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Validation of the simultaneous determination of methylprednisolone and methylprednisolone acetate in human plasma by high-performance liquid chromatography

N. K. Hopkins*

The Upjohn Company, 7215-24-2, Kalamazoo, MI 49001 (USA)

C. M. Wagner, J. Brisson and T. E. Addison

Biopharmaceutics Department, Hazleton Wisconsin, Inc., Madison, WI 53707 (USA)

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ABSTRACT

A reversed-phase high-performance liquid chromatographic procedure was developed that accurately quantitates methylprednisolone (MP) and methylprednisolone acetate (MPA) in human plasma over the range 2.00–50.0 ng/ml. The internal standard, fluorometholone, was added to an aliquot of sodium fluoride–potassium oxalate-derived plasma. Samples were prewashed with hexane and extracted twice with methylene chloride. The extracts were dried with anhydrous sodium sulfate, centrifuged, and the organic layer separated and dried under nitrogen. The samples were reconstituted in mobile phase and washed an additional time with hexane before 100 μl were injected onto a Beckman/Altex Ultrasphere ODS column with ultraviolet absorbance detection at 254 nm. Composition of the mobile phase was acetonitrile–water–glacial acetic acid (33:62:5, v/v/v). Calibration curves were obtained by unweighted, linear regression of peak-height ratios of MP (or MPA)/internal standard *versus* theoretical concentrations of MP or MPA using a Hewlett-Packard 3357 Laboratory Automation System. Extraction efficiencies for MP and MPA over the linear range were 86.4 and 84.7%, respectively. This method was succesfully implemented for the analysis of specimens generated from a single-dose bioavailability and safety study for a new formulation of Depo-Medrol sterile aqueous suspension.

INTRODUCTION

Methylprednisolone acetate (MPA) is a synthetic anti-inflammatory glucocorticoid often administered as an aqueous suspension by intramuscular (i.m.) injection. MPA has been used extensively for treatment of endocrine, rheumatic, and hematological disorders, collagen diseases, as well as other indications. It has been reported that administration of MPA results in the hydrolysis of the ester to the pharmacologically active alcohol, methylprednisolone (MP) [1–3]. This hydrolysis probably occurs during absorption or in the liver before it reaches circulation. It has also been suggested that if the ester were to persist systemically, that serum cholinesterases would aid in the enzymatic hydrolysis of MPA to MP [4,5].

Much of the support data collected during the 1960–1970s to register and market MPA (as Depo-Medrol) was provided by use of radioimmunoassay (RIA) procedures [6]. Although the RIA reported quantitation to sub-nanogram levels, the specificity of the RIA procedure was ques-

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tionable, *i.e.*, inability to distinguish between MP, MPA and other related metabolites [7]. In order to support a safety and bioavailability study that administered a relatively low dose of MPA (40 mg, i.m.), it was anticipated that low ng/ml quantitation would be required for both MP and MPA. An alternative methodology to the RIA was investigated when several of the critical reagents, *e.g.*, specific anti-MPA antiserum and radiolabel-MPA, were not readily available, and synthesis of these reagents could be costly and take considerable time to produce.

Modifications to published [8,9] or existing proprietary high-performance liquid chromatographic (HPLC) procedures were pursued at Hazleton Wisconsin with the completed modifications described in this manuscript. In unpublished experiments, it was demonstrated that if MPA were added to whole blood containing sodium fluoride–potassium oxalate (NaF–K oxalate) or heparin or EDTA, that NaF–K oxalate best preserved MPA throughout sample processing. It was therefore felt that use of NaF–K oxalate in the blood collection process would preserve any low MPA levels present, if any of the MPA (ester) persisted in circulation. By demonstrating the ability to quantitate low ng/ml levels of both MP and MPA, data obtained from the sum of these components may closely approximate the RIA data. The method validations for both MP and MPA are presented in this report.

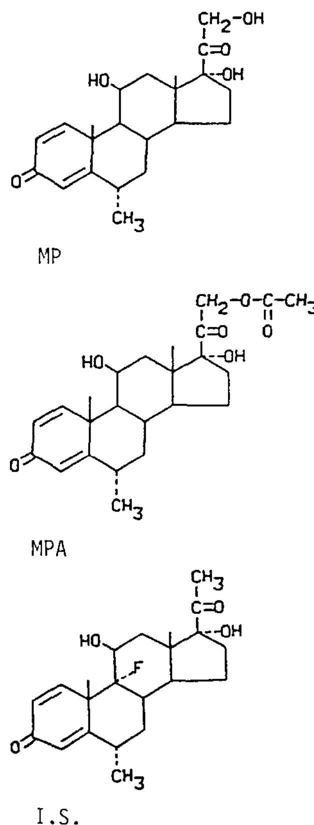

Fig. 1. Chemical structures of MP, MPA and fluorometholone, the internal standard. 11β,17α,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione (MP), 11β,17α,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione, 21 acetate or methylprednisolone acetate or Depo-Medrol (MPA) and 9α-fluoro-11β,17α-dihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione or fluorometholone, the internal standard (I.S.).

EXPERIMENTAL

Reagents and materials

MP, MPA and fluorometholone were control reference standards from Upjohn (Kalamazoo, MI, USA), with purity of 99.0, 99.5 and 99.4%, respectively. Fig. 1 displays the chemical structures for the compounds of interest. The analytical-grade glacial acetic acid was purchased from Mallinckrodt (Paris, KY, USA). The hexane (ultraviolet grade) and methylene chloride were purchased from Burdick and Jackson Labs. (Muskegon, MI, USA) and the acetonitrile (HPLC grade) was purchased from Fisher Scientific (Springfield, NJ, USA). Water used in this assay was deionized and then processed through a Milli-Q water purification system (Millipore, Bedford, MA, USA). The NaF–K oxalate-derived plasma (25 mg NaF and 20 mg K oxalate per 10 ml whole blood) was purchased from Interstate Blood Bank (Memphis, TN, USA).

Apparatus and conditions

The mobile phase composition for MP and MPA determination was acetonitrile–water–glacial acetic acid (33:62:5, v/v/v). The solution was prepared and then degassed by helium purge. A flow-rate of 1 ml/min was used. The ultraviolet detector was set at 254 nm, with sensitivity set at

0.005 a.u.f.s. All experiments were run at ambient temperature.

A solvent delivery system from Scientific Systems (State College, PA, USA) was used in conjunction with a Gilson Model 231 autoinjector, a Model 7010 Rheodyne valve, and a Kratos Spectroflow 773 detector. The column used was a Beckman/Altex Ultrasphere ODS, 5 μm, 250 mm × 4.6 mm I.D., coupled with a RP-18 Spheri-5, 5 μm, 30 mm × 4.6 mm I.D. guard column from Brownlee Labs. (Santa Clara, CA, USA). Data were recorded by a Hewlett-Packard 3357 Laboratory Automated System.

Preparation of stock solutions and standards

Stock solutions of MP, MPA and fluorometholone, the internal standard, all at concentrations of 1.00 mg/ml, were prepared and diluted in acetonitrile. Appropriate dilutions of the MP and MPA stock solutions were made in order to prepare daily calibration curves consisting of points targeted at 1.00, 2.00, 3.00, 4.00, 5.00, 7.00, 10.0, 25.0, and 50.0 ng/ml. Quality control pools containing both MP and MPA were prepared in human plasma with concentrations targeted at 4.00, 15.0 and 30.0 ng/ml.

Sample preparation

Plasma (2 ml) containing 500 ng of the internal standard, was extracted with 5 ml of hexane by shaking at 60 cycles per min (cpm) for 10 min on a horizontal shaker. The hexane and plasma layers were separated by centrifugation (1000 *g*, 5 min) and the hexane layer was aspirated and discarded. The compounds of interest were extracted from the plasma into 8 ml of methylene chloride by shaking at 60 cpm for 10 min on a horizontal shaker. The layers were separated by centrifugation (1000 *g*, 5 min) and the methylene chloride layer was transferred to a clean test tube. The remaining plasma was extracted a second time with 8 ml of methylene chloride.

The two methylene chloride portions were combined and 0.3 g of anhydrous sodium sulfate was added. The methylene chloride–sodium sulfate mixture was shaken on a horizontal shaker at 200 cpm for 5 min, then separated by centrifugation (1000 *g*, 5 min). The methylene chloride layer was transferred to conical centrifuge tubes and evaporated under a stream of nitrogen in a 40°C water bath. The residues were reconstituted in 200 μl of mobile phase and washed once with 2 ml of hexane by mixing on a vortex mixer for 1 min. The hexane and mobile phase were separated by centrifugation (1000 *g*, 5 min) and the hexane layer was aspirated and discarded. A 100-μl aliquot of the reconstituted sample was injected onto the HPLC system.

RESULTS AND DISCUSSION

As stated, the initial goals were to develop an HPLC procedure that was sensitive enough to monitor the low concentrations of MP and MPA that may be present in plasma after a 40-mg i.m. injection. If any MPA were to persist in circulation a very low level of detection would be required. Plasma collected in tubes containing NaF–K oxalate were used to preserve any MPA still present. However, when approximately 20% of the clinical specimens screened for both MP and MPA were found to have MPA levels below the limit of quantitation (2.00 ng/ml), quantitation of MPA was discontinued. Concentrations of MP above 2.00 ng/ml were accurately quantitated by this procedure.

Linearity

MP and MPA calibration curves were prepared fresh daily by spiking stock solutions into blank human plasma. The resulting calibration curves were linear over the concentration range 2.00–50.0 ng/ml. Quantitation of plasma samples was accomplished by unweighted linear regression analysis of peak-height ratios *versus* theoretical concentration. During the validation exercises, the correlation coefficient averaged 0.9991 for both compounds. The average slope for MP was 0.0798 and for MPA was 0.0294. The *y*-intercept for MP and MPA was not found to be significantly different from zero at $\alpha = 0.01$.

Accuracy and precision

The accuracy and precision of the assay was

determined by comparing the mean and percentage coefficient of variation (C.V.) of measured concentrations with the theoretical concentrations of MP and MPA in spiked plasma quality control pools. Aliquots from large plasma pools were prepared and frozen until analysis. Intra-day variability data are presented in Table I, where $n = 6$ for each quality control pool. Inter-day variability data were collected for each pool during the analysis of the clinical protocol where $n = 58$ over thirty analytical runs for MP and $n = 14$ over seven analytical runs for MPA, as shown in Table II.

During validation exercises, only the 4.00 ng/ml MP quality control pool exhibited an inter-day mean bias exceeding 10% from target (−11.1%). However, during the analysis of the clinical protocol, the 4.00 ng/ml MP quality control pool reported an improved mean bias of −3.0% from the theoretical concentration. During the validation exercises the 4.00 ng/ml MPA quality control pool resulted in a C.V. of 14.3%. This was the greatest variability exhibited in any of the quality control pools. During protocol analysis, improvements in the mean bias and precision were observed for all quality control pools. The improvements were possibly due to new pool preparations, a larger number of determinations making up each pool mean and C.V. plus additional experience working with the method.

Limit of detection and limit of quantitation

A 1.00 ng/ml calibration standard was included in the evaluations of MP and MPA during an initial set of experiments. Because of high variability (C.V. ⩾ 35%) this calibration standard point was excluded from the reportable range of the standard curves. The 1.00 ng/ml calibration standard, which could reliably be distinguished from zero, was classified as the lower limit of detection for both assay procedures.

The useful limit of quantitation was set at 2.00

TABLE I

INTRA-DAY VARIABILITY

Quality control pool target concentration (ng/ml)	MP ($n = 6$)			MPA ($n = 6$)		
	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Bias (%)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Bias (%)
4.00	3.62 ± 0.457	12.6	−9.6	3.43 ± 0.215	6.27	14.16
15.0	14.4 ± 0.33	2.3	−4.3	14.4 ± 0.37	2.6	− 4.0
30.0	29.2 ± 0.80	2.7	−2.6	28.5 ± 0.77	2.7	− 5.2

TABLE II

INTER-ASSAY VARIABILITY

Quality control pool target concentration (ng/ml)	MP[a]			MPA[b]		
	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Bias (%)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Bias (%)
4.00	3.88 ± 0.317	8.17	−3.0	3.63 ± 0.334	9.20	−9.25
15.0	14.7 ± 0.53	3.60	−2.0	14.8 ± 0.844	5.69	−1.33
30.0	29.5 ± 0.90	3.10	−1.7	29.2 ± 0.833	2.85	−2.67

[a] MP quality control pools were run ($n = 58$) on thirty different days.
[b] MPA quality control pools were run ($n = 14$) on seven different days.

ng/ml for both analytes after conducting validation exercises. At that concentration, the mean bias for the back-calculated values for the MP and MPA calibration standards was −4.7 and −13.1% with C.V.s of 17.8 and 9.1%, respectively, where $n = 4$ determinations. However, summaries of the 2.00 ng/ml calibration standards for MP and MPA during the analysis of clinical protocol resulted in mean biases of less than −4.8% for either analyte and C.V.s of 10.4 and 14.1%, respectively.

Fig. 2 is a representative chromatogram of the 3.0 ng/ml calibration standard for MP and MPA.

Absolute recovery

Absolute recoveries were determined by comparing the peak heights of the extracted calibration standards or internal standard to those of unextracted standards prepared in mobile phase. The mean recoveries were all very similar at 86.4, 83.7 and 85.5% for MP, MPA and the internal standard, respectively.

Selectivity

During an initial phase of the validation, one set of chromatographic conditions were evaluated. However, when the analysis of authentic plasma samples from the clinical protocol was initiated, an endogenous interference was detected. The composition of the mobile phase was modified by replacing the methanol with acetonitrile to improve the selectivity of the mobile phase. The subsequent re-validation was abbreviated since results correlated well with the initial validation data. With the modifications, the interference was successfully moved out of the chromatographic window of the internal standard. All 0-h samples for the various treatments were blank and were reported to be lower than the limit of quantitation for MP (Fig. 3). No other drugs were administered in the clinical protocol and no other steroids or endogenous substances were evaluated for possible co-elution in this system. Fig. 4 is a chromatogram of the 7.00 ng/ml calibration standard indicating good separation of the peaks of interest.

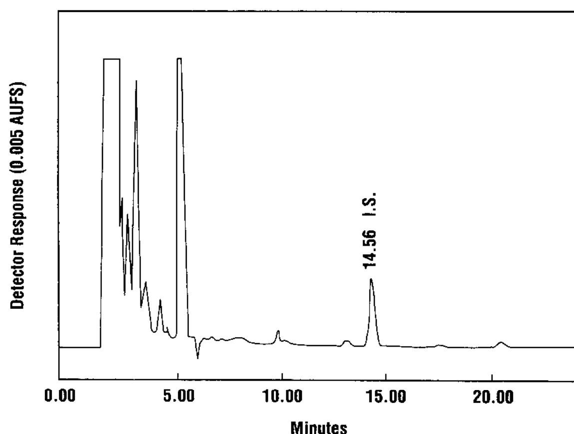

Fig. 3. Typical chromatogram of a 0-h plasma specimen showing no interfering peaks in the areas of interest.

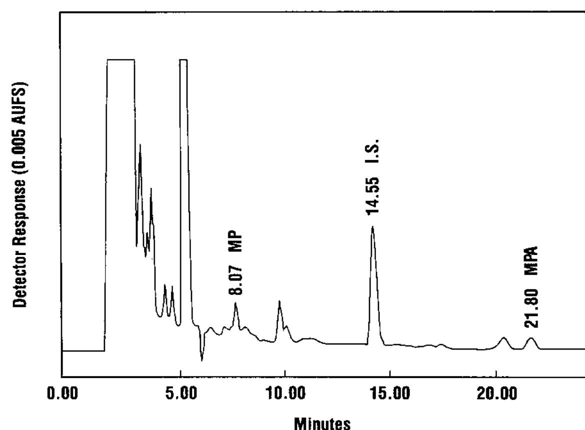

Fig. 2. Representative chromatogram of human plasma spiked with the I.S. and 3.0 ng/ml MP and MPA.

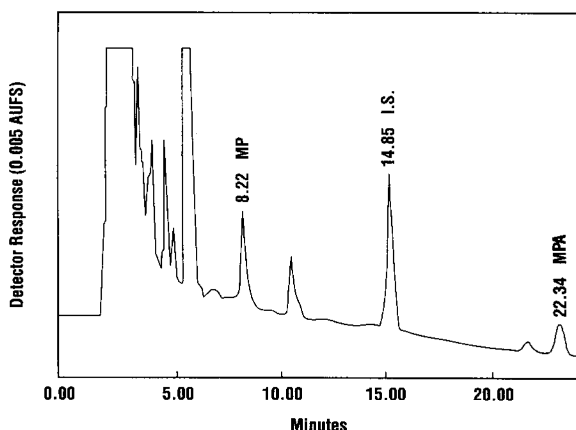

Fig. 4. Representative chromatogram of human plasma spiked with I.S. and 7.00 ng/ml MP and MPA.

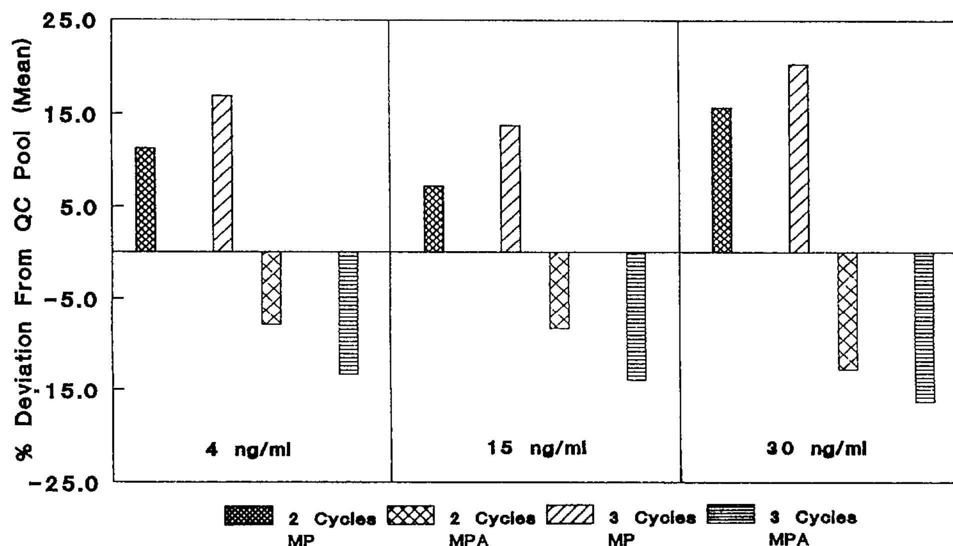

Fig. 5. Quality control (QC) pools prepared at target concentrations of 4.0, 15.0 and 30.0 ng/ml of both MP and MPA. Even in NaF–K oxalate-stabilized plasma, there is conversion of MPA to MP after two and three freeze–thaw cycles.

Stability

Samples collected during the clinical protocol could not be quantitated for MPA because of the complete conversion of MPA (ester) to its active form MP (alcohol) which was presumed to occur *in vivo*. Possible conversion of MPA to MP during processing of the blood samples could have been further evaluated by tedious and expensive gas chromatographic–mass spectrometric procedures, but exceeded the scope of this project. Quality control pool data indicated good stability of both MP and MPA for six months if samples are maintained at or below −20°C.

To further investigate compound stability in plasma, quality control samples were subjected to numerous freeze–thaw cycles prior to analysis. Quality control pools were prepared in plasma derived from blood collected into tubes containing NaF–K oxalate. Since all study samples are frozen and thawed at least one time before analysis, quality control data in Fig. 5 are expressed relative to one freeze–thaw cycle. There is strong

TABLE III

QUALITY CONTROLS THAWED AND PLACED AT ROOM TEMPERATURE FOR 24 h PRIOR TO ANALYSIS

Quality control pool target concentration (ng/ml)	MP ($n=3$)		MPA ($n=3$)	
	Concentration found (mean ± S.D.) (ng/ml)	Bias[a] (%)	Concentration found (mean ± S.D.) (ng/ml)	Bias[a] (%)
4.00	4.57 ± 0.224	28.7	2.07 ± 0.147	−45.1
15.0	17.5 ± 0.06	26.8	15.8 ± 0.32	9.7
30.0	37.2 ± 0.49	35.8	25.2 ± 0.32	−12.8

[a] Bias relative to quality control pool established mean.

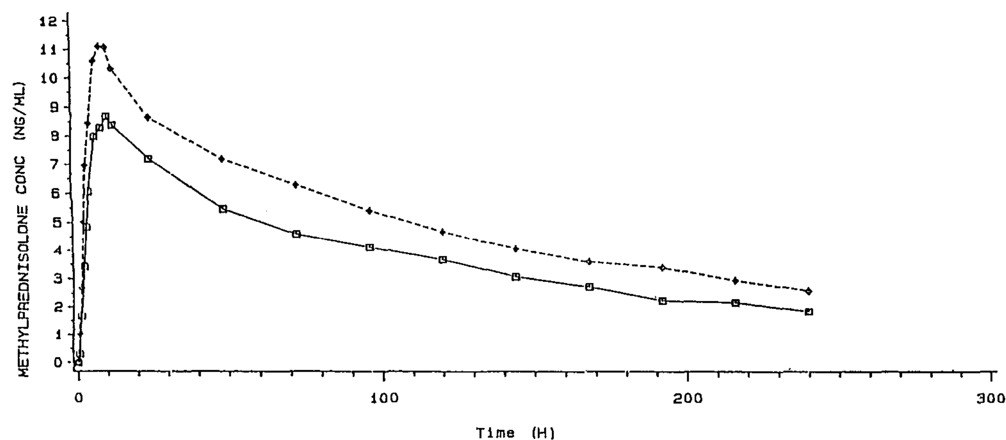

Fig. 6. Plasma concentrations of MP following i.m. administration of 40 mg Depo-Medrol marketed formulation (□); new formulation (◇).

indication that even in NaF–K oxalate-stabilized plasma, there is conversion of MPA to MP during the freeze–thaw process. All of the quality control pools indicate increasing bias with an increasing number of freeze–thaw cycles. If specimens are allowed to sit at room temperature for 24 h before processing, MP concentrations may increase as much as 35.0%, while MPA concentrations may decrease by up to 45.0% as shown in Table III.

CONCLUSION

This analytical procedure provides a relatively simple, sensitive method for the simultaneous quantitation of both MP and MPA in human plasma. The ruggedness of the method has been demonstrated by its use for the analysis of daily calibration standards, quality control specimens, and clinical protocol specimens over a six-month period of time. Fig. 6 presents a summary of the resulting pharmacokinetic profile of MP found in normal volunteers after i.m. administration of 40 mg Depo-Medrol. Use of blood collected into NaF–K oxalate may not be necessary since it appears that administered MPA is efficiently converted to the active parent drug, MP *in vivo*.

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