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Simultaneous analysis of dextran-methylprednisolone succinate, methylprednisolone succinate, and methylprednisolone by size-exclusion chromatography

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Abstract

An analytical HPLC method is reported for simultaneous measurement of low (1.0–100 μg ml^{-1}) concentrations of dextran-methylprednisolone succinate (DEX-MPS) and its degradation products methylprednisolone hemisuccinate (MPS) and methylprednisolone (MP). The analytes were detected at 250 nm after resolution using a size exclusion column with a mobile phase of KH_2PO_4 (10 mM): acetonitrile (3:1) and a flow rate of 1 ml min^{-1}. The resolution of MP and MPS peaks was substantially affected by the pH of the mobile phase; while MP and MPS co-eluted at pH 3.4, they were baseline-resolved at $pH \geq 5$. Linear relationships ($r \geq 0.997$) were found between the detector response and the concentrations of the analytes (1.0–100 μg ml^{-1} for MP and MPS and 2.5–100 μg ml^{-1} for DEX-MPS). Intra- and inter-run error ($< 13\%$) and precision (CV of $\leq 6\%$) data indicated that the assay could accurately and precisely quantitate all three components in the examined concentration range. The application of the assay to determination of degree of substitution, purity, and stability of DEX-MPS was also demonstrated.

Keywords: Dextran-Methylprednisolone; Methylprednisolone succinate; Methylprednisolone; High-performance size-exclusion chromatography; Validation; Stability

1. Introduction

Dextran polymers have been suggested as macromolecular carriers for delivery of drugs and therapeutic agents [1,2]. Recently [3,4], dextrans have been investigated for delivery of steroids to the colon. Additionally, our preliminary studies [5] indicate that dextran-methylprednisolone succinate (DEX-MPS) conjugates may be of some value for sustained delivery of methylprednisolone (MP) to specific organs such as the liver.

The conjugation of MP to dextran is achieved through a succinate linker, resulting in a double ester in the conjugate [3]. It has been proposed [3] that the hydrolysis of DEX-MPS would lead to

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formation of MP and MPS, the latter being further hydrolyzed to MP. Therefore, for stability and purity investigations, it is desirable to quantitate all three possible components of DEX-MPS in the sample. Reversed-phase chromatography [6,7] has been used to quantitate MP and MPS simultaneously. However, the same methods cannot be used to separate and quantitate the conjugate as well. In this article, a size-exclusion chromatographic (SEC) method is described which can simultaneously quantitate DEX-MPS, MPS, and MP. Additionally, validation of the method for low concentrations of the components and application of the method to stability and purity analysis of DEX-MPS are reported.

2. Experimental

2.1. Chemicals

Dextran with an average molecular weight of 73 000, 6α-methylprednisolone (MP), 1,1′-carbonyldiimidazole (CDI), triethylamine, and glacial acetic acid were obtained from Sigma (St. Louis, MO). 6α-methylprednisolone 21-hemisuccinate (MPS) was 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. Synthesis of DEX-MPS

DEX-MPS was synthesized (Fig. 1) based on a published method [3] with minor modifications. Briefly, 0.3 g of MPS was dissolved in 3 ml dimethylsulfoxide (DMSO) and 360 mg CDI was added and allowed to react with MPS at room temperature for 30 min. Next, 40 ml of a 5% (w/v) solution of dextran in DMSO and 3.5 ml of triethylamine were added, and the mixture was left at room temperature for 24 h. The DEX-MPS conjugate was then purified [3] and stored at −20°C as a powder.

The purity of the powder was determined using the size exclusion chromatographic (SEC) method. The degree of substitution of methylprednisolone on DEX-MPS was determined by hydrolysis of DEX-MPS under basic conditions: To 5 mg of the conjugate were added 5 ml of 0.1 M NaOH and 3 ml of methanol. After leaving at room temperature for 5 min, 100 μl of the sample was micropipeted into a microcentrifuge tube containing 100 μl of 0.1 M HCl. An aliquot (40 μl)

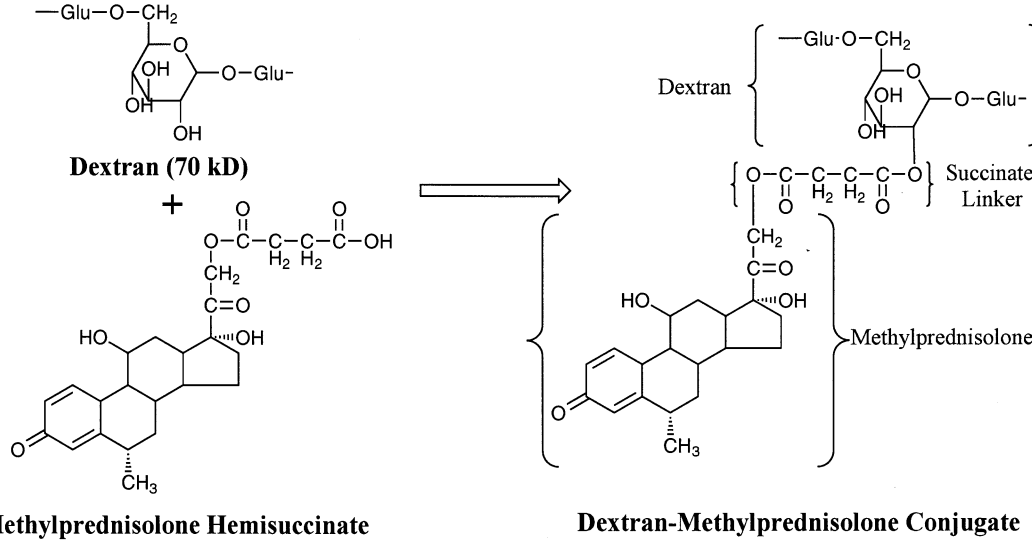

Fig. 1. Synthesis and structure of DEX-MPS.

was then injected into the HPLC. The concentration of the released MP was determined based on MP standard solutions.

2.3. Chromatography

Dextran-methylprednisolone succinate and its degradation and/or impurity products were analyzed at ambient temperature utilizing a 30 cm × 7.8 mm analytical, gel chromatography column (PolySep-GFC; Phenomenex, Torrance, CA). The mobile phase consisted of KH_2PO_4 (10 mM): acetonitrile (3:1) and was pumped at a flow rate of 1.0 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).

In addition to the above gel filtration chromatography, a reversed-phase chromatographic method was also used to validate the degree of substitution of DEX-MPS obtained using the SEC method. The separation was achieved on a 10 cm × 4.6 mm column (Partisil 5 ODS3; Whatman, Clifton, NJ) preceded by a 5-cm guard column, using the above HPLC system. The mobile phase consisted of water: acetonitrile: acetic acid (65:35:0.1) and was pumped at a flow rate of 1 ml min^{-1}.

2.4. Standard solutions

Stock solutions of MP (0.2 mg ml^{-1}) were prepared in methanol:water (1:24) and kept at 4°C, whereas the stock solution of MPS (1 mg ml^{-1}; MP equivalent) was prepared in methanol and stored at −20°C. The standards of MP and MPS (0, 1.0, 2.5, 5.0, 10, 25, 50, and 100 μg ml^{-1}) were prepared daily by diluting the stock solutions with the mobile phase. For DEX-MPS, standard solutions (0, 2.5, 5.0, 10, 25, 50, and 100 μg ml^{-1}) were prepared daily by dissolving the powder in the mobile phase. Calibration curves were constructed by plotting the peak areas against the concentration in the sample using a weight of 1/concentration^2.

2.5. Effects of mobile phase pH and acetonitrile content on resolution

The effect of mobile phase pH on the resolution of MP, MPS, and DEX-MPS was investigated at pH values of 3.4, 3.7, 4.0, 4.2, 5.0, 7.0, and 8.4 using 10 mM phosphate or acetate buffer or their components while keeping the aqueous: acetonitrile ratio (3:1) constant. Additionally, the effect of acetonitrile content was investigated at acetonitrile concentrations of 0, 10, 15, 20, 25, 30, 35, and 40% in the mobile phase with a 10 mM KH_2PO_4 as the aqueous part. After establishing equilibrium with each mobile phase, the same sample containing approximately 66 μg ml^{-1} of MP, MPS, and DEX-MPS was injected and the chromatograms were recorded.

2.6. 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 [8]. Briefly, quality control samples were run along with calibration standards at the lowest concentration (1.0 μg ml^{-1} for MP and MPS and 2.5 μg ml^{-1} for DEX-MPS), middle of the curve (25 μg ml^{-1}), and highest concentration (100 μg ml^{-1}) in the calibration curve. The concentrations of the quality control samples were then determined against the calibration curve and used for calculation of %CV and %error. To avoid overestimation of detector response to MP and MPS because of possible hydrolysis of DEX-MPS and/or MPS during sample preparation and analysis, separate calibration curves and quality control samples were run for MP, MPS, and DEX-MPS.

2.7. Application to stability studies

Dextran-methylprednisolone succinate was dissolved in 10 mM KH_2PO_4 (pH 5.0) or Na_2HPO_4 (pH 8.4) at a concentration equivalent to 100 μg ml^{-1} MP. The solutions were then left in glass vials at room temperature on a laboratory bench without any light control. Samples (100 μl) were taken at 0, 3, 6, 10, 24, 48, 72, and 96 h and

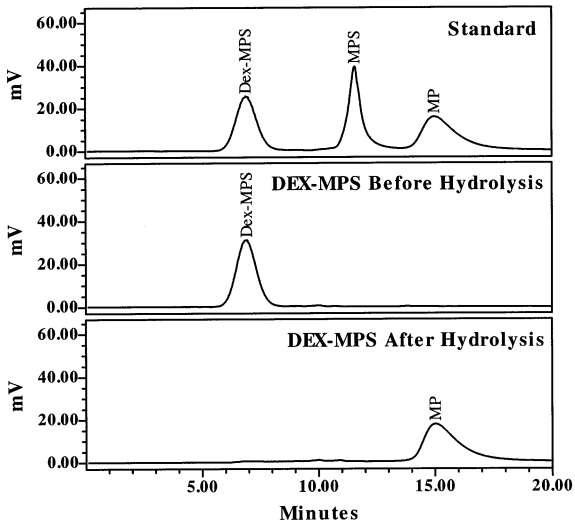

Fig. 2. Chromatograms of a standard solution containing DEX-MPS, MPS, and MP (top) and DEX-MPS before (middle) and after (bottom) base hydrolysis using the SEC method with a mobile phase of KH_2PO_4 (10 mM): acetonitrile (3:1).

added to 100 μl of mobile phase before the injection into the HPLC for MP, MPS, and DEX-MPS determination.

3. Results

The chromatogram of a sample containing DEX-MPS, MPS, and MP, analyzed with a mobile phase of KH_2PO_4 (10 mM): acetonitrile (3:1) is shown in Fig. 2. Also depicted in Fig. 2 are chromatograms of DEX-MPS samples before and after base hydrolysis for determination of degree of MP substitution. Under these conditions, DEX-MPS, MPS, and MP were baseline resolved with retention times of $\sim$7.0, 12, and 15 min, respectively.

As illustrated in Fig. 2, the DEX-MPS sample before hydrolysis was relatively free of MP and MPS impurities; the total percentage of free MP and MPS in the purified samples was $< 5\%$ of the MP content of the powder.

Based on the released MP, the degree of MP substitution of the synthesized polymer was $\sim$ 8.5%. Additionally, the area of the released MP peak was the same as the area of the DEX-MPS peak before hydrolysis (Fig. 2), indicating that the degree of substitution may be determined directly from the area of DEX-MPS peak without hydrolysis. The substitution degree obtained by the SEC method ($\sim$ 8.5%) was comparable with that determined using a reversed phase assay in which MP and MPS eluted at 5.0 and 8.5 min, respectively (Fig. 3).

The retention times of DEX-MPS and MP remained relatively constant when the pH of the mobile phase was changed from 3.4 to 8.4 (Fig. 4). However, the retention time of MPS exhibited substantial dependence on the pH of the mobile phase (Fig. 4). At a pH of 3.4, MPS co-eluted with MP as a single peak with a resolution of zero. However, as the pH increased from 3.4 to 7, the retention time of MPS progressively decreased, resulting in a baseline resolution of MPS and MP at pH values of 5 or above. A further increase in pH from 7 to 8, on the other hand, resulted in an increase in the MPS retention time and, subsequently, a decrease in the resolution of MPS and MP. The resolution of DEX-MPS and MPS decreased as the pH increased from 3.4 to 8.4 (Fig. 4).

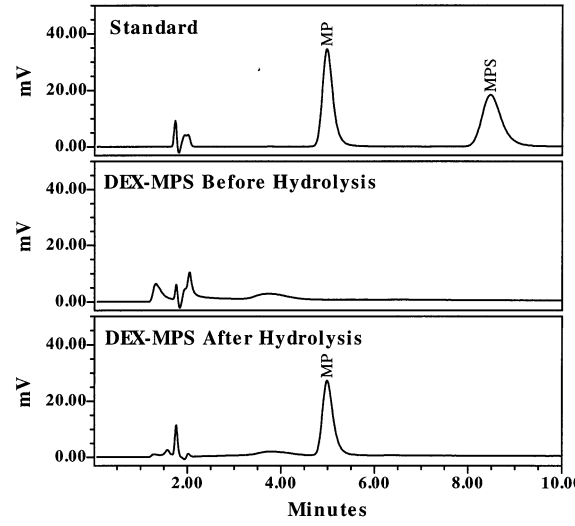

Fig. 3. Chromatograms of a standard solution containing MP and MPS (top) and DEX-MPS before (middle) and after (bottom) base hydrolysis using the reversed-phase HPLC method.

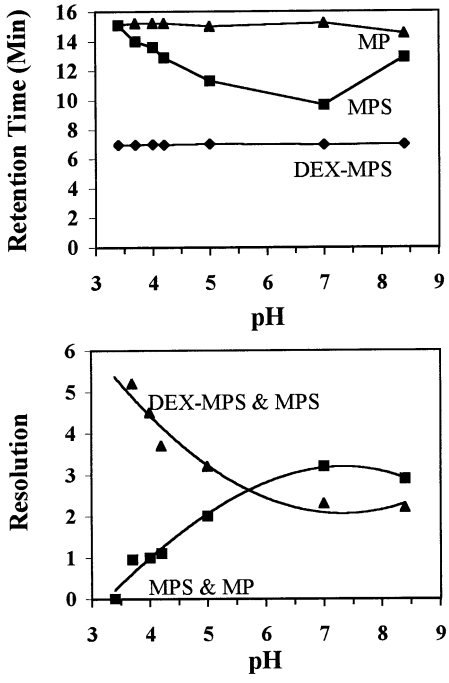

Fig. 4. Effects of mobile phase pH on the retention time (upper panel) and resolution (lower panel) of DEX-MPS, MPS, and MP. The aqueous: acetonitrile ratio (3: 1) was kept constant.

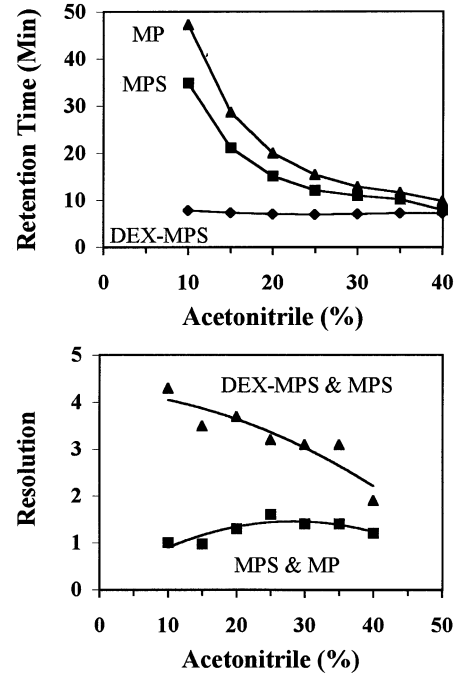

Fig. 5. Effects of the acetonitrile content of the mobile phase on the retention time (upper panel) and resolution (lower panel) of DEX-MPS, MPS, and MP. The aqueous part was 10 mM KH_2PO_4.

The resolution and retention time data as a function of percentage of acetonitrile in the mobile phase are depicted in Fig. 5 for acetonitrile range of 10–40%; the data in the absence of acetonitrile in the mobile phase are not reported because MP and MPS did not elute during a 60-min analysis using a 100% aqueous mobile phase. The retention time of DEX-MPS exhibited minimal dependence on the acetonitrile content percentage. However, the retention times of both MPS and MP decreased with an increase in the acetonitrile content (Fig. 5), resulting in changes in the resolution of the components (Fig. 5).

The responses of the detector to analytes were linear (r range: 0.997–1.00) over the range of 2.5–100 μg ml^{-1} for DEX-MPS and 1–100 μg ml^{-1} for MP and MPS (Fig. 6). The slopes of the regression lines were less than 15% different for the three components, indicating that the detector response for all three components is almost the same. Additionally, when the DEX-MPS and MPS were analyzed separately, no hydrolysis to MPS and/or MP was detected during the sample preparation and analysis.

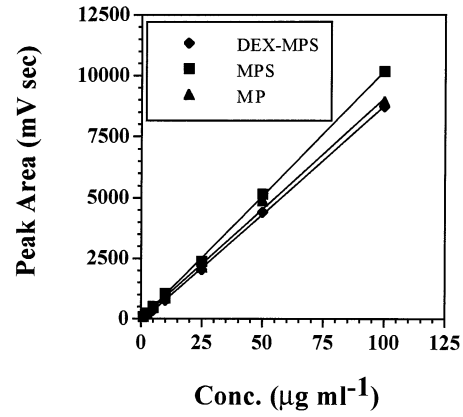

Fig. 6. Calibration standards of DEX-MPS (◆), MPS (■), and MP (▲); concentrations are based on MP equivalence.

Table 1
Intra-run accuracy and precision of the assay ($n = 5$)[a]

Added concentration (μg ml^{-1})	Calculated concentration (μg ml^{-1}) (%error)			%CV		
	MP	MPS	DEX-MPS	MP	MPS	DEX-MPS
1	1.04 (3.80)[a]	1.01 (1.32)		2.4	2.4	
2.5			2.82 (12.8)			0.79
25	22.9 (−8.56)	25.9 (3.60)	25.2 (0.960)	3.6	1.4	0.35
100	93.8 (−6.22)	93.0 (−6.96)	107 (6.80)	0.36	1.4	0.78

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

The intra- and inter-run validation data for all 3 components are reported in Tables 1 and 2, respectively. Both intra- and inter-run errors were $< 13\%$ for all the components, including the lowest concentrations of 1 (MP and MPS) and 2.5 (DEX-MPS) μg ml^{-1} (Tables 1 and 2). The precision of the assay is demonstrated by a CV of $\leq 6\%$ (Tables 1 and 2).

The application of the assay to stability studies is demonstrated by simultaneously quantitating all three components in a preliminary study at pH values of 5.0 and 8.4 (Fig. 7). The conjugate was relatively stable at pH 5.0, with $> 95\%$ remaining intact at 96 h. On the other hand, substantial hydrolysis occurred at pH 8.4: the decline in the concentration of DEX-MPS was associated with an increase in the concentrations of free MP and MPS in the sample (Figs. 7 and 8). However, the shape and retention times of peaks remained relatively constant during 96 h. After 96 h of incubation at pH 8.4, MP, MPS, and DEX-MPS constituted, respectively, 57, 26, and 18% of the total MP content of the sample (Figs. 7 and 8).

4. Discussion

Reversed- or normal-phase chromatography may be used for separation of relatively small molecules such as MP and MPS. These separations are based on the differences in the affinity of the analytes for the packing material and the mobile phase. On the other hand, SEC is based on separation of the analytes according to their molecular size. Whereas separation of a macromolecule such as DEX-MPS from a small molecule such as MP or MPS is expected using SEC, simultaneous resolution of all three components based only on size principles would be unlikely. This is because while the differences between the molecular weights (MWs) of DEX-MPS ($> 70\,000$) and MP (374) or MPS (475) are substantial, the MWs of MP and MPS are relatively close to each other. Therefore, in addition to size exclusion interactions, other mechanism(s) should be in place in order to resolve all three components.

Our studies with different mobile phases showed that the retention times of both MP and MPS were sensitive to the percentage of acetonitrile in the mobile phase (Fig. 5), suggesting hydrophobic interactions between these analytes and the column bedding. We took advantage of these interactions to resolve MP and MPS by manipulating the pH of the mobile phase: while at a pH value of 3.4, the MP and MPS co-eluted as a single peak, at pH values ≥ 5, the two peaks were baseline resolved (Fig. 4).

The substantial pH effect observed here (Fig. 4) may be explained by the fact that MPS is a weak acid with a pKa of 4.5–4.6 [6]. Therefore, at pH 3.4, the drug is mostly in the unionized form which co-elutes with MP. However, at pH 5 or above, MPS is mostly ionized with higher degrees of water solubility and thus less hydrophobic interaction with the column, resulting in earlier elution from the column. On the other hand, because MP is not ionized in the mobile phase, its retention time is not pH sensitive (Fig. 4). The pH effect in our SEC method is in agreement with the findings of Anderson and Taphouse [6] for a reversed phase chromatography; similar to our

Table 2
Inter-run accuracy and precision of the assay ($n = 5$)[a]

Added concentration (μg ml^{-1})	Calculated concentration (μg ml^{-1}) (%error)			%CV		
	MP	MPS	DEX-MPS	MP	MPS	DEX-MPS
1	1.07 (7.20)[a]	1.08 (8.00)		1.2	4.0	
2.5			2.71 (8.48)			3.4
25	26.9 (7.44)	25.4 (1.52)	25.4 (1.76)	2.7	3.8	2.7
100	95.1 (−4.9)	93.0 (−6.98)	106 (6.40)	6.0	2.7	3.2

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

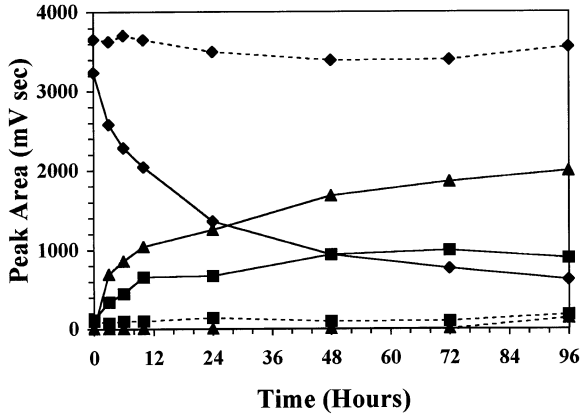

Fig. 7. Stability of DEX-MPS at pH 5.0 (dashed lines) and 8.4 (solid lines); (◆), DEX-MPS; (▲), MP; (■), MPS; each point is an average of two measurements.

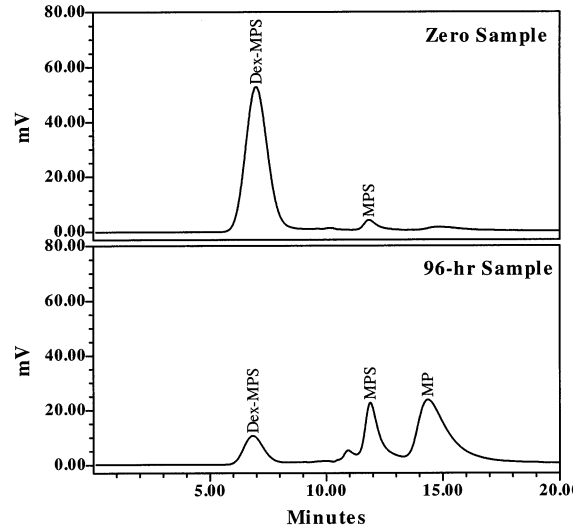

Fig. 8. Chromatograms of a DEX-MPS sample immediately (upper panel) and 96 h (lower panel) after dissolving the powder in a pH 8.4 solution.

study, the retention time of MPS declined with an increase in the pH of the mobile phase, while the retention time of MP remained relatively constant in their study (Fig. 1 in Ref. [6]).

The commercially available MPS is a 21-ester which in aqueous solutions may undergo a reversible acyl migration to a 17-ester [6]. However, it has been shown that the 21 ester is thermodynamically more stable. A previous reversed-phase HPLC assay [6] was able to detect a small peak of the 17-ester in the presence of high concentrations of the 21-ester. In our stability studies at pH 8.4, in addition to the three known components, an unknown peak was detected which eluted before the MPS peak (Fig. 8). This peak which had a peak area of approximately 10% of the known MPS may have been the 17-ester of MP. Nevertheless, because of lack of availability of a standard of the 17-ester, the identity of this peak was not confirmed.

The stability findings reported in Fig. 7 are consistent with the findings of McLeod et al. [3] who used a reversed-phase HPLC in their studies for measurements of MP and MPS. Similar to our results, these authors demonstrated that the hydrolysis of both DEX-MPS and MPS increased substantially when the pH was increased from 3 to 9. However, it should be noted that, similar to other available HPLC methods [6,7], the HPLC method used by McLeod et al. [3] is not capable of quantitating DEX-MPS. Therefore, the available HPLC methods cannot be used in investiga-

tions that require direct measurement of the conjugated polymer.

In conclusion, a validated analytical size-exclusion chromatographic method is reported here which can simultaneously quantitate low concentrations of DEX-MPS conjugate and its potential degradation products MPS and MP. The method is suitable for determination of the degree of substitution, purity, and stability of DEX-MPS.

Acknowledgements

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