ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
16 (1997) 499–506

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Quantitation and determination of molecular weight distribution of residual water soluble extractable polyamines in DMP 504, a poly-alkylamine bile acid sequestrant, by aqueous high performance size exclusion chromatography[1]

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Received 8 July 1996; received in revised form 11 March 1997; accepted 19 March 1997

Abstract

A high performance size exclusion chromatography (HPSEC) method with refractive index detection for quantitation and molecular weight determination of extractable water soluble polyamines (SPA) in a novel proprietary polymeric pharmaceutical compound (DMP 504) is described. The extracted polyamines are synthetic impurities as well as potential degradation products of the polymer. Data for calibration, precision, solution stability, and limits of detection and quantitation are presented to validate the suitability of this method for its intended purpose. Response linearity for the poly(2-vinylpyridine) standard is demonstrated between 10 and 1000 μg ml^{-1}, equivalent to 0.005% and 0.5% (w/w) in DMP 504. Molecular weight distributions of SPA extracted from DMP 504 are presented. No increase in SPA is detected in DMP 504 over 6 months storage at room temperature. © 1997 Elsevier Science B.V.

Keywords: Aqueous size exclusion chromatography; Polyamines; Quantitation; Stability; Molecular weight; Bile acid sequestrant

1. Introduction

DMP 504 is a novel proprietary bile acid sequestrant currently under development by the DuPont Merck Pharmaceutical Company, intended for the reduction of serum cholesterol levels. The polymer is an insoluble, cross-linked condensation copolymer of 1,10-dibromodecane and 1,6-diaminohexane, in the hydrochloride form. The empirical chemical formula, $(C_{18}H_{40}N_2Cl_2)_n$, was determined by elemental analysis and represents a non-stoichiometric condensation product. The ex-

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[1] This manuscript was presented as a poster at the ACS Middle Atlantic Regional Meeting, Villanova University, Villanova, Pennsylvania in May, 1996.

PII S0731-7085(97)00081-2

tractable soluble polyamines (SPA) comprise the relatively low molecular weight fraction that is soluble in water. The SPA are monitored to evaluate product quality, purity and stability.

Compendial methods used for analysis of cholesterol reducing, pharmaceutical anion exchange polymers, such as colestipol hydrochloride and cholestyramine resin, were evaluated. The water soluble fraction in colestipol hydrochloride, a copolymer of diethylenetriamine and 1-chloro-2,3-epoxypropane, is determined by gravimetric analysis [1]; the dialyzable quaternary amines present in cholestyramine resin, a styrene-divinylbenzene copolymer, are determined using dialysis [2]. These methods, however, are not suitable for characterization of SPA in DMP 504. A gravimetric analysis does not provide the required method selectivity and sensitivity; a dialysis method does not allow adequate sample throughput. Therefore, an aqueous size exclusion chromatographic (SEC) method was developed. Aqueous SEC has been used to determine the molecular weight distribution (MWD) of cationic polyelectrolytes [3–5], and water soluble anionic polymers in oil field brines [6].

Although DMP 504 is insoluble in both aqueous and organic solvents, the low molecular weight fraction (polyamines) can be dissolved in aqueous media, extracted from the drug substance, and concentrated using liquid-liquid extraction. The sample solution is then analyzed by high performance size exclusion chromatography (HPSEC).

2. Experimental

2.1. Instrumentation

A Waters high performance liquid chromatogaphy (HPLC) system consisting of a model 510 pump, WISP model 712 autosampler, model 680 automated gradient controller, column temperature control module and model 410 differential refractometer were used to obtain the method validation and assay results. A Hewlett-Packard model HP1090 HPLC consisting of a model HP1047A refractive index detector, DR-5 ternary solvent delivery system, HPLC^{3D} ChemStation (DOS Series), autosampler and column oven were used for the method development work. Chromatographic data were collected and analyzed using either the Fisons Multichrom™ data system or HPLC^{3D} ChemStation (DOS Series).

2.2. Chemicals

Methanol and methylene chloride, obtained from EM Science, and phosphoric acid (Fisher) were HPLC grade. Sodium hydroxide (EM Science) was reagent grade. HPLC grade water was obtained from a Milli-Q® water purification system. Poly(2-vinylpyridine) (PVP) cationic molecular weight standards (PVP KIT, American Polymer Standards Corporation) were used to prepare the molecular weight calibration curve. DMP 504 drug substance was manufactured at The DuPont Merck Pharmaceutical Company.

2.3. SEC Columns

A PL aquagel-OH guard column (7.5 cm × 7.5 mm) and PL aquagel-OH 40, -OH 50 and -OH 60 SEC columns (30 cm × 7.5 mm i.d., 8 μm) were obtained from Polymer Laboratories Inc. The effective molecular weight range for the PL aquagel-OH 40 (40 Å) column is approximately 200–100 000 based on polyethylene oxide (PEO). The molecular weight range (PEO) of the PL aquagel-OH 50 (50 Å) and PL aquagel-OH 60 (60 Å) columns are 20 000–1 000 000 and 100 000–20 000 000, respectively.

2.4. Procedure

An accurately weighed quantity ($\sim$1 g) of drug substance was suspended in 100 ml of water in a separatory funnel. The dispersion was rendered alkaline (pH of approximately 13) with 2 ml of 50% (w/w) sodium hydroxide solution. The aqueous dispersion was extracted with three 50 ml portions of methylene chloride. Each organic portion was filtered through a glass funnel lined with filter paper (Whatman No. 1). The

combined organic fractions were evaporated to dryness. The residue was reconstituted in 5.00 ml of solvent (10:90, methanol: 0.2% (v/v) phosphoric acid in water) and filtered through a 0.45-μm nylon filter (Gelman Acrodisc, part no. 4438).

The PVP molecular weight calibration stock standards were prepared at 1 mg ml^{-1} by separately dissolving approximately 10 mg of the 3, 7, 13, 35, 100, 150 and 300 kDa PVP standards in 1.00 ml of methanol and diluting to 10.0 ml with 0.2% (v/v) phosphoric acid. Working MW calibration standards were prepared at 0.1 mg ml^{-1} by suitably diluting the stock standards with solvent. The 100 kDa PVP standard solution (1 mg ml^{-1}) was used without further dilution. The 3 kDa PVP quantitation standards were similarly prepared at concentrations of 80, 200 and 800 μg ml^{-1}.

The separation was carried out by injecting 50 μl of each standard and sample solution into two PL aquagel-OH 40 size exclusion columns connected in series. The mobile phase consisted of 0.1% (v/v) phosphoric acid in water. Column flow rate and temperature were maintained at 1.0 ml min^{-1} and 40°C, respectively. The internal temperature of the refractive index detector was maintained at 35°C. Method development work was performed using a 25 μl injection volume, a flow rate of 1.5 ml min^{-1} and a single PL aquagel-OH 40 column. Determination of the high molecular weight SPA fraction utilized PL aquagel-OH 40, -OH 50 and -OH 60 columns connected in series, a flow rate of 1.0 ml min^{-1} and an injection volume of 25 μl. All analyses utilized a PL aquagel-OH guard column connected in series with the analytical column(s).

A molecular weight calibration curve was prepared by cubic polynomial regression of $\log_{10} M_p$ (molecular weight corresponding to the peak retention time) versus the peak retention time for each PVP standard. Weight average molecular weight ($\bar{M}_w$) and number average molecular weight ($\bar{M}_n$) were calculated for each sample [7]. Quantitation of weight percent SPA, using peak areas, was based upon a three point linear regression calibration curve prepared from the 3 kDa PVP standard.

3. Results and discussion

3.1. Method development

The PL aquagel-OH column was selected be-

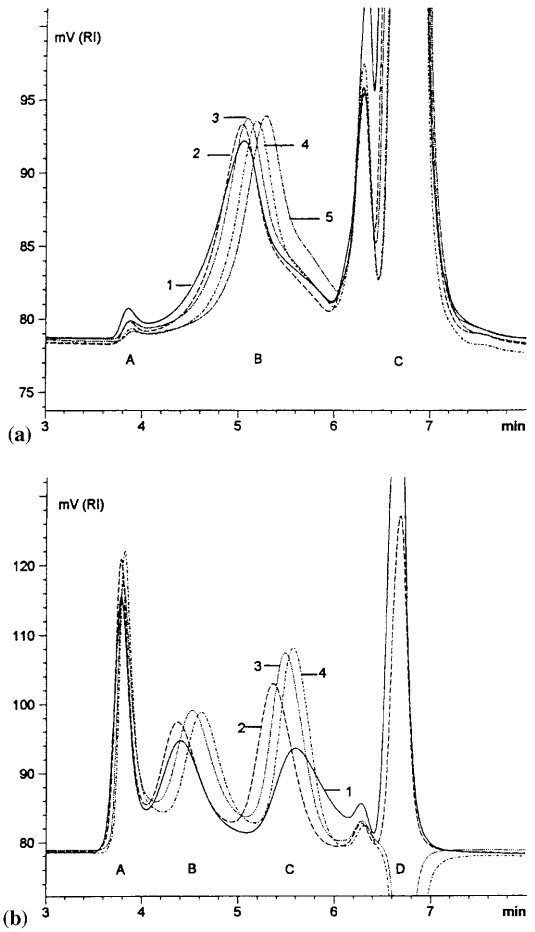

Fig. 1. (A) Overlaid chromatograms showing the effect of mobile phase phosphoric acid concentration on retention time and peak shape of SPA. Chromatograms were obtained using 0.01% (1), 0.03% (2), 0.05% (3), 0.10% (4) and 0.20% (5) phosphoric acid in the mobile phase. Band A is the column exclusion volumn, band B is SPA; band C is phosphoric acid and other small polar compounds. (B) Overlaid chromatograms showing the effect of mobile phase phosphoric acid concentration on retention time and peak shape of PVP MW standards. Chromatograms were obtained using 0.01% (1), 0.03% (2), 0.10% (3), and 0.20% (4) phosphoric acid in the mobile phase. Band A is 300K PVP, band B is 35K PVP, band C is 3K PVP; band D is phosphoric acid and other small polar compounds.

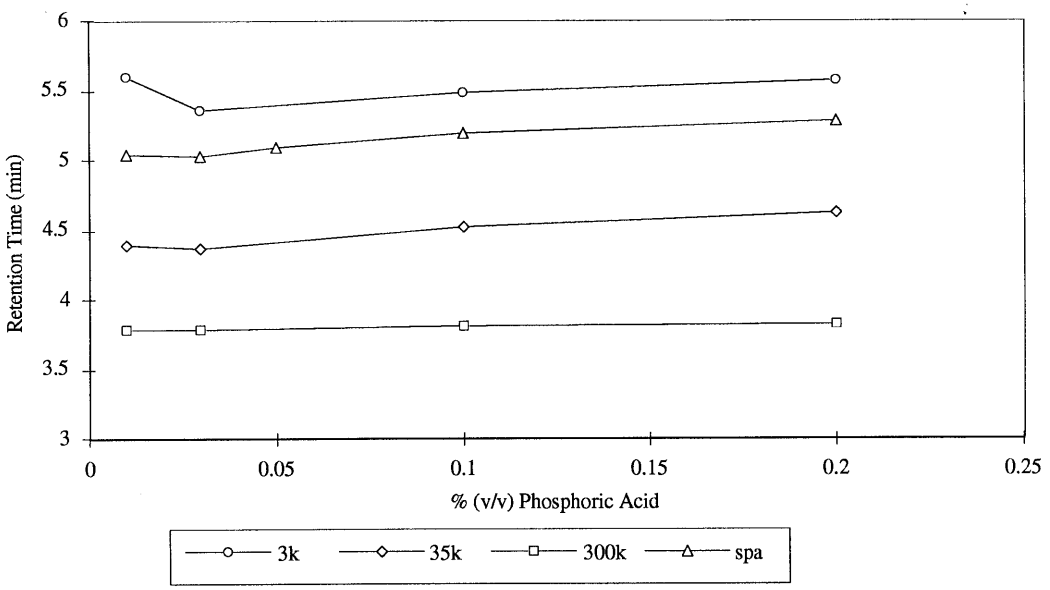

Fig. 2. Plot showing the effect of mobile phase phosphoric acid concentration on the retention time of an extracted soluble polyamine sample and the 300, 35 and 3 KDa PVP molecular weight standards.

cause it is a polymeric rather than a silica based column. While suppression of secondary interactions with silica based columns is possible [8,9], a polymeric column reduced the likelihood that ion exclusion [10–12], classical amine peak tailing or electrostatic interaction of SPA with the anionic silica would interfere with the analysis.

Phosphoric acid (0.1% v/v) was selected as the mobile phase based on its ability to buffer the mobile phase and to protonate the extracted SPA and PVP standards. Phosphoric acid concentration and flow rate were studied to determine their effect on the chromatographic separation. PVP molecular weight standards and an extracted SPA sample were eluted from the column using mobile phases consisting of phosphoric acid concentrations between 0.0 and 0.20% (v/v). The results show that phosphoric acid concentration has a small but systematic effect on retention time. For a mobile phase consisting of neutral-water, the extracted SPA and PVP MW standards were not adequately protonated and were retained on the column. Fig. 1 shows overlaid chromatograms of a SPA sample and overlaid chromatograms of the 300, 35 and 3 kDa PVP standards obtained at various concentrations of phosphoric acid. Fig. 2 shows a plot of their respective peak retention times versus phosphoric acid concentration. Reasonable care in controlling the phosphoric acid concentration in the mobile phase effectively eliminated this source of chromatographic variability.

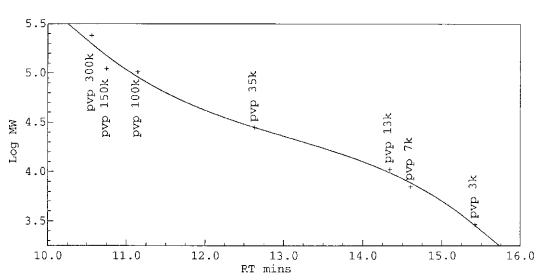

Fig. 3. PVP molecular weight calibration plot of $\log_{10}$ Mp versus retention time obtained using two PL aquagel-OH 40 columns, a flow rate of 1.0 mL/min and a mobile phase of 0.1% (v/v) phosphoric acid.

An experiment was performed to verify that differences in flow rate would not seriously distort peak shape or change the elution volume of the polyamines and PVP standards. Samples and molecular weight standards (300, 35 and 3 kDa)

Table 1
Method precision of six replicate DMP 504 drug substance preparations (Lot Q9214-48) assayed for %SPA (w/w), $\bar{M}_n$ and $\bar{M}_w$

Sample number	% (w/w) Polyamine	$\bar{M}_n$	$\bar{M}_w$
1	0.115	4600	7200
2	0.109	4800	7300
3	0.116	4600	7200
4	0.116	4600	7300
5	0.118	4800	7400
6	0.120	4700	7300
Mean	0.116	4700	7300
SD	0.004	98	75
%RSD ($n = 6$)	3.2%	2.1%	1.0%

were analyzed at flow rates between 0.75 and 1.5 ml min^{-1}. Chromatograms obtained at these flow rates nearly overlay when plotted on an elution volume scale. This indicates that experiments performed at different flow rates (e.g. 1.0 ml min^{-1} and 1.5 ml min^{-1}) can be directly compared. Note, of course, that flow rate must be carefully controlled to obtain accurate molecular weight determinations.

PVP was chosen as a secondary MW calibration standard since no primary SPA standard is available. PVP and SPA are both cationic polyelectrolytes and are readily soluble in the sample solvent of dilute phosphoric acid, methanol and water. In addition, PVP has a good UV chromophore. This simplified some of the initial screening of columns and mobile phase

Table 2
Soluble polyamine results in four lot numbers of DMP 504 drug substance analyzed for %SPA (w/w), $\bar{M}_n$ and $\bar{M}_w$

DMPC lot number	% (w/w)	$\bar{M}_n$	$\bar{M}_w$
Q9214-48	0.11	5500	8400
Q9214-49	0.04	(6600)	(18000)
Q9214-50	0.07	5000	8300
Q9214-51	0.09	(6400)	(14000)

Results in parenthesis are not accurate $\bar{M}_n$ and $\bar{M}_w$ values because the sample exceeds the molecular weight range of the SEC column. The results indicate that the distribution contains a larger fraction of high MW species than lot numbers Q9214-48 and Q9214-50.

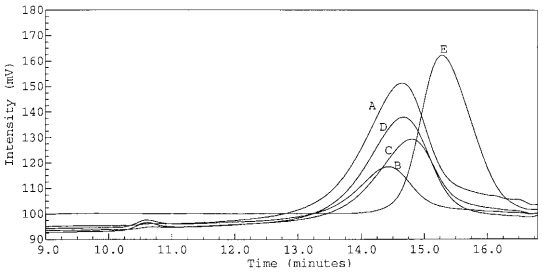

Fig. 4. Comparison of the molecular weight distribution for the extracted soluble polyamines in DMP 504 drug substance, lot numbers Q9214-48 (A), Q9214-49 (B), Q9214-50 (C) and Q9214-51 (D) and the 3K PVP standard (E). Analytical conditions are the same as Fig. 3.

solvents since the equilibrium time for the UV detector is much less than for the RI detector.

The 3 kDa PVP standard was selected as a secondary reference standard for the quantitative analysis based on similar detector responses obtained for bis-hexamethylene triamine (Bis-HMTA) and the 3 kDa PVP standard using RI detection. Bis-HMTA was not suitable as a reference standard because it was not chromatographically resolved from the solvent peak.

3.2. Calibration

A representative PVP molecular weight calibration curve is shown in Fig. 3. The 300, 150 and 100 kDa standards eluted in a narrow interval between 10.6 and 11.2 min because they exceeded the molecular weight range for PL aquagel-OH 40 column. A bimodal column set (i.e. PL aquagel-OH 40 and -OH 60) that would extend the upper MW range was not utilized because the wider molecular weight distribution would make accurate detection, integration and quantitation of the SPA peak difficult. In addition, most of the extractable soluble polyamines had a relative MW of less than 100 000.

A relative response factor between 3 kDa PVP and bis-HMTA was determined to demonstrate the suitability of using 3 kDa PVP as a secondary reference standard. The linear regression of response versus amount was determined between 40 and 1000 μg ml^{-1} (3 kDa PVP) and between 11

and 400 μg ml^{-1} (Bis-HMTA). The relative response factor between these analytes was determined to be 1.06 and was calculated by dividing the 3 kDa PVP slope by the slope determined for Bis-HMTA. Regression analysis of response versus concentration was additionally demonstrated at nine concentrations of the 3 kDa PVP molecular weight quantitation standard between 10 and 1000 μg ml^{-1}.

3.3. Precision

Precision of the HPLC system was demonstrated by preparing six 3 kDa PVP standard preparations at 200 μg ml^{-1}, equivalent to 0.1% (w/w), and calculating the relative standard deviation of their peak areas. The mean and RSD for peak area responses from six standard preparations were 3 629 021 mV and 1.0%, respectively. Precision of the method was measured by calculating the RSD of six replicate sample extractions analyzed for percent (w/w) SPA, $\bar{M}_w$ and $\bar{M}_n$. The relative standard deviations determined for a DMP 504 drug substance sample containing 0.12% (w/w) polyamine were 3.2, 1.0 and 2.1% for SPA, $\bar{M}_w$ and $\bar{M}_n$, respectively. The mean, RSD and individual results of six DMP 504 replicate drug substance preparations are shown in Table 1. The RSD obtained for system and method precision was less than 5% for all determinations. This is adequate precision for quantitation and molecular weight determination of extractable soluble polyamines in DMP 504.

3.4. Determination limits

The limits of quantitation and detection were experimentally determined by preparing serial dilutions of the 3 kDa PVP standard. The limit of quantitation was determined to be 10 μg ml^{-1}, 0.005% (w/w), based on the 3 kDa standard peak area signal-to-noise ratio of 15:1, with a RSD of 4.0%. The limit of detection was experimentally determined to be 5 μg ml^{-1}, 0.0025% (w/w), based on a signal-to-noise ratio of 7:1. The limit of detection criterion of 7:1 was selected because SPA peak widths are typically very large. Determination limits for SPA will vary according to the molecular weight distribution of the sample. However, this method provides adequate sensitivity to quantify SPA at the level typically observed in DMP 504.

3.5. Solution stability

PVP molecular weight standard solutions and 3 kDa PVP quantitation standards solutions were shown to be stable for at least 1 month at room temperature. The absolute peak retention times for PVP molecular weight standard solutions did not vary by more than 0.06 min from the initial analysis. The peak areas of the 3 kDa PVP quantitation standards were 97.1, 98.3 and 99.5% of the peak areas determined at time zero for the 0.04, 0.1, and 0.4% standards, respectively. The

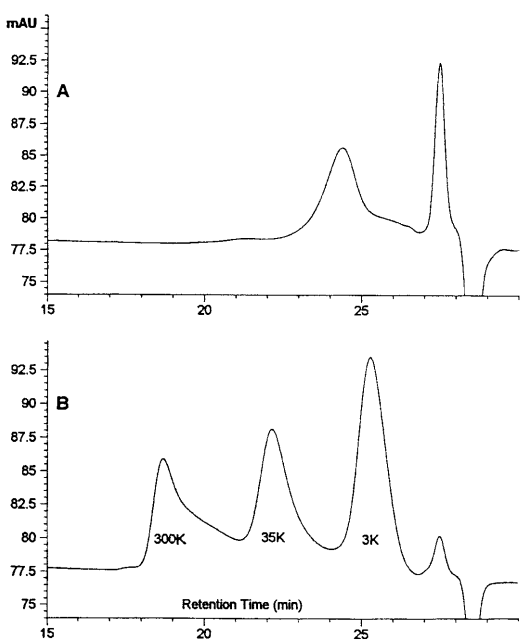

Fig. 5. Chromatograms of the extracted soluble polyamines in lot number Q9214-49 (A) and the 300, 35 and 3 kDa PVP molecular weight standards (B) obtained using PL aquagel-OH 40, -OH 50 and -OH 60 columns in series; a flow rate of 1.0 mL/min; and a mobile phase of 0.1% (v/v) phosphoric acid. The band at the column exclusion volume is not observed in Fig. 5A. This shows that the early eluting band in chromatograms B and D in Fig. 4 (lots Q914-49 and -51, respectively) represents the high MW fraction and not a secondary column interaction.

Table 3
Stability data for DMP 504 drug substance, lot number Q9214-49, at storage conditions of between 0 and 6 months

	Months				
	0	1	2	3	6
% (w/w)					
25°C/50% r.h.	0.04	0.04	0.05	0.06	0.04
40°C		0.04		0.06	
40°C/75% r.h.		0.06		0.13	
50°C		0.04		0.06	
600 ft-c		0.04		0.10	
$\bar{M}_w$					
25°C/50% r.h.	18000	18000	18000	18000	10000
40°C		14000		12000	
40°C/75% r.h.		6300		7000	
50°C		12000		12000	
600 ft-c		9900		8100	
$\bar{M}_n$					
25°C/50% r.h.	6600	5500	6200	3400	2800
40°C		3800		2900	
40°C/75% r.h.		3100		2000	
50°C		4900		2900	
600 ft-c		4200		2200	

peak areas of sample solutions were 96% of the initial peak area after 5 days storage at room temperature.

3.6. Analysis of DMP 504 for SPA

Results for SPA, $\bar{M}_w$ and $\bar{M}_n$ in four lot numbers of DMP 504 analyzed with this method are shown in Table 2. High $\bar{M}_w$ values for lot numbers Q9214-49 and Q9214-51 are due to the small peak that elutes in the high MW region of the chromatogram (Fig. 4). This peak represents the high molecular weight polyamines, which elute in the exclusion volume of the column. Since the high molecular weight SPA are compressed into a single band, $\bar{M}_w$ and $\bar{M}_n$ are not accurate representations of the MWD for lot numbers Q9214-49 and Q9214-51. Their MWD exceeds the molecular weight range of the PL-aquagel-OH 40 column. However, this method was sufficient for monitoring relative changes in the MWD over time. Compression of the high molecular weight fraction into a single narrow band was actually an advantage because it facilitated peak integration for the quantitative aspect of the method. This narrow band was easier to observe and integrate than a continuous distribution containing low levels of high molecular weight SPA.

It was verified that this band is the high MW SPA and not due to secondary interation (e.g. reversed-phase like absorption, ionic interaction, etc.) by injecting a sample and the 3, 35 and 300 kDa PVP standards into a PL-aquagel-OH 40 column connected in series with PL-aquagel-OH 50 and -OH 60 columns. As expected, the high molecular weight peak was not observed in the sample chromatogram since it was now part of a continuous molecular weight distribution (Compare Fig. 5(a) and Fig. 1(a)). The highest molecular weight standard (300 kDa) elutes in a much broader band and shows more even spacing with the 3 and 35 kDa PVP standards (Compare Fig. 5(b) and Fig. 1(b)).

Results for DMP 504, lot number Q9214-49, stored for 6 months at 25°C/50% relative humidity (r.h.), and for 3 months at 40°C/75% r.h., 40°C, 50°C and 600 ft-c., are shown in Table 3. The data demonstrate that DMP 504 did not degrade into SPA over 6-months storage at room temperature. An increase in SPA concentration for samples stored at 40°C/75% r.h. and 600 ft-c. showed that degradation of DMP 504 into SPA had occurred at these accelerated conditions (Fig. 6). $\bar{M}_w$ and $\bar{M}_n$ were shown to decrease at accelerated storage conditions and 6 months 25°C/50% r.h., indicating that the MWD contains a larger fraction of low molecular weight species.

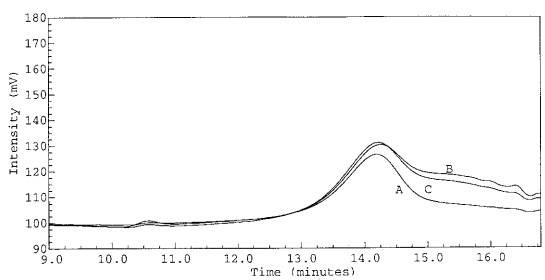

Fig. 6. Comparison of the molecular weight distribution for the extracted soluble polyamines in DMP 504 drug substance, lot number Q9214-49, at 3 months storage at 40°C (A), 40°C /75% r.h. (B) and 600 ft-c (C).

4. Conclusion

Data for calibration, system precision, method precision, solution stability and limits of detection and quantitation have been presented to support the suitability of size exclusion chromatography as a method for quantifying and profiling the molecular weight distribution of extractable soluble polyamines in DMP 504 drug substance. No increase in SPA was found in DMP 504 over 6 months storage at room temperature.

Acknowledgements

The authors are grateful to Dr Howard Barth, of the DuPont Corporate Center for Analytical Sciences, to Dr James A. Shea, Dr Paul Hovsepian and Dr Christopher Riley of The DuPont Merck Pharmaceutical Co. for review of the manuscript and helpful discussions, and to Mr Gary Cantrell of The DuPont Merck Pharmaceutical Co. for technical advice and assistance. The authors would also like to acknowledge Dr Glenn A. Sherwood, Jr. of DuPont Agricultural Products for helpful technical discussions.

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