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Simultaneous analysis of methylprednisolone, methylprednisolone succinate, and endogenous corticosterone in rat plasma

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Abstract

A reversed-phase HPLC method is reported for simultaneous quantitation of methylprednisolone (MP), MP succinate (MPS), and endogenous corticosterone (CST) in plasma of rats. Additionally, the 11-keto metabolite of MP (methylprednisone, MPN) is resolved from the other analytes. After addition of internal standard (triamcinolone acetonide; IS) and an initial clean up step, the analytes of interest are extracted into methylene chloride. The steroids are then resolved on a reversed-phase polymer column using a mobile phase of 0.1 M acetate buffer (pH 5.7): acetonitrile (77:23) which is pumped at a flow rate of 1.5 ml min^{-1}. Sample detection was accomplished using an UV detector at a wavelength of 250 nm. All the five components (MPS, MP, MPN, CST and IS) were baseline resolved from each other and other components of plasma. Linear relationships were found between the steroids: IS peak area ratios and plasma concentrations in the range of 0.1–4 μg ml^{-1} for MP and MPS and 0.1–1.0 μg ml^{-1} for MPN and CST. The assay is accurate as intra- and inter-run error values were $< \pm 8\%$ for all the components. Further, the intra- and inter-run CVs of the assay were $< 16\%$ at all the concentrations and for all the components. The application of the assay was demonstrated after the injection of a single 5 mg kg^{-1} (MP equivalent) dose of MPS or a macromolecular prodrug of MP to rats.

Keywords: Methylprednisolone succinate; Methylprednisolone; Methylprednisolone prodrugs; Corticosterone; Methylprednisone; Reversed-phase liquid chromatography

1. Introduction

Methylprednisolone (MP) is a corticosteroid which is used primarily for its antiinflammatory and immunosuppressive effects. Because of its low water solubility, a sodium succinate salt of MP (MPS) is used as an injectable dosage form. Therefore, it is necessary to quantitate both MPS

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and MP in the biological samples after the administration of MPS. Such an assay is also needed after the administration of prodrugs which release both MP and MPS in vivo.

In addition to MPS and MP, an ideal assay should also quantitate endogenous corticosteroids, such as cortisol in man and corticosterone (CST) in rats. This is because pharmacodynamic evaluation of corticosteroid drugs, such as methylprednisolone, should also consider the levels of endogenous corticosteroids, which are normally inhibited by exogenous administration of these hormones [1]. Additionally, because MP is converted to its keto acid metabolite, methylprednisone (MPN) [2], the metabolite should also be quantitated or at least be resolved from the other analytes in the sample.

A few HPLC assays [2–6] have been reported for the measurement of MP in biological samples. Additionally, two of these assays [2,5] have measured the concentrations of MPS indirectly after its hydrolysis to MP, requiring sample preparation and analysis twice. A third assay [4], quantitated both MP and MPS simultaneously in human plasma. However, neither the MP metabolite (MPN) nor the endogenous steroids were resolved in this assay. In this work, a direct HPLC assay is reported for the determination of MPS, MP, and CST in plasma of rats simultaneously. Additionally, the metabolite of MP (MPN) is resolved from the other analytes.

2. Experimental

2.1. Chemicals

MP, CST, internal standard (triamcinolone acetonide, IS), bovine serum albumin (fraction V), and frozen blank rat plasma were obtained from Sigma Chemical (St. Louis, MO). 6α-Methylprednisolone 21-hemisuccinate (MPS) and MPN were purchased from Steraloids (Wilton, NH). For chromatography, HPLC grade acetonitrile (Mallinckrodt Chromar HPLC) was obtained from VWR Scientific (Minneapolis, MN). All other reagents were analytical grade and obtained through commercial sources.

2.2. Calibration standards

Because blank rat plasma contains endogenous CST, the calibration standards were prepared in an albumin solution or in blank rat plasma stripped of endogenous CST by the use of charcoal as described before by Haughey and Jusko [6]. Albumin solution was prepared by dissolving bovine serum albumin (4.5%, w/v) in a pH 7.4 phosphate buffer.

Stock solutions (1 mg ml^{-1}) of MP, MPS, CST and MPN were prepared in methanol and kept at −20°C. The stock solutions were diluted daily with distilled water before addition to the blank plasma or albumin solution. Calibration curves were constructed by plotting the peak area ratios of the analytes to IS against the concentration in the sample (0.1–1 μg ml^{-1} for MPN and CST and 0.1–4 μg ml^{-1} for MPS and MP) using a weight of inverse concentration. Concentrations of MP or MPS in each calibration standard were 4 fold higher than those of MPN or CST (Table 1). For IS, a stock solution (0.2 mg ml^{-1} in methanol) was diluted with 10% glacial acetic acid to make a final concentration of 5 μg ml^{-1}.

Table 1
Calibration standards

	Standard no.							
	1	2	3	4	5	6	7	8
Concentration (μg ml^{-1})								
MP and MPS	0	0.1	0.2	0.4	1.0	2.0	3.0	4.0
CST and MPN	0	0.025	0.05	0.1	0.25	0.50	0.75	1.0

2.3. Sample preparation

Plasma (0.1–0.5 ml) was added to a glass test tube and the volume was adjusted to 0.5 ml using the albumin solution. To each tube were added 100 μl of IS (5 μg ml^{-1} in 10% glacial acetic acid) and 10 ml of hexane. The samples were then mixed using a rotary mixer at a rate of 20 rpm for 10 min. After centrifugation of the samples for 5 min, the hexane layer was discarded. The analytes were then extracted into 8 ml of methylene chloride after mixing the samples at a rate of 20 rpm for 20 min. After centrifugation for 10 min, the top aqueous layer was discarded and methylene chloride was transferred to clean tubes containing 100 μl of glacial acetic acid. Thereafter, methylene chloride was evaporated under a nitrogen stream in a water bath at a temperature of 40°C. The analytes were then dissolved in 200 μl of mobile phase, vortex-mixed for 10 s, transferred to microcentrifuge tubes, and centrifuged for 3 min. After transferring the samples to autosampler inserts, 50–100 μl were injected into the HPLC.

2.4. Chromatography

The samples were analyzed at ambient temperature utilizing a 15 cm × 4.1 mm polystyrene-divinyl benzene copolymer (5 μm) column (PRP-1; Hamilton, Reno, Nevada), preceded by a 5-cm guard column packed with pellicular C_{18} material. The mobile phase consisted of 0.1 M acetate buffer (pH 5.7) and acetonitrile (77:23) which was pumped at a flow rate of 1.5 ml min^{-1}.

The HPLC instrument consisted of a 590 pump (Waters; Milford, MA), a SIL-9A autosampler (Shimadzu Scientific; Columbia, MD), and a 484 UV detector (Waters) set at a wavelength of 250 nm. The chromatographic data was managed using the Millennium software (Waters).

2.5. Absolute recovery

The efficiency of the extraction method to recover the steroids from plasma was tested ($n = 4$) at plasma concentrations of 1 μg ml^{-1} for MP and MPS and 0.25 μg ml^{-1} for MPN and CST. The extracted samples were processed as described in Section 2.3 with exact volumes (6 ml) of methylene chloride evaporated. The peak areas of the steroids in the extracted samples were then compared with those after the injection of the unextracted samples dissolved in the mobile phase.

2.6. Comparison of standards prepared in albumin and stripped plasma

Calibration standards were prepared in both albumin solution and steroid-stripped rat plasma and analyzed using the above system. The effects of matrix (plasma or albumin solution) on the analyte:IS peak area ratios were then analyzed using factorial ANOVA.

2.7. Assay validation

The intra- and inter-run precision and accuracy of the assay were determined by % CV and % error, respectively ($n = 5$), based on reported guidelines [7]. Briefly, each set of quality control samples was run along with a calibration standard. The concentrations of the quality control samples were then determined against the calibration curve and used for calculation of % CV and % error, the latter being calculated by the following equation:

$$\%\ \text{error} = \frac{\text{calculated conc.} - \text{added conc.}}{\text{added conc.}} \times 100$$

The quality controls were run at concentrations of 0.1, 0.4, 1 and 4 μg ml^{-1} for MP and MPS and at concentrations of 0.1, 0.25 and 1 μg ml^{-1} for MPN and CST.

2.8. Application of the assay

To demonstrate the application of the assay to MPS injection, both right and left jugular veins of a 290-g, male, Sprague–Dawley rat were cannulated under a ketamine: xylazine (80:12 mg kg^{-1}) anesthesia. Three hours after recovery from anesthesia, a MPS solution, containing 6.35 mg MPS, 1.6 mg NaH_2PO_4 and 17.5 mg Na_2HPO_4 per ml of distilled water, was injected into the right jugular vein catheter at a dose of 1 ml kg^{-1} (5 mg/kg

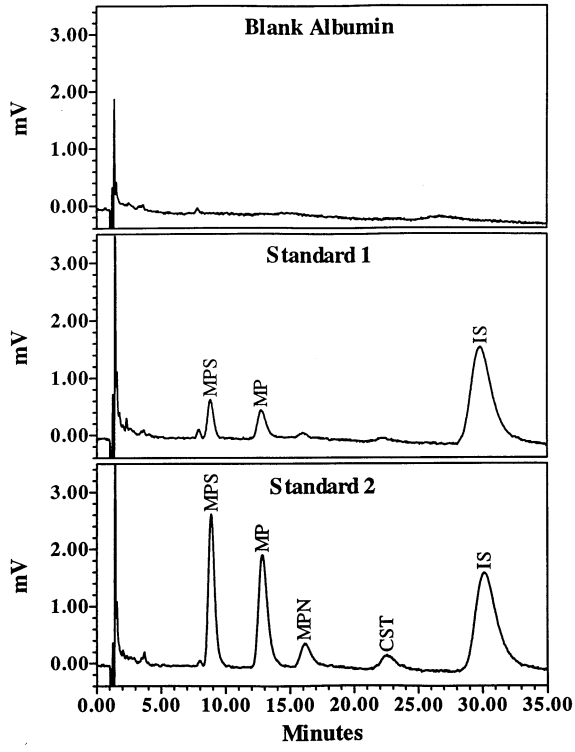

Fig. 1. Chromatograms of albumin standards: top, blank; middle, standard 1 containing 0.1 μg ml^{-1} of MP and MPS plus 0.025 μg ml^{-1} MPN and CST (below limit of quantitation); bottom, standard 2 containing 0.4 μg ml^{-1} of MP and MPS and 0.1 μg ml^{-1} of MPN and CST.

MP). Blood samples ($\sim$0.5 ml) were withdrawn from the left jugular vein catheter at 0 (before drug administration), 2, 5, 10, 20 and 30 min. Additionally, at 40 min after the drug injection, the rat was euthanized in a CO_2 chamber and 2 ml of blood was taken. After centrifugation of the blood in heparinized microcentrifuge tubes, the plasma samples (0.25 ml for 0–30 min samples and 0.5 ml for the 40-min sample) were subjected to the assay described above.

The application of the assay was also tested for a macromolecular prodrug of MP, prepared by conjugating MPS to dextran with a molecular weight of 70 000, as described before [8]. The conjugate contained 8.5% (w/w) MP. Dextran-methylprednisolone succinate (5 mg kg^{-1}, MP equivalent) was injected into the tail vein of rats, and animals were euthanized at 15 min ($n=4$) and 24 h ($n=4$) after the prodrug injection. Blood samples were taken by cardiac puncture, centrifuged at 4°C in heparinized microcentrifuge tubes, and 0.5 ml of the resultant plasma was subjected to the assay.

3. Results

Chromatograms of a blank albumin solution, an albumin standard containing 0.1 μg ml^{-1} of MP and MPS, and a standard containing 0.4 μg ml^{-1} of MP and MPS and 0.1 μg ml^{-1} of MPN and CST are illustrated in Fig. 1. Under the chromatographic conditions used, MPS, MP, MPN, CST, and IS eluted at 8.7, 13, 16, 22 and 30 min, respectively; all peaks were baseline resolved (Fig. 1).

The chromatograms of a blank rat plasma before and after treatment with charcoal and a sample taken 20 min after the injection of a single 5 mg kg^{-1} (MP equivalent) dose of MPS are depicted in Fig. 2. As demonstrated in this figure, charcoal treatment completely eliminated the CST peak (Fig. 2, top). Additionally, all the peaks of interset were completely resolved from endogenous peaks present in the plasma.

The relationships between peak areas of MP, MPS, MPN, or CST over that of IS and the concentrations of the steroids in the samples are reported in Table 2 for five calibration curves used for the inter-run validation of the assay; the concentration ranges were 0.1–4 μg ml^{-1} for MPS and MP and 0.1–1 μg ml^{-1} for MPN and CST. The relationships were linear for all the four components with r^2 values of $\geq$0.99 (Table 2). Further, the response of the detector was very similar for all the steroids as evidenced by the similarity of the slopes for different steroids (Table 2).

The effect of matrix (stripped rat plasma or the albumin solution) on the calibration curves are reported in Table 3. The calibration curves using plasma and albumin were virtually indistinguishable from each other for all four components ($P=0.959$) (Table 3).

The results of assay validation are reported in Tables 4 and 5 for intra- and inter-run data,

respectively. The accuracy of the assay is demonstrated by error values of $< \pm 8\%$ (Tables 4 and 5) for all the four components and at all the concentrations tested. The reproducibility of the assay is also illustrated in Tables 4 and 5 by CV values of $< 16\%$. Based on these data, the lower limit of quantitation of the assay is set at 0.1 μg ml^{-1} for all the four components.

The extraction efficiency of the method was 90, 83, 95 and 86% for MP, MPS, MPN, and CST, respectively.

The plasma concentration-time courses of MPS, MP, and endogenous CST after a single 5 mg kg^{-1} dose (MP equivalent) of MPS are illustrated in Fig. 3. The plasma concentration of MPS declined very rapidly during the first 5 min, and, thereafter, the decline was relatively slower with a half-life of ~ 10 min. The concentrations of MP increased very rapidly and reached its maximum after 5 min with an elimination half-life (9.4 min) very similar to that of MPS. The concentrations of CST remained relatively constant during the sampling period (Fig. 3). No detectable concentration of MPN was observed with this assay.

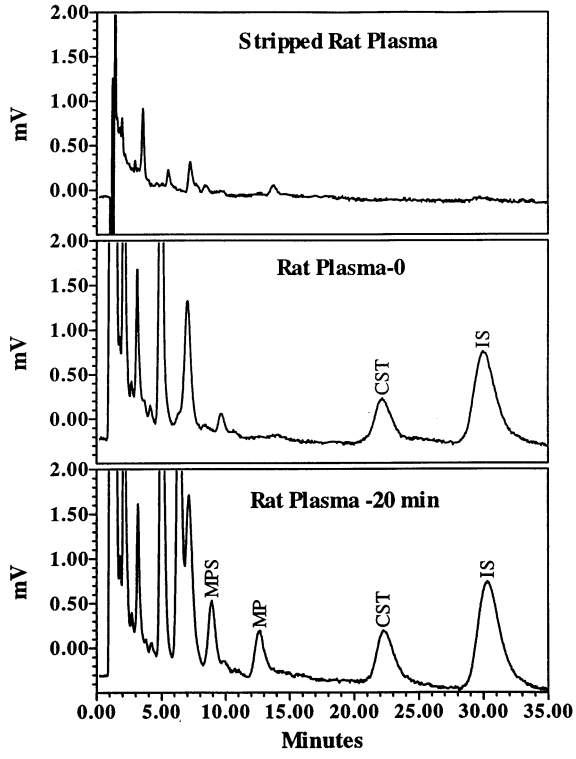

Fig. 2. Chromatograms of a charcoal-treated blank rat plasma (top) and non-treated plasma samples taken before (middle) and 20 min after (bottom) a single dose (5 mg kg^{-1}, MP equivalent) of MPS.

Fifteen minutes after the injection of a single dose (5 mg kg^{-1}; MP equivalent) of the macromolecular prodrug, the plasma concentrations of MP and CST were 0.291 ± 0.255 and 0.570 ± 0.083 μg ml^{-1}, respectively. The concentrations of MPN were below the detection limit in most samples, and no MPS was detected in any of the 15-min samples. Twenty-four hours after the administration of the prodrug, none of the steroids, including the endogenous CST, was detected in the plasma samples.

4. Discussion

Previously reported HPLC assays have described the measurement of MP in plasma of humans [3–5], rabbits [2], and rats [6]. In addition to quantitating MP, some of these assays are also capable of measurement of MPN (11-keto metabolite of MP) [2,6] and/or endogenous steroids [2,3,5,6]. Among these methods, the method of Haughey and Jusko [6] appears to be the most sensitive and, therefore, most appropriate for studies involving small volumes of plasma in rats. However, if the administered drug is MPS, the concentrations of both MP and MPS cannot be measured using this method.

The concentrations of MPS were measured indirectly by Ebling et al. [2,5] who analyzed plasma samples twice, before and after hydrolysis of MPS to MP. The concentrations of MPS were then determined by subtracting the original MP concentrations (before hydrolysis) from the total MP concentration (after hydrolysis). However, there are situations where this indirect method may not be applicable. For example, we have previously prepared a macromolecular prodrug of MP (dextran methylprednisolone succinate) which is hydrolyzed to MP and MPS [8]. The methods [2,5] previously used for the hydrolysis of MPS to MP could also hydrolyze this prodrug. Therefore,

Table 2
The relationship between analyte: internal standard peak area ratio and the added concentration for inter-run data [peak area ratio = intercept + (slope × conc.)]

	Calibration no.	Intercept	Slope	r^2	Standard error
MPS					
	1	−0.0218	1.20	0.997	0.0789
	2	−0.0170	1.16	0.995	0.103
	3	0.00323	1.04	0.994	0.0991
	4	−0.0241	1.16	0.999	0.0524
	5	−0.00966	1.11	0.999	0.0496
Mean		−0.0139	1.13	0.997	0.0766
S.D.		0.0110	0.06	0.002	0.0251
MP					
	1	−0.0196	1.29	0.998	0.0712
	2	−0.0123	1.24	0.996	0.0982
	3	0.00292	1.16	0.998	0.0709
	4	−0.0144	1.25	1.00	0.0323
	5	−0.0114	1.20	0.999	0.0451
Mean		−0.0110	1.23	0.998	0.0635
S.D.		0.0084	0.05	0.001	0.0256
MPN					
	1	−0.0168	1.35	0.997	0.0469
	2	0.00168	1.35	0.993	0.0750
	3	0.0149	1.18	0.997	0.0400
	4	0.0146	1.30	0.997	0.0460
	5	0.0212	1.18	1.00	0.0462
Mean		0.00712	1.27	0.997	0.0508
S.D.		0.0151	0.09	0.002	0.0138
CST					
	1	−0.00969	1.30	0.995	0.0589
	2	−0.00199	1.28	0.987	0.0958
	3	0.00291	1.17	0.998	0.0333
	4	−0.00664	1.27	0.999	0.0310
	5	−0.00541	1.22	0.999	0.0280
Mean		−0.00416	1.25	0.996	0.0494
S.D.		0.00482	0.05	0.005	0.0287

these methods could not be applied to situations requiring direct measurement of MP and MPS (without the hydrolysis of the sample). Additionally, indirect measurements of MPS may be less accurate, especially in samples containing low concentrations of MPS and high concentrations of MP. Therefore, our method may be more appropriate for situations where direct measurement of both MP and MPS is necessary.

The only reported [4] HPLC method for the direct measurement of MP and MPS is applied to human plasma. This method uses a C_{18} reversed-phase column and a mobile phase of aqueous acetonitrile and glacial acetic acid to resolve MP and MPS. Because the pH of this mobile phase is lower than the pK_a of MPS (4.5–4.6 [9]), the succinate elutes after the MP peak in this system [4]. We attempted to use this method for the measurements of MP and MPS in rat plasma. However, both endogenous CST and MPN interfered with the MP and/or MPS peaks. After extensive evaluations, we substituted a polystyrene-divinyl benzene copolymer column for the C_{18} column and used a mobile phase with a pH (5.6) above the pK_a (4.5–4.6) of MPS. This chromatographic system resulted in baseline reso-

lution of MPS, MP, MPN, CS, and IS as demonstrated in Fig. 1, with MPS peak eluting first in the chromatogram.

During our search for an optimum mobile phase, we first used a mobile phase containing 25 mM potassium dihydrogen phosphate. Such a system resulted in complete resolution of all the peaks. However, the MPS peak eluted between the MPN and CST peaks, while the order of elution for other peaks was similar to that shown in Figs. 1 and 2. The problem was, however, that the MPS peak was so sensitive to a slight change in the pH of the mobile phase and/or the sample that at times it co eluted with MPN or CST peak.

Table 3
The relationship between analyte: internal standard peak area ratio and the added concentration [peak area ratio = intercept + (slope × conc.)] when stripped rat plasma or an albumin solution was used as a matrix[a]

Drug	Matrix	Intercept	Slope	r^2	Standard error
MPS	Plasma	−0.00574	1.05	0.997	0.0681
	Albumin	−0.0211	1.14	0.997	0.0867
MP	Plasma	−0.00953	1.26	0.999	0.0572
	Albumin	0.00160	1.24	0.995	0.0785
MPN	Plasma	−0.000892	1.25	1.00	0.0121
	Albumin	0.0150	1.20	0.991	0.0734
CST	Plasma	−0.00720	1.21	0.995	0.0572
	Albumin	0.00182	1.20	0.996	0.0513

[a] No significant difference between plasma and albumin solution ($P = 0.959$; ANOVA).

Table 4
Intra-run accuracy and precision of the assay ($n = 5$)[a]

Added conc. (μg ml^{-1})	Calculated conc. (μg ml^{-1}) (%error)				%CV			
	MP	MPS	MPN	CST	MP	MPS	MPN	CST
0.10	0.0944 (−5.6)[a]	0.107 (7.0)	0.107 (7.0)	0.0960 (−4.0)	5.3	16	9.9	13
0.25	–	–	0.260 (3.8)	0.234 (−6.6)	–	–	4.2	7.6
0.40	0.404 (0.88)	0.411 (2.8)	–	–	5.7	12	–	–
1.0	1.05 (5.0)	1.03 (3.0)	1.02 (1.6)	1.04 (3.9)	3.7	4.7	4.4	4.2
4.0	4.13 (3.3)	4.06 (1.6)	–	–	3.6	3.7	–	–

[a] Values in parentheses are % error of the assay.

Table 5
Inter-run accuracy and precision of the assay ($n = 5$)[a]

Added conc. (μg ml^{-1})	Calculated conc. (μg ml^{-1}) (%error)				%CV			
	MP	MPS	MPN	CST	MP	MPS	MPN	CST
0.10	0.103 (3.2)[a]	0.102 (2.4)	0.104 (3.6)	0.107 (7.0)	6.6	9.3	5.9	9.2
0.25	–	–	0.244 (−2.3)	0.231 (−7.6)	–	–	1.8	7.0
0.40	0.399 (−0.25)	0.397 (−0.85)	–	–	0.89	2.6	–	–
1.0	0.985 (−1.5)	0.976 (−2.4)	0.944 (−5.6)	0.962 (−3.8)	4.2	4.7	5.1	4.9
4.0	3.85 (−3.7)	3.79 (−5.2)	–	–	3.6	6.9	–	–

[a] Values in parentheses are % error of the assay.

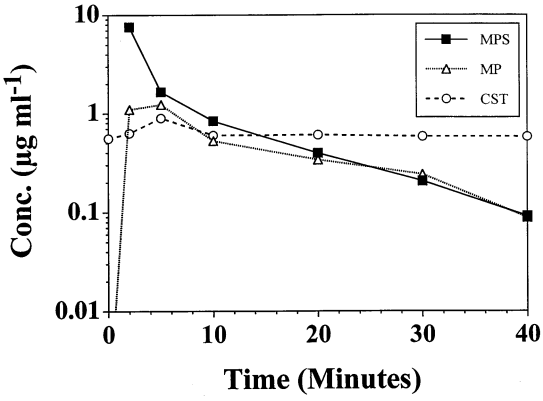

Fig. 3. Plasma concentration–time courses of methylprednisolone (MP; △), MP succinate (MPS; ■), and endogenous corticosterone (CST; ○) after iv administration of a single 5 mg kg^{-1} (MP equivalent) dose of MPS to a rat.

Therefore, we decided to use a stronger buffer at a pH sufficiently higher than the pK_a of MPS (100 mM acetate buffer, pH 5.7). Using this system, the ionized MPS eluted first among the other analytes (Figs. 1 and 2) and its retention time was very stable.

Haughey and Jusko [6] quantitated plasma concentrations of MP, MPN, and CST after the iv administration of 10 mg kg^{-1} MP to a rat. They reported that the plasma concentrations of MPN in the rat were ten fold lower than those of the parent drug, MP. In our study after the injection of 5 mg kg^{-1} (MP equivalent) MPS, we were not able to quantitate MPN. This was not unexpected because of the lower MP concentrations in our study (Fig. 3) and a higher limit of quantitation of our assay (0.1 μg ml^{-1}). Although not quantitated in the rat samples, MPN is well resolved from the other analytes in our assay (Fig. 1). Therefore, it does not interfere with the quantitation of MPS, MP, or CST. Nevertheless, if MPS measurements are not necessary (such as after the injection of MP), the assay developed by Haughey and Jusko [6] is more suitable for delineation of the pharmacokinetics of MP and its metabolite MPN in rats because it has a higher sensitivity.

In our studies, when glass test tubes without any coating were used, the recovery of steroids was not reproducible. However, when the tubes were coated with a silicone solution (Sigmacote, Sigma) or were acid washed before use, the results were more reproducible. Other investigators [5] have also reported the use of acid-washed glass tubes when dealing with MP, presumably for the same reason. However, the most convenient and reproducible results were obtained when 100 μl of glacial acetic acid was added to methylene chloride before its evaporation (see Section 2.3). Addition of acetic acid to methylene chloride before evaporation eliminated the need for coating the tubes or an acid-wash procedure.

In conclusion a reversed-phase HPLC method is reported here which can directly quantitate the plasma concentrations of MPS, MP, and CST in rat plasma. The assay is especially suitable after the administration of MPS or other prodrugs of MP when direct measurements of MP and MPS in the same chromatogram is necessary.

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