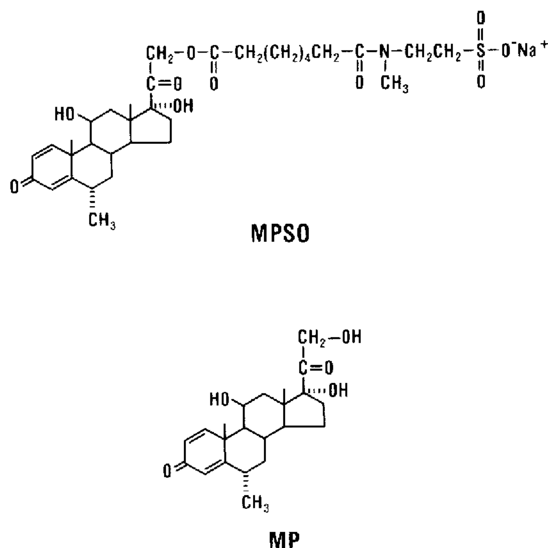

Fig 1 Structures of MPSO and MP

et al. [8] 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 rather time-consuming and laborious Previously we developed an assay method for the determination of MP and MPSO in human plasma [9] This plasma ion-pair extraction procedure [9] was revised for the analysis of MPSO and MP in human urine, and the simple, sensitive and rapid method is described in this paper.

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 and disodium salt of ethylenediaminetetraacetic acid (EDTA) with purity 99+% were 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 Water for Irrigation, USP (American McGaw, Irvine, CA, U.S.A.).

Chromatographic equipment and conditions

An LDC Constametric III pump and an LDC Spectomonitor III variable-wavelength ultraviolet detector or Krato 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}, 250 mm$\times$4.5 mm I D. column (5 μm particle size) (IBM Instruments, Wallingford, CT, U S A.) fitted with an RP-8, Spheri-5, 3-cm guard column (Brownlee Labs , Santa Clara, CA). 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 (71.2 18 8 10.0, v/v) containing 75 μl of 0.1 *M* hydrochloric acid and 0.45 g of TEACl per liter 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 and column temperature was ambient The sample injection volume was 50 μl and the response parameter was set to peak heights

Under these chromatographic conditions, the retention time of MPSO, MP

and internal standard was 14.8, 22.4 and 31 4 min, respectively Small changes in the composition of mobile phase and the history of the column can cause minor changes in retention times Old columns may have shorter retention times than new columns.

Human study protocol and sample handling

A single-dose intravenous tolerance study [10] of MPSO (total doses of 10, 40, 100, 250, 500, 1000, 1500, 20000 and 3000 mg MP equivalents) was conducted 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.

The volume of each volunteer's output of urine was measured immediately upon voiding and the volume and time of voiding were recorded for drug analysis A 25-ml aliquot of urine was placed in a polyethylene bottle containing 2 5 ml of an aqueous solution of 15 mg/ml Na_2EDTA (equivalent to 1.5 mg Na_2EDTA per ml of urine). The closed container was shaken to mix with the Na_2EDTA and all samples were stored at $-70°C$ as soon as possible for future assay.

Preparation of stock solutions

A stock solution containing about 200 μg/ml MPSO and about 100 μg/ml MP was prepared in acetonitrile–methanol. An internal standard solution of about 7 μg/ml propyl *p*-hydroxybenzoate 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 urine standards

The stock solution of MP and MPSO (20 ml) was pipetted into a 100-ml volumetric flask and then diluted with acetonitrile

Aliquots of the calibration standard solution and 100 μl standard solution were pipetted into each of a series of centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen. The centrifuge tubes were chilled in an ice bath for about 5 min and then 0.1-ml aliquots of 15 mg/ml Na_2EDTA solution, 1-ml aliquots of blank human urine and 250-μl aliquots of 1 0 *M* TEACl solution were added to each centrifuge tube and immediately vortexed for 8–10 s. Immediately, 5 ml chloroform were added and vortexed for about 5 s. The

steps from the chilled tube stage to the first chloroform extraction were completed for each tube before beginning the same steps for the next tube. All tubes were well shaken in a horizontal shaker for 15 min at slow speed The samples were then centrifuged at 2500 *g* for 10 min and the chloroform layer was transferred to a clean, labeled centrifuge tube The aqueous layer was immediately re-extracted with 5 ml chloroform as before, and the combined chloroform extracts were evaporated to dryness with nitrogen at room temperature. The sides of each tube were rinsed with 1 ml chloroform and evaporated just to dryness under nitrogen The dried extracts were reconstituted with 200–500 μl of mobile phase by vortexing for 8–10 s, and 50 μl were injected into the high-performance liquid chromatographic (HPLC) system for analysis

Preparation of unknown urine samples

The urine samples were prepared by pipetting 100 μl of stock internal standard solution into a series of 15-ml centrifuge tubes and evaporating to dryness with nitrogen at room temperature. Dried centrifuge tubes were chilled in an ice-bath for about 5 min. Then 1 1-ml aliquots of urine sample and 250 μl of 1.0 *M* TEACl solution were added to each centrifuge tube and vortexed for at least 10 s. Samples were immediately extracted with 5 ml chloroform, and subsequent centrifugation, evaporation and reconstitution steps were carried out as described for the urine standards above. This procedure is suitable for MPSO doses below the 0.5 g MP equivalents. Samples obtained following doses of 0 5–1 g equivalents of MP were assayed by diluting a 0.055-ml quenched human urine sample with 1.045 ml of blank human urine of the same subject. Similarly, for doses of 1.5, 2 and 3 g, the 0 022-ml quenched urine sample was diluted with 1.078 ml blank urine from the same subject The diluted samples and undiluted samples (predose and all post 6 h) were processed as described above.

Calculations

The calculation of MP and MPSO in urine samples was accomplished by using the appropriate slopes obtained by linear regression analysis of peak-height ratios versus concentration data. For doses below 0 5 g, the amounts of MP and MPSO in urine were calculated by determining the concentrations per milliliter of urine, multiplied by the total urine volume collected over each time interval. Urine specimens from subjects receiving 0 5-g doses and higher were diluted before analysis. Appropriate dilution factors were used to calculate MP and MPSO concentrations per milliliter of urine. These concentrations were multiplied by the total urine volume collected over each time interval to calculate the amount of drug excreted over each time interval.

RESULTS AND DISCUSSION

Chromatographic analysis

Several different columns, guard columns and a number of mobile phase compositions were tested for the selection of the one that would yield optimum

resolution of drug-related material from coextracted endogenous components Optimum resolution of MP, MPSO and internal standard from endogenous urine components was provided by an IBM C_{18}, 250 mm×4 5 mm I.D column (5 μm particle size) equipped with a C_8 30 mm×4 6 mm I.D Brownlee guard column (5 μm particle size) and a mobile phase composed of water–acetonitrile–isopropanol (71 2 18.8 10 0, v/v) containing 75 μl of 0 1 *M* hydrochloric acid and 0 450 g of TEACl per liter Most of the steps involved in this assay are similar to those reported for the analysis of MPSO and MP in human plasma [9]. Modifications were made in sample stabilization, size of the sam-

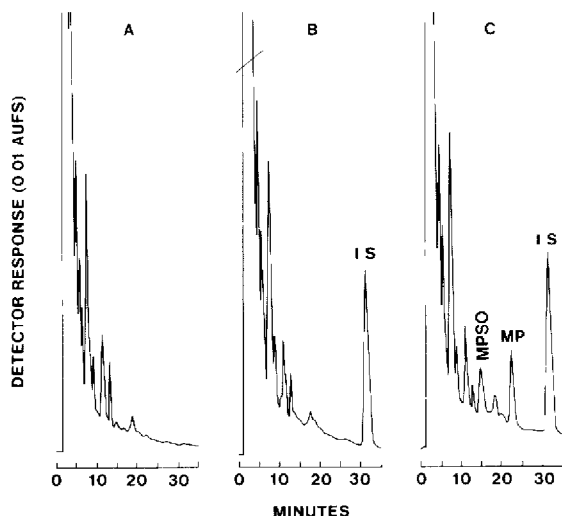

Fig 2 Chromatograms of human urine (A) Blank urine, (B) urine containing internal standard (I S), (C) urine containing MPSO, MP and I S

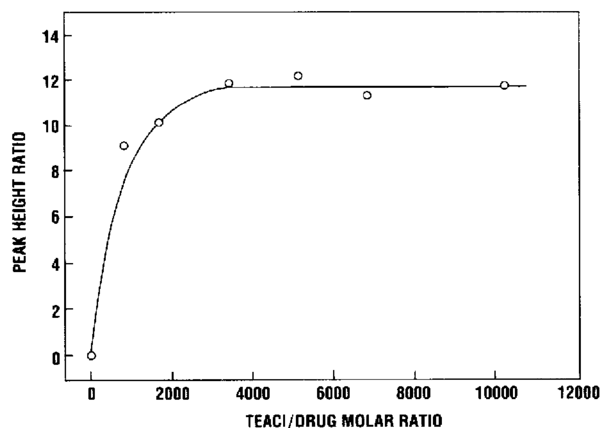

Fig 3 Effect of TEACl on MPSO extraction

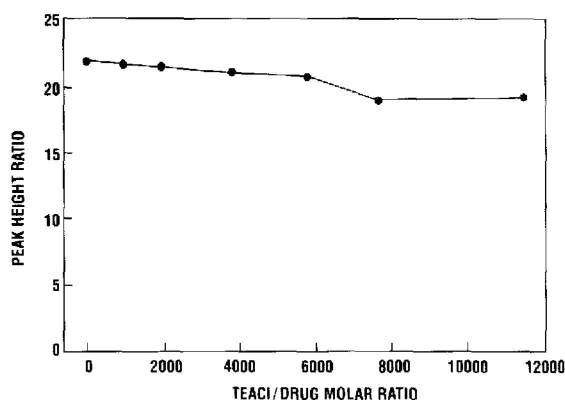

Fig 4 Effect of TEACl on MP extraction

ple analyzed, solvent compositions and amount of TEACl used during sample extraction The assay then provided complete resolution of MPSO, MP and internal standard from urine endogenous peaks, with retention times of 14.8, 22.4 and 31 4 min, respectively Typical chromatograms of the blank human urine, urine containing spiked internal standard and urine containing MPSO, MP and internal standard are shown in Fig 2 Fig 3 shows the effect of varying amounts of ion-pairing agent (TEACl) on the extent of formation of the TEACl ion pair with MPSO A molar ratio of drug to TEACl of 1 4300 gave optimum ion-pair formation of MPSO in human urine Fig. 4 shows that use of TEACl ratios up to 1:4300 had no effect on the extraction of MP from urine. The data points in Figs. 3 and 4 represent single measurements with no replicates

Extraction efficiency

Blank urine samples (1 ml) were spiked with internal standard and known concentrations of MP from 0.13 to 19 5 μg/ml and MPSO from 0 25 to 39 25 μg/ml These samples were extracted according to the procedure described above for urine standards. The absolute extraction efficiencies of MP and MPSO from human urine were calculated by comparing the absorbance of known concentrations of MP and MPSO reference standards in mobile phase. The results demonstrated an average (±95% confidence limit) absolute extraction efficiency of 91 7±3 83% for MP over a concentration range of 0.25–39.25 μg/ml. The average (±95% confidence limit) absolute extraction efficiency of MPSO was 90 7±5.65% over a concentration range of 0.13–19 54 μg/ml in human urine

Linearity of response and detection

Linear regression analyses of calibration curve data for MP and MPSO in human urine indicated no significant deviations from linearity for MP up to

about 200 μg/ml and for MPSO up to about 80 μg/ml The slope intercept and correlation coefficient 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 of standard curves prepared for MP and MPSO were better than 0.999 over a five-week period at five different times. The assay detection limits for MP and MPSO in urine were approximately 8 and 25 ng/ml, respectively, when 1 0 ml of urine was analyzed. The detection limits for both compounds were calculated based on a signal-to-noise ratio of 3 1 and 100-μl injection volume 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 urine Samples were prepared on three different days during a two-week period at various levels (within the standard curve range) and were treated as unknowns in the HPLC analysis. Intra-day and inter-day accuracy and precision results of MP and MPSO from human urine are summarized in Table I

Stability of MPSO in human urine

The stability of MPSO was determined both with and without Na_2EDTA at $-70°C$ storage The data showed that MPSO-spiked urine samples were stable in the presence of 1.5 mg Na_2EDTA per ml urine and at storage at $-70°C$ for at least three months The extracted urine samples in dried form prior to reconstitution were stable for about one week at 4°C; after reconstitution with the mobile phase, the urine samples were stable for at least 48 h at room temperature.

TABLE I

INTRA-DAY AND INTER-DAY ACCURACY AND PRECISION OF MP AND MPSO FROM HUMAN URINE

Compound	Concentration added (μg/ml)	Recovery (mean ± S D , n=3) (%)	
		Intra-day	Inter-day
MP	0 313	108 1±3 67	108 6±4 89
	9 53	97 2±6 03	97 6±6 34
MPSO	0 638	102 2±1 64	101 7±8 34
	19 23	98 3±1 97	98 0±5 49

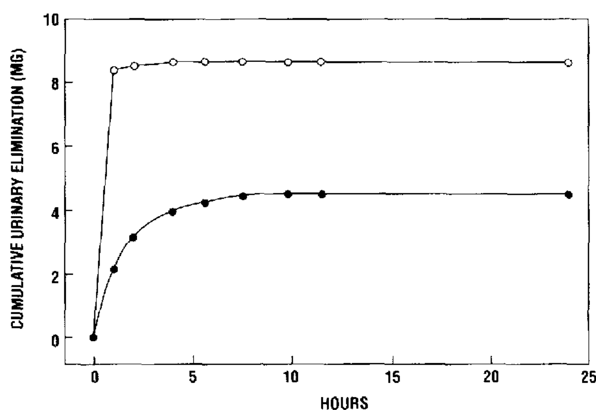

Fig 5 Cumulative urinary elimination of MPSO (○) and MP (●) following intravenous administration of MPSO equivalent to 100 mg MP in subject No 9

TABLE II

PHARMACOKINETIC PARAMETERS FOR MP IN HUMAN URINE AFTER VARIOUS INTRAVENOUS DOSES OF MPSO

Subject No	Dose (mg)[a]	K_{elim} (h^{-1})	$t_{1/2\ elim}$ (h)	Percentage of dose
2	10	0 677	1 02	2 7
5	10	0 352	1 96	2 8
1	40	0 559	1 24	4 3
9	100	0 527	1 31	4 5
31	1000	0 394	1 8	8 6
32	1000	0 367	1 88	8 3

[a]MPSO equivalent of MP

Accountability of the methodology

The utility of the analytical method for bioavailability and pharmacokinetic studies was demonstrated by monitoring cumulative urinary elimination profiles of MP and MPSO in humans. A typical example of urinary elimination of MP and MPSO in one volunteer who received MPSO in a dose of 100 mg MP equivalents is shown in Fig 5. The elimination rate constant (K_{elim}) and its associated half-life ($t_{1/2\ elim}$) of MP were obtained using semilogarithmic plots of amount remaining to be eliminated in urine ($U\infty - U_t$) versus time. Table II shows pharmacokinetic parameters for MP in human urine, by subject, after various intravenous doses of MPSO

CONCLUSION

The results obtained from the human volunteers indicated that the developed analytical method has the necessary sensitivity to detect levels of MP

and MPSO extracted from urine. The results from the volunteers suggest that methodology is quite suitable for the analysis of pharmacokinetic parameters

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REFERENCES

1 I H Itkin and M S Menzel, J Allergy, 45 (1970) 146
2 S L Spector, F H Katz and R S Fall, J Allergy Clin Immunol, 54 (1974) 367
3 R S Zieger, M Schatz, W Sperling, R A Simon and D D Stevenson, J Allergy Clin Immunol, 66 (1980) 438
4 S J Szefler, J Q Rose, E F Ellis, S L Spector, A W Green and W H Jusko, J Allergy Clin Immunol, 66 (1980) 447
5 B D Anderson, R A Conradi, C H Spilman and A D Forbes, J Pharm Sci, 74 (1985) 365
6 P Rohdewald, J Rehder, G Drehsen, G Hochhaus, H Derendorf and H Mollmann, J Pharm Biomed Anal, 3 (1985) 565
7 J A Shah and D J Weber, J Chromatogr, 344 (1985) 41
8 W F Ebling, S J Szefler and W J Jusko, J Chromatogr, 305 (1984) 271
9 J A Shah, D J Weber and B E Bothwell, J Chromatogr, 414 (1987) 1
10 J T VanderLugt, The Upjohn Company, 1984, personal communication