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Note

Simultaneous analysis of methylprednisolone hemisuccinate, cortisol and methylprednisolone by normal-phase high-performance liquid chromatography in human plasma

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Methylprednisolone (MP) has particular utility in high doses (pulse therapy) in cadaveric renal transplantation to prevent and treat rejection, for acute bronchospasm (status asthmaticus), in the emergency treatment of septic shock, and in cardiac bypass. Its low water solubility, however, prevents it from being directly injected intravenously. Esterification of MP with acids such as phosphoric acid and succinic acid results in water-soluble prodrugs that can be administered intravenously. Upon entry into the body, these prodrugs are supposed to hydrolyze rapidly by carboxylesterase enzymes to form the parent MP alcohol, which is the biologically active species [1]. Alterations in ester disposition due to drug interactions and/or disease states are of clinical concern. Therefore, the ability to measure simultaneously the ester prodrug and the parent alcohol to examine their disposition would be valuable.

Different methods have been reported for the simultaneous determination of alcohols and esters of corticosteroids [2–5]. However, no normal-phase high-performance liquid chromatographic (HPLC) assay to directly and simultaneously analyze methylprednisolone 21-hemisuccinate (MPHS), endogenous cortisol (C), and MP in human plasma have been reported. Most reversed-phase assays either lack the sensitivity or exhibit long retention times. One method [6] for the simultaneous determination of these compounds in human plasma required the concentration of MPHS to be measured indirectly as the difference between MP concentrations from hydrolyzed and unhydrolyzed samples.

This report describes a simple, sensitive and rapid method using normal-phase HPLC for the simultaneous determination of MPHS, C and MP in human plasma.

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EXPERIMENTAL

Apparatus

The HPLC system utilized in this procedure consisted of a Waters Model M45 solvent delivery system and a Waters 440 UV absorbance detector (Waters Assoc., Milford, MA, U.S.A.). Injections were made onto the system with a Model 7125 universal loop injector (Rheodyne, Berkeley, CA, U.S.A.). The UV absorbance of all steroids was measured at 254 nm. Three Zorbax SIL Reliance 5 (Dupont, Wilmington, DE, U.S.A.) cartridge columns (80 mm×4 mm cartridge, 5 μm particle size) and a guard column cartridge (5-μm Zorbax SIL, 12.5 mm×4 mm) and equipped with a 70 mm×6 mm stainless-steel Whatman pre-column were used to separate the compounds. The pre-column consisted of HC-Pellocil (37–53 μm particles) (Whatman, Clifton, NJ, U.S.A.). Peak-height measurements were done with a Model 3392A Hewlett-Packard integrator (Avondale, PA, U.S.A.).

Chemicals and reagents

The ethyl acetate and hexane used in the extraction procedure and methylene chloride used in the mobile phase were of HPLC grade and obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The sulfuric acid and nitric acid were of reagent grade and anhydrous sodium sulfate used in the extraction procedure were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). The pure ethyl alcohol (U.S.P.) employed in the mobile phase was obtained from AAPER Alcohol & Chemical (Shelbyville, KT, U.S.A.). The glacial acetic acid, used in the mobile phase, was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Pharmaceutical-grade decolorizing carbon, Norit "A", was purchased from Amend Drug and Chemical (Irving, NJ, U.S.A.). The MP, C, and prednisone were purchased from Sigma (St. Louis, MO, U.S.A.). MPHS was obtained from Steraloids (Wilton, NH, U.S.A.).

Standard preparation

Preparation of C-stripped plasma was described previously [6,7]. A 1-ml volume of this plasma was added to acid-washed glass extraction tubes with PTFE-lined screw caps (20 mm×150 mm). MPHS, C, and MP in acetonitrile–methanol (1:1, v/v) were added to provide standard concentrations of 50–1600 ng/ml. A 0.1-ml volume of 3 *M* sulfuric acid was added to acidify the plasma. The internal standard, prednisone [ca. 300 ng in 50 μl of acetonitrile–methanol (1:1)] was mixed with the sample. Then 15 ml of hexane–ethyl acetate (1:1) were added, and the tubes were shaken for 20 min. The tubes were centrifuged, the aqueous layer was frozen at −70°C and the upper organic layer was decanted into clean tubes. The organic phase was then washed with 1 ml of 1 *M* nitric acid. After freezing the aqueous phase and decanting the organic phase into clean tubes, 1 g of anhydrous sodium sulfate was added to dry the organic phase. The latter was evaporated to dryness at 30°C under a nitrogen gas stream.

Chromatography

The residue was reconstituted with approximately 100 μl of mobile phase for injection. The mobile phase consisted of a methylene chloride–hexane–ethanol–glacial acetic acid (69:26:2.3:1, v/v) mixture and was pumped through the column at a flow-rate of 2.0 ml/min.

Steroid recovery

The assay recovery of each steroid was assessed at 100, 500, and 1000 ng/ml in the following manner. Five plasma samples (1 ml) containing each steroid were extracted and injected onto the chromatograph. Five injections of the same amounts of steroid (100, 500, and 1000 ng) in mobile phase were directly injected. The peak heights of the steroids in both sets of samples were measured. The assay recovery of each steroid was calculated using the following equation:

$$\text{Recovery} = \frac{\text{peak height, extracted drug}}{\text{mean peak height, direct injection}} \times 100\%$$

Reproducibility

The within-day and between-day reproducibility of the assay was assessed for MPHS, C, and MP at 100 and 1000 ng/ml concentrations.

Human study

To illustrate the ability of this assay to simultaneously measure MPHS, C, and MP, the pharmacokinetics were studied in a 35-year-old normal male volunteer. The study dose consisted of 30 mg MP sodium succinate (Solu-Medrol, Upjohn, Kalamazoo, MI, U.S.A.) administered by bolus injection via an intravenous catheter at 9 a.m. Heparinized blood samples (10 ml) were obtained from another intravenous catheter (contralateral arm) prior to and at selected times following the intravenous corticosteroid dose. Blood samples were immediately centrifuged, 0.1 ml of 3 *M* sulfuric acid were added to each milliliter of plasma to stabilize and increase extraction efficiency of the prodrug [3], and the samples were stored at $-70\,^{\circ}$C and analyzed within a one-week period.

RESULTS

Fig. 1a represents a chromatogram resulting from the HPLC analysis of a blank natural human plasma obtained at 8 a.m. before administration of 40 mg of MPHS in a normal subject with a C concentration of 139 ng/ml. Fig. 1b illustrates the response to steroid concentrations of approximately 500 ng/ml MPHS, C, and MP and 300 ng/ml internal standard (I.S.), prednisone, in charcoal-stripped human plasma from which endogenous steroids were removed. Each steroid eluted with distinct separation at baseline. A chromatogram resulting from the HPLC analysis of plasma obtained 0.5 h after the intravenous injection of 40 mg of MPHS to a human subject is shown in Fig. 1c. This chromatogram represents concentrations of 235 ng/ml for MPHS, 117 ng/ml for C, and 289 ng/ml for MP. Each steroid of interest is clearly defined.

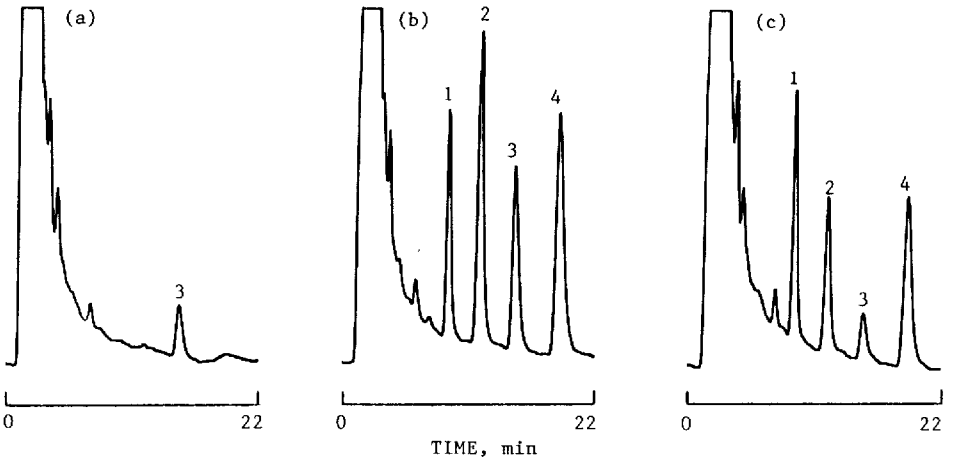

Fig. 1. Chromatograms of (a) blank plasma extract, (b) cortisol-stripped plasma spiked with 500 ng of methylprednisolone hemisuccinate, cortisol, and methylprednisolone and 300 ng of internal standard, prednisone, and (c) plasma extract obtained 0.5 h after a single 40-mg intravenous dose of methylprednisolone hemisuccinate. Peaks: 1 = internal standard, prednisone; 2 = methylprednisolone hemisuccinate; 3 = cortisol; 4 = methylprednisolone.

TABLE I

STEROID EXTRACTION RECOVERIES AT THREE CONCENTRATIONS

Steroid	Recovery (mean ± S.D.) (%)			
	100 ng/ml	500 ng/ml	1000 ng/ml	Mean
Methylprednisolone hemisuccinate	69.0 ± 3.5	67.1 ± 3.3	66.4 ± 3.5	67.5
Cortisol	53.7 ± 2.8	52.1 ± 4.2	53.6 ± 3.3	53.1
Methylprednisolone	63.4 ± 1.1	60.8 ± 4.3	60.4 ± 4.8	61.5

The extraction recoveries at three steroid concentrations are presented in Table I. The mean assay recovery ranged from 53.1% for C to 67.5% for MPHS and was independent of concentration. The assay sensitivity of this method was determined to be 10, 10, and 15 ng/ml for MPHS, MP and C, respectively, when using sample volumes of 1 ml of charcoal-stripped human plasma. Calibration curves of peak-height ratio (peak height of MPHS, C, or MP over peak height of internal standard) versus steroid concentration were linear over the range 50–1600 ng/ml with the greatest detector response observed for MPHS judging from the slopes of the calibration plot.

The within-day and between-day variability of the assay for MPHS, C, and MP are presented for high and low steroid concentrations in Table II. The coefficients of variation were less than 7% for each steroid examined.

Acidification of plasma with sulfuric acid had little effect on the extractability of MP, C, and I.S., but significantly improved extraction efficiency of MPHS.

TABLE II

INTRA-DAY AND INTER-DAY COEFFICIENTS OF VARIATION

All variability statistics are based on six measurements.

Steroid	Coefficient of variation (%)			
	Within		Between	
	100 ng/ml	1000 ng/ml	100 ng/ml	1000 ng/ml
Methylprednisolone hemisuccinate	5.4	3.2	3.7	4.4
Cortisol	5.2	3.5	3.4	1.9
Methylprednisolone	5.9	2.8	6.5	2.3

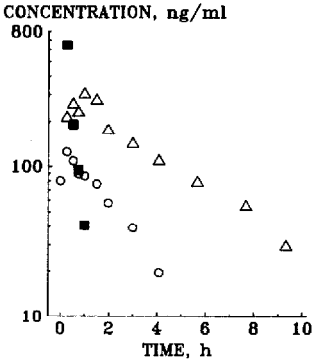

Fig. 2. Plasma methylprednisolone hemisuccinate (■), methylprednisolone (△), and endogenous cortisol (○) concentrations after a single intravenous dose of 30 mg of methylprednisolone hemisuccinate given to a 35-year-old normal male volunteer at 9 a.m.

Washing the extract with 1 *M* nitric acid provides a method of circumventing interference if the problem arises and does not affect the stability of MPHS and the steroids examined. Less than 1% hydrolysis of MPHS to MP was observed when samples were acidified with sulfuric acid and stored at −70°C for one week. A 3% hydrolysis was observed without acid under the same condition.

Fig. 2 depicts the pharmacokinetic profile of the steroids after an intravenous bolus dose of 30 mg MPHS given to a 35-year-old male subject at 9 a.m. The prodrug MPHS disappears from the plasma very rapidly with a terminal half-life ($t_{1/2}$) of about 12 min. The free alcohol (MP) is formed rapidly from MPHS reaching maximal concentrations at about 1 h and then declines with a terminal $t_{1/2}$ of about 2.7 h. The endogenous C disappears in a monoexponential fashion with a $t_{1/2}$ of around 1.9 h and the normal circadian rhythm of C is absent [6–8].

DISCUSSION

This HPLC technique provides a sensitive, selective, efficient, and reproducible means of determining concentrations of the ester prodrug, MPHS, and its

parent compound, MP, as well as the endogenous C in human plasma. The procedure facilitates the characterization of MPHS and its pharmacologically active parent compound, MP. Further, it allows examination of the effect these steroids have on circulating C concentrations. The data in Fig. 2 were obtained in a healthy male volunteer who received a 30-mg Solu-Medrol intravenous bolus. These and other pharmacokinetic data confirm the expected disposition characteristics of these corticosteroids and the effect on C secretion.

This assay was developed for pharmacokinetic studies of MPHS in normal volunteers and has subsequently been used in repeated studies in six subjects. As with any other assays, cautions must be exercised for possible interference in future applications to different disease states or in the presence of xenobiotics administered concomitantly. Corticosteroids are used for a wide array of chronic diseases with the co-administration of numerous other drugs. It is thus impossible to test all possible interferences, but future analytical applications should be viewed on a case by case basis. We are applying this assay to disposition studies of MPHS and MP in cardiac bypass patients and liver disease patients who are on spironolactone and have found no interferences.

Previous analytical techniques apparently lack the desired assay sensitivity and do not have the ability to simultaneously measure MPHS, MP, and the endogenous C [2,3]. Lawson [3] reported a rapid method for the measurement of MP and MPHS in human plasma, but only after very high doses of MPHS, i.e. 1–2 g, and did not show any results for the low dose range as reported here. In addition, Lawson [3] employed the sodium salt of MPHS dissolved in water as stock standards. We found that when mixed and extracted immediately, significant amounts of MP were present in both Solu-Medrol and A-Methapred (Abbott, North Chicago, IL, U.S.A.) consistent with the results reported by other investigators [5]. To circumvent this hydrolysis problem, we dissolved pure MPHS in organic solvent as described in the Experimental section, which produces stability of MPHS for at least six months upon storage at $-20\,^{\circ}C$. Shah and Weber [4] described a sensitive reversed-phase assay for MP and MPHS in dog plasma, but did not apply the technique to human samples. Rohdewald et al. [5] reported a reversed-phase assay for MP, MPHS, and C in human plasma, but their detection limits for MP and MPHS were 100 and 200 ng/ml, respectively. Thus our assay methodology provides a significantly improved and useful approach to evaluate simultaneously the disposition of the ester prodrug, MPHS, the parent drug, MP, and endogenous C in humans.

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