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High-performance size-exclusion chromatographic analysis of dextran–methylprednisolone hemisuccinate in rat plasma

R. Mehvar*

School of Pharmacy, Texas Tech University Health Sciences Center, 1300 Coulter, Amarillo, TX 79106, USA

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Abstract

A size exclusion chromatographic method is presented for the measurement of the concentrations of a macromolecular prodrug of methylprednisolone (MP), dextran–methylprednisolone succinate (DEX–MPS), in rat plasma. After precipitation of the plasma (100 μl) proteins with perchloric acid, the samples are injected into a size exclusion column with a mobile phase of water:acetonitrile:glacial acetic acid (75:25:0.2) and a flow-rate of 1 ml/min. The DEX–MPS conjugate, detected at 250 nm, elutes at a retention time of ~6.5 min, free of endogenous peaks. Excellent linear relationships (r^2=0.997) were found between the detector response and the concentrations of DEX–MPS in the range of 2–100 μg/ml (MP equivalent), with intra- and inter-run C.V.s of <6% and error values of <5%. The application of the assay was also demonstrated by measurement of the plasma concentrations of DEX–MPS after single 5 or 10 mg/kg doses of the conjugate administered intravenously to rats. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Dextran; Methylprednisolone

1. Introduction

Dextran polymers have been investigated as macromolecular carriers for delivery of drugs and proteins after the systemic administration of the dextran–drug conjugates [1,2]. Additionally, conjugates of dextrans with non-steroidal [3,4] and steroidal [5,6] antiinflammatory drugs have been studied for local delivery of these drugs to the colon. Our studies [7,8] with dextrans of different molecular weights (MWs) have indicated that the tissue distribution of dextran carriers is dependent on the MW of the polymer. Therefore, dextrans of different MWs may be useful for delivery of drugs to different tissues after the systemic administration of the conjugate [7].

Recently [9], a conjugate of the polymer with methylprednisolone (MP), dextran–methylprednisolone succinate (DEX–MPS), was synthesized and characterized. The conjugate is intended for systemic delivery of MP. Therefore, it is necessary to quantitate the concentrations of the conjugate in biological samples. In this article, an analytical size-

*Tel.: +1-806-356-4000, ext. 337; fax: +1-806-356-4034.
E-mail address: rmehvar@cortex.ama.ttuhsc.edu (R. Mehvar)

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exclusion chromatographic method is presented which can quantitate the concentrations of DEX–MPS in rat plasma.

2. Experimental

2.1. Chemicals

Dextran with an average MW of 73 000, 6α-methylprednisolone (MP), 1,1′-carbonyldiimidazole (CDI), triethylamine, and glacial acetic acid were obtained from Sigma Chemical (St. Louis, MO, USA). 6α-methylprednisolone 21-hemisuccinate was purchased from Steraloids (Wilton, NH, USA). For chromatography, HPLC grade acetonitrile (Mallinckrodt Chromar HPLC) was obtained from VWR Scientific (Minneapolis, MN, USA). All other reagents were analytical grade and obtained through commercial sources.

2.2. Synthesis of DEX–MPS

Dextran–methylprednisolone succinate (DEX–MPS) was synthesized from dextran 70 000 and methylprednisolone hemisuccinate as described before [9]. The degree of substitution of MP and the purity of the prodrug were also examined as explained before [9].

2.3. Calibration standards

Because of the ease of availability and a lower cost, preparation of calibration standards in human plasma, instead of rat plasma, was considered. In a preliminary study, calibration standards containing 2–100 μg/ml DEX–MPS (MP equivalent) were prepared in both human and rat plasma. Samples were then subjected to the HPSEC assay described here. The calibration curves using human and rat plasma were almost identical (a difference of 2.2% in the slope). Therefore, validation experiments were conducted using the human plasma.

For preparation of calibration curves, a stock solution of DEX–MPS (1 mg/ml, MP equivalent) in distilled water was prepared daily. Blank human plasma was then spiked with the stock solution to produce calibration standards containing 0, 2, 4, 10, 20, 40, and 100 μg/ml MP in the form of DEX–MPS.

2.4. Sample preparation

To 100 μl of plasma in siliconized microcentrifuge tubes were added 50 μl of 0.4 *M* phosphate buffer (pH 7.0) and 50 μl of methanol. Samples were then briefly vortex-mixed and 20 μl of a 20% (v/v) perchloric acid (70%) was added to precipitate proteins. After vortex mixing for 5 s, the samples were centrifuged in a Marathon 16 KM microcentrifuge (Fisher Scientific; Chicago, IL, USA) at 10 000 rpm for 3 min. The resultant supernatants were then transferred to autosampler inserts, and a 40 μl aliquot was injected into the HPLC.

2.5. Chromatography

Dextran–methylprednisolone succinate was separated from the endogenous material and quantitated using a 30 cm×7.8 mm analytical, gel chromatography column (PolySep-GFC; Phenomenex, Torrance, CA, USA). The mobile phase consisted of water:acetonitrile:glacial acetic acid (75:25:0.2) and was pumped at a flow-rate of 1.0 ml/min. The prodrug was detected at a UV wavelength of 250 nm.

The HPLC instrument (Waters; Milford, MA, USA) consisted of a 501 pump, a 717 autosampler, and a 486 UV detector. The chromatographic data was managed using the Millennium software (Waters).

2.6. Recovery

To determine the recovery of DEX–MPS from plasma after protein precipitation, plasma samples ($n=5$) containing 100 μg/ml DEX–MPS were subjected to the above assay and the peak areas were determined. The peak areas of these samples were then compared with those containing an equal concentration of DEX–MPS in distilled water, injected directly into the HPLC.

2.7. Stability of samples during storage and injection

To determine the stability of DEX–MPS in plasma samples during storage, blank plasma samples were spiked with DEX–MPS to produce a concentration of 10 μg/ml (MP equivalent). The samples were then stored at −20°C or −80°C. Samples (n=3) were analyzed before storage and at 1, 3, 6, and 11 weeks after storage. The peak areas of DEX–MPS in the stored samples were compared with those of the fresh samples.

To determine the stability of DEX–MPS in the precipitating media, a set of calibration standards, containing all standards, was prepared and analyzed as described above. Additionally, the samples were left in the autosampler (room temperature) for 24 h and reinjected. The peak areas of the samples after the initial (time zero) and second (after 24 h) injections were compared to determine the stability of the samples while waiting for analysis.

2.8. Assay validation

The validity of the assay was investigated by determination of the accuracy and precision of the assay based on the reported guidelines [10]. The intra-run validity was determined by analyzing five replicates of quality control samples at each concentration of 2 (low), 20 (intermediate), and 100 (high) μg/ml against a calibration standard in the range of 2 to 100 μg/ml. For inter-run validity, one quality control sample at each concentration of 2, 20, or 100 μg/ml was analyzed against a calibration standard at 5 different occasions. The accuracy and precision values were then calculated by percent errors and C.V.s, respectively.

2.9. Application

To determine the application of the assay to measurement of DEX–MPS in plasma in a destructive sampling design, single i.v. bolus doses (5 mg/kg; MP equivalent) of DEX–MPS were injected into the tail vein of adult male Sprague–Dawley rats (n=3). At 10 min and at 2 and 24 h after the injection, animals were euthanized using CO_2. Blood was obtained by cardiac puncture into heparinized syringes, and plasma was stored for analysis. Plasma collected from a vehicle-injected rat served as blank. The destructive sampling method is necessary for future pharmacodynamic studies, using spleens for a lymphocyte proliferation assay. Additionally, a single i.v. bolus dose (10 mg/kg; MP equivalent) of DEX–MPS was injected to a rat via a jugular vein catheter, and serial blood samples (0.25 ml) were obtained at 0 (before the drug injection) and 5 min and at 1, 2, 3, 5, and 8 h after the drug injection. After centrifugation, plasma was used for analysis. The serial sampling method is needed for characterization of the pharmacokinetics of DEX–MPS in the same rat.

3. Results

Chromatograms of a blank plasma and the lowest standard in the calibration curve (2 μg/ml) are depicted in Fig. 1. Additionally, chromatograms of plasma samples taken from rats before (blank) and at 10 min and 2 h after the administration of a single 5 mg/kg dose of DEX–MPS are demonstrated in Fig. 2. Dextran–methylprednisolone succinate eluted at ~6.5 min and was well separated from the endogen-

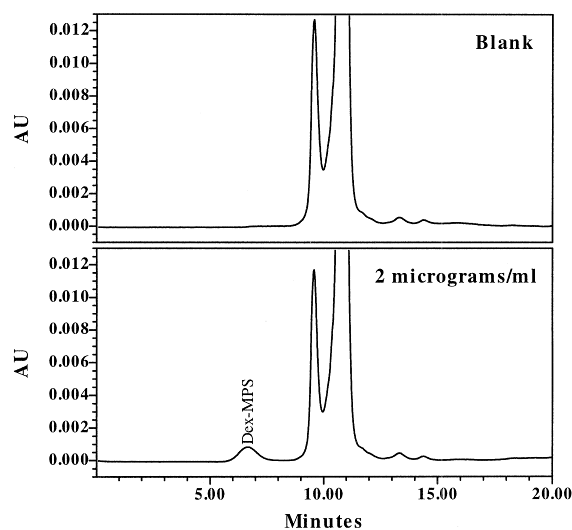

Fig. 1. Chromatograms of a blank plasma (top) and a plasma standard (bottom) spiked with 2 μg/ml of DEX–MPS (MP equivalent).

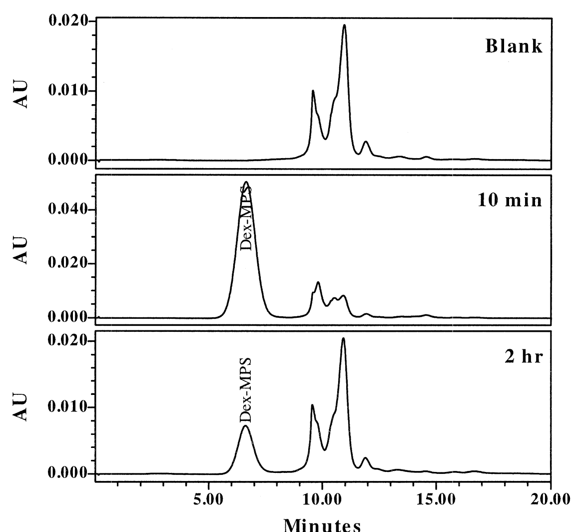

Fig. 2. Chromatograms of plasma samples taken from rats before (top) and at 10 min (middle) and 2 h (bottom) after the i.v. injection of a single 5 mg/kg dose (MP equivalent) of DEX–MPS.

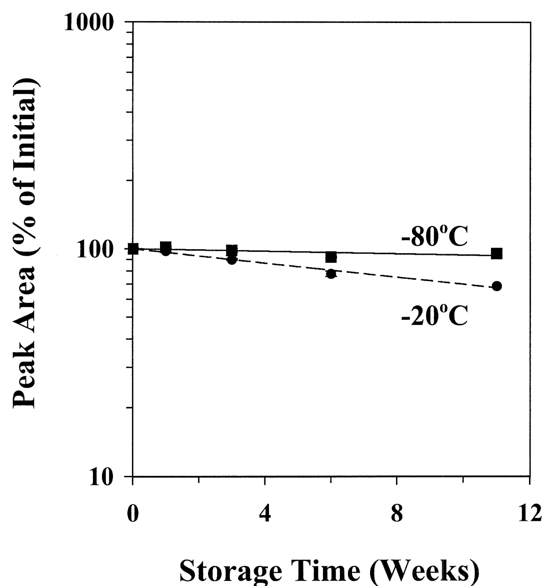

Fig. 3. The effect of storage of plasma samples, at −20°C or −80°C, on the peak area of DEX–MPS as a function of time. Standard deviation bars are within the size of the symbols.

ous peaks in plasma (Figs. 1 and 2); all the endogenous peaks eluted after the DEX–MPS peak.

Table 1 lists the response of the detector to various concentrations of DEX–MPS in the sample for 5 different calibration curves. The relationship between the peak area of DEX–MPS and the detector was linear ($r^2 \geq 0.997$, Table 1) over the studied range of 2 to 100 μg/ml. Additionally, the intercept values (Table 1) were less than 10% of the peak area of the lowest concentration (2 μg/ml) used in the calibration curve.

Table 1
The relationship between the DEX–MPS peak area and the added concentration for inter-run data [Peak Area=Intercept+(Slope× Conc.)]

Calibration #	Intercept	Slope	r^2	Standard errors	
				Intercept	Slope
1	5999	27235	0.998	525	11.7
2	5709	27415	0.999	508	11.3
3	5915	27522	0.997	738	16.4
4	5388	28324	0.999	404	8.99
5	5062	27975	0.999	509	11.3

The results of the assay validation for both intra- and inter-run experiments are presented in Table 2. Excellent accuracy of the assay is demonstrated by error values of<5% for all the concentrations (even at the lowest concentration of 2 μg/ml). The assay is also deemed precise because the C.V. values are <6% for both intra- and inter-run data (Table 2). Based on the data presented in Table 2, the limit of quantitation of the assay is 2 μg/ml.

Fig. 3 depicts the peak areas of DEX–MPS in

Table 2
Intra- and inter-run accuracy and precision of the assay (n=5)

Added Conc. (μg/ml)	Calculated Conc. (μg/ml)		% Error		% C.V.	
	Intra-run	Inter-run	Intra-run	Inter-run	Intra-run	Inter-run
2.0	1.98	1.91	−0.98	−4.49	1.46	5.46
20	19.4	19.2	−2.9	−4.11	1.73	3.67
100	101	100	1.0	0.38	2.13	3.10

plasma samples stored at −20°C or −80°C for 11 weeks. The first-order degradation half life values of DEX–MPS were 19.3 and 112 weeks at −20°C or −80°C, respectively. The peak area of DEX–MPS in samples stored for 11 weeks at −80°C was more than 90% of the fresh sample. However, after 11 weeks of storage at −20°C, the peak areas were around 70% of the original values.

Fig. 4 illustrates the peak areas of DEX–MPS for standards analyzed immediately after the sample preparation and again after a 24 h wait in the autosampler (room temperature). A 24 h delay between the preparation of samples and their injection into the HPLC did not have a substantial effect on the peak areas at all the tested concentrations (Fig. 4). Additionally quality control samples analyzed with the calibration standards resulted in virtually identical values at both times (data not shown).

The concentrations (MP equivalent) of DEX–MPS in the plasma of rats injected with a single 5 mg/kg dose of DEX–MPS were 133 and 16.1 μg/ml at 10 min and 2 h after the prodrug injection, respectively. However, at 24 h after the injection, the DEX–MPS concentration was below the limit of quantitation.

The plasma concentration-time course of DEX–MPS after a single i.v. dose of 10 mg/kg is depicted in Fig. 5. The prodrug concentrations at all the sampling times were above the lower limit of quantitation of the assay (Fig. 5). Additionally, the

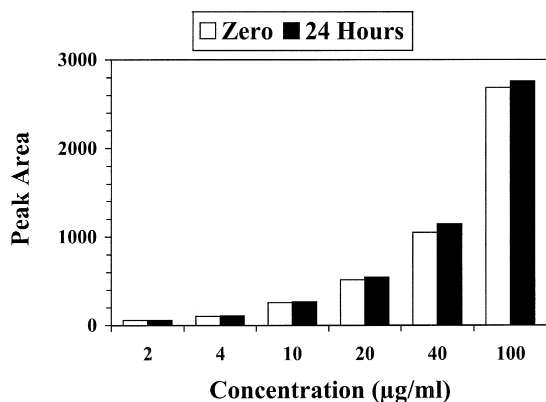

Fig. 4. A comparison of peak areas of DEX–MPS peak for the same samples injected immediately (Zero) and also after a 24 h wait (24 h) in the autosampler (room temperature).

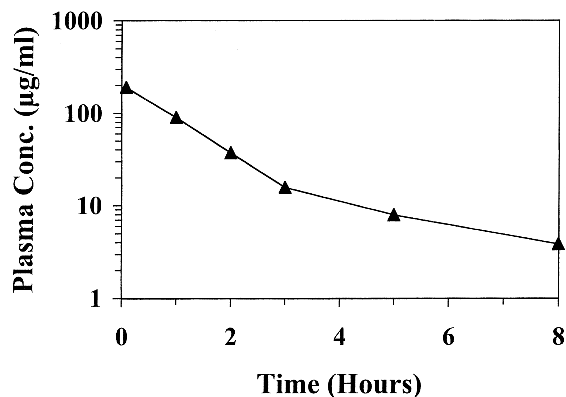

Fig. 5. Plasma concentration-time course of DEX–MPS in plasma of a rat injected with a single 10 mg/kg dose (MP equivalent) of the prodrug.

plasma concentrations of the prodrug demonstrated a multiexponential decline with a more rapid decline during the first 3 h after the injection (Fig. 5). The estimated clearance and terminal half life of DEX–MPS in the rat were 36 ml/h/kg and 2.5 h, respectively.

The recovery of DEX–MPS from plasma after protein precipitation was 83.4±0.49 (mean±SD).

4. Discussion

Dextrans are macromolecules with a high degree of water solubility [1]. Therefore, liquid-liquid extraction of the dextran prodrugs from aqueous biological samples, such as plasma, is not feasible. Instead, direct methods using precipitation of sample proteins should be considered. Previous studies with dextrans have used 20% (w/v) trichloroacetic acid for the precipitation of plasma proteins in determination of fluorescein dextrans [11,12] and a dextran–naproxen ester prodrug [13]. However, the use of trichloroacetic acid in the present study resulted in significant loss of DEX–MPS during the protein precipitation procedure. After testing various protein precipitants (e.g., methanol, acetonitrile, zinc sulfate, and perchloric acid), it was noticed that a combination of methanol and perchloric acid would result in the highest recovery (83%) of DEX–MPS from plasma.

One of the issues to be considered during method

development for ester prodrugs is the stability of the prodrug in the precipitating media. For instance, Larsen [13] indicated that a dextran–naproxen ester conjugate degraded in the precipitating media (trichloroacetic acid) with a first order half life of 5.8 h. Therefore, it was concluded [13] that in order to obtain reproducible results, the sample has to be injected within 5 min of protein precipitation.

In our studies, buffer was added to plasma samples, before the addition of perchloric acid, to increase the stability of DEX–MPS. Initial studies, using perchloric acid in the absence of any buffer added to plasma, showed a degradation half life of ~17 h for DEX–MPS after protein precipitation. However, when 50 μl of a 0.4 *M* phosphate buffer (pH 7.0) was added to 100 μl plasma before the addition of methanol and perchloric acid, DEX–MPS was stable in the precipitation media for at least 24 h (Fig. 4). A reduction in the volume or the strength of perchloric acid, instead of the addition of buffer, was not satisfactory because it resulted in cloudiness of the samples left in the autosampler. Nonetheless, considering the run time for each sample (20 min), the method reported here allows simultaneous preparation and automatic injection of at least 70 samples.

Our laboratory recently reported [9] a size exclusion chromatographic method capable of simultaneous quantitation of DEX–MPS, MP, and MPS in non-biological samples. The assay was used for the determination of degree of substitution, stability, and purity of DEX–MPS [9]. This study [9] indicated that the retention time of MPS is extremely sensitive to the pH of the mobile phase; at pH 3.4 or lower, the MP and MPS peaks co-eluted. However, as pH increased from 3.4 to 7, the retention time of MPS progressively decreased, resulting in a baseline resolution at pH 5 or above. In application of this method to determination of DEX–MPS in plasma, it was noticed that endogenous material in plasma would interfere with the DEX–MPS peak at pH values necessary for the resolution of MP and MPS. Therefore, a mobile phase containing acetic acid was used in this study which resulted in excellent separation of endogenous peaks from the DEX–MPS peak (Figs. 1 and 2). However, the MP and MPS peaks elute together as a single peak at a retention time of 14.5 min using this mobile phase (data not shown). Nevertheless, the sensitivity of the current assay is not appropriate for simultaneous determination of MPS and/or MP which are expected to have low blood concentrations.

In conclusion, a size exclusion chromatographic method is reported for determination of the plasma concentrations of a dextran–methylprednisolone conjugate. The assay is precise and accurate in the concentration range of 2–100 μg/ml (MP equivalent). Additionally, the assay can be applied to the measurement of the plasma concentrations of the conjugate after the administration of i.v. doses of 5 or 10 mg/kg of the prodrug to rats.

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