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Determination of *M*/*G* ratio of propylene glycol alginate sodium sulfate by HPLC with pre-column derivatization

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ABSTRACT

A reliable high performance liquid chromatography with pre-column derivatization method was developed for the determination of the mannuronic acid (*M*)/guluronic acid (*G*) ratio of propylene glycol alginate sodium sulfate (PSS). The hydrolysis conditions of PSS were investigated by four degradation methods based on the degree of destruction of *M* and *G*, and the chromatographic separation conditions were also optimized. A satisfactory resolution of *M* and *G* was achieved with a KP-C18 column using 0.1 mol/L phosphate buffer (pH 7.0)-acetonitrile (83/17, v/v) as a mobile phase, after PSS was hydrolyzed with 0.1 mol/L sulfuric acid and labeled with 1-phenyl-3-methyl -5-pyrazolone. The *M*/*G* ratio of PSS determined by this method was in good accordance with that obtained by the ^{1}H NMR method with a desulfurization strategy. Our method is rapid, sensitive, accurate and reproducible. The limit of detection was found to be 0.25 μg/mL for *M* and 0.40 μg/mL for *G*.

1. Introduction

Alginate is a linear polysaccharide composed of (1→4) linked β-D-mannuronic acid (*M*) and its C-5 epimer α-L-guluronic acid (*G*) (Rahelivao, Andriamanantoanina, Heyraud, & Rinaudo, 2013). Alginate and its sulfated derivatives have various biological activities, such as anti-inflammatory and anticoagulant actions (Zhao et al., 2007; Fan et al., 2011), and they have been extensively used as wound dressing materials (Pawar & Edgar, 2012). Propylene glycol alginate sodium sulfate (PSS), a heparinoid-active sulfated polysaccharide (Fig. 1) prepared by chemical sulfation of low-molecular-weight alginate, has been commonly used as an anti-cardiovascular disease drug in China for nearly 30 years (Li, Su, & Guan, 2012). However, the activities of PSS are influenced by the uronic acid composition (*M*/*G* ratio), degree of sulfate substitution and weight-averaged molecular weight (Fan et al., 2011; Lin et al., 2007). The *M*/*G* ratio in PSS plays an important role in its anticoagulation, blood lipid regulation activities and side effects. There is a need to develop a reliable and sensitive method for determination of the *M*/*G* ratio of PSS for quality control and clinical purposes.

Nuclear magnetic resonance (NMR) as a simple and non-destructive method has been widely used for determining the *M*/*G* ratio in alginate (Heyraud et al., 1996; Burana-osot et al., 2009; Aida, Yamagata, Watanabe, Richard, & Smith, 2010). However, it is not suitable for the analysis of sulfated alginate derivatives because the presence of sulfate groups affect the resolution of anomeric proton signals. The commonly used method for *M*/*G* ratio determination of alginate by anion exchange liquid chromatography is rapid and simple (Gacesa, Squire, & Winterburn, 1983). However, the lactone forms of uronic acids interfere with the quantification of *M* and *G*. It is also not suitable for the microanalysis of alginate sulfated derivatives in biological samples.

Here, we report a reversed-phase high performance liquid chromatographic (HPLC) method using 1-phenyl-3-methyl-5-pyrazolone (PMP) as a pre-column derivatization reagent for the separation and analysis of *M* and *G*. This method is rapid, accurate and sensitive for determination of the *M*/*G* ratio in PSS, and it is suitable for quality control and microanalysis of PSS.

2. Experimental

2.1. Chemicals

M and G monosaccharide standards were provided by the School of Medicine and Pharmacy at the Ocean University of China (Qingdao, China). PMP was purchased from Sigma–Aldrich (Shanghai,

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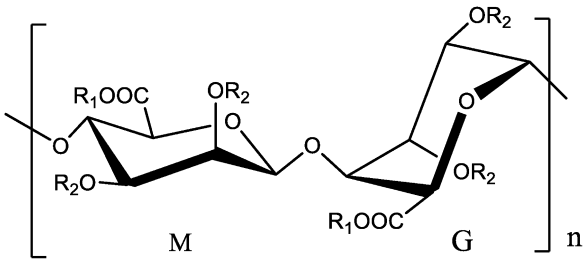

Fig. 1. Chemical structure of propylene glycol alginate sodium sulfate (PSS). R_1 = Na, $CH_2CH(OH)CH_3$; R_2 = H, SO_3Na.

China). HPLC-grade acetonitrile was purchased from Merck KGaA (Germany). Propylene glycol alginate sodium sulfate (PSS) was provided by Qingdao Chiatai Haier Pharmaceutical Co., Ltd (Qingdao, China). All other chemicals and solvents were of analytical grade unless otherwise specified.

2.2. Hydrolysis of PSS

The hydrolysis of PSS was carried out by four commonly used different degradation methods for polysaccharides as follows: (1) PSS was dissolved in dilute sulfuric acid (0.1 mol/L) at a concentration of 5 mg/mL, hydrolyzed in a sealed glass ampoule at 120 °C for 4 h, and then neutralized to pH 7.0 with NaOH after it was cooled to room temperature; (2) PSS was suspended in 2 mol/L trifluoroacetic acid (TFA) at a concentration of 5 mg/mL, hydrolyzed in a sealed glass ampoule at 110 °C for 6 h. The TFA was removed by rotary evaporation and washed out by water (Wang, Zhao, Yu, Li, & Hao, 2009; Dai et al., 2010); (3) PSS was dissolved in 0.1 mol/L HCl at a concentration of 20 mg/mL, hydrolyzed under microwave irradiation (1600 W) at 130 °C for 15 min (Hu et al., 2013), and then neutralized with NaOH; and (4) PSS was hydrolyzed by the conventional acid hydrolysis method (with 80% H_2SO_4 at 20 °C for 18 h and then 2 mol/L H_2SO_4 at 100 °C for 6 h) as described by Haug and Larsen (1962). All experiments were operated in parallel three times, and all hydrolysates were diluted to the same concentration before derivatization.

2.3. Derivatization procedure

The derivatization reactions of PSS hydrolysate, *M* and *G* monosaccharide standards with PMP were carried out essentially as previously described (Honda et al., 1989; Wang et al., 2009). Briefly, 100 μL of 0.3 mol/L NaOH and 120 μL methanolic solution of PMP (0.5 mol/L) were added to 100 μL of the sample solution, and the mixture was heated to 70 °C and incubated for 1 h. The reaction mixture was neutralized with 100 μL of 0.3 mol/L HCl after it was cooled to room temperature and was then extracted with chloroform (500 μL each) three times. The aqueous layer was filtered through a 0.22 μm micron membrane filter before HPLC analysis.

2.4. HPLC analysis

The Agilent 1260 HPLC system consisted of a binary pump (G1311C), an autosampler (G1329B), a variable wavelength detector (VWD, G1314F) and a system controller. The data were collected using an Open LAB CDS Chemstation Edition (version C.01.05) provided by the Agilent Company (USA). A KP-C18 column (150 × 4.6 mm, 5 μm), purchased from Zhongran Technology Co., Ltd. (Huaian, China), was used for HPLC analysis. Chromatographic separation of PMP derivatives was carried out using 0.1 mol/L phosphate buffer (pH 7.0) and acetonitrile at a ratio of 83:17 (v/v, %) as a mobile phase at a flow rate of 0.8 mL/min. The temperature of the column was maintained at 30 °C and detected by VWD at 245 nm.

2.5. Determination of the M/G ratio of PSS

PSS was hydrolyzed and derivatizated with PMP, and then separated at the optimal chromatographic conditions as mentioned above. The *M*/*G* ratio of PSS was calculated by comparing the peak area values of *M* or *G* to each calibrated standard curve. Calibration curves of *M* and *G* were constructed by plotting the peak areas against the concentrations of their monosaccharide standards on each analysis day using freshly prepared calibration standards.

2.6. Desulfation of PSS

Desulfation of PSS was performed according to the method described by Falshaw and Furneaux (1998). Briefly, PSS (30 mg) was dissolved in water and passed through an ion-exchange column (732 resin, H^+ form), and was then neutralized with pyridine to pH 7.0 and lyophilized to give a pyridinium salt. The salt was dissolved in 10 mL of dimethyl sulfoxide (DMSO) containing 10% (v/v) of anhydrous methanol and 1% pyridine. After stirring at 100 °C for 4 h, the reaction mixture was adjusted to pH 9.0 and dialyzed. The purified desulfated product was recovered by alcohol precipitation and desulfation was confirmed by the disappearance of sulfate peaks in its infrared spectrum.

2.7. ^1H NMR analysis

The *M*/*G* ratio of PSS, the desulfurization product of PSS and its intermediate (low-molecular-weight alginate) were compared with a nuclear magnetic resonance (NMR) method. Briefly, the sample (20 mg) was freeze-dried three times with 1 mL of D_2O (99.96%) to remove all exchangeable protons. The ^1H NMR spectra were recorded at 20 °C on a Bruker Avance 500 MHz spectrometer using deuterated acetone as an internal standard. The *M*/*G* ratio was calculated as previously reported (Rahelivao et al., 2013) and three peak areas (P_1, signal at 5.1 ppm, H_1-G; P_2, signal at 4.7 ppm, H_1-M and H_5-GM; P_3, signal at 4.5 ppm, H_5-GG) defined the M/G ratio (*M*/*G* ratio = $[P_2 - P_1 + P_3]/P_1$).

3. Results and discussion

3.1. Optimization of separation conditions

The separation conditions of *M* and *G* derivatives were investigated by changing the pH value of the phosphate buffer, the proportion of acetonitrile, the flow rate and loading quantity of the sample based on previous trials (Wang et al., 2009). Results showed that the retention behavior and resolution of *M* and *G* standard PMP derivatives were significantly affected by the pH value of the phosphate buffer and the proportion of acetonitrile. The separation degree of the two PMP derivatives was improved and the retention time was reduced with pH values increasing from 6.5 to 7.4, when the acetonitrile proportion remained at 17% (Fig. 2A). However, the column pressure increased rapidly at high pH values. The elution of *M* and *G* derivatives was earlier but the degree of separation was lower when the proportion of acetonitrile increased from 15% to 20% (Fig. 2B). The peaks of the two PMP derivatives were broad and even tailed at low proportions of acetonitrile, and 17% (v/v) of acetonitrile in the mobile phase provided the best separation degree. The separation degree of two PMP derivatives was improved slightly when the flow rate decreased from 1.0 mL/min to 0.5 mL/min (Fig. 2D), while the loading quantity of the sample had no significant effect on the separation degree of *M* and *G* derivatives (Fig. 2C). Considering the separation degree, peak shape, column pressure and analysis efficiency altogether, the optimal chromatographic conditions were chosen as follows (with a separation degree above 2.0): the mobile phase should be 0.1 mol/L

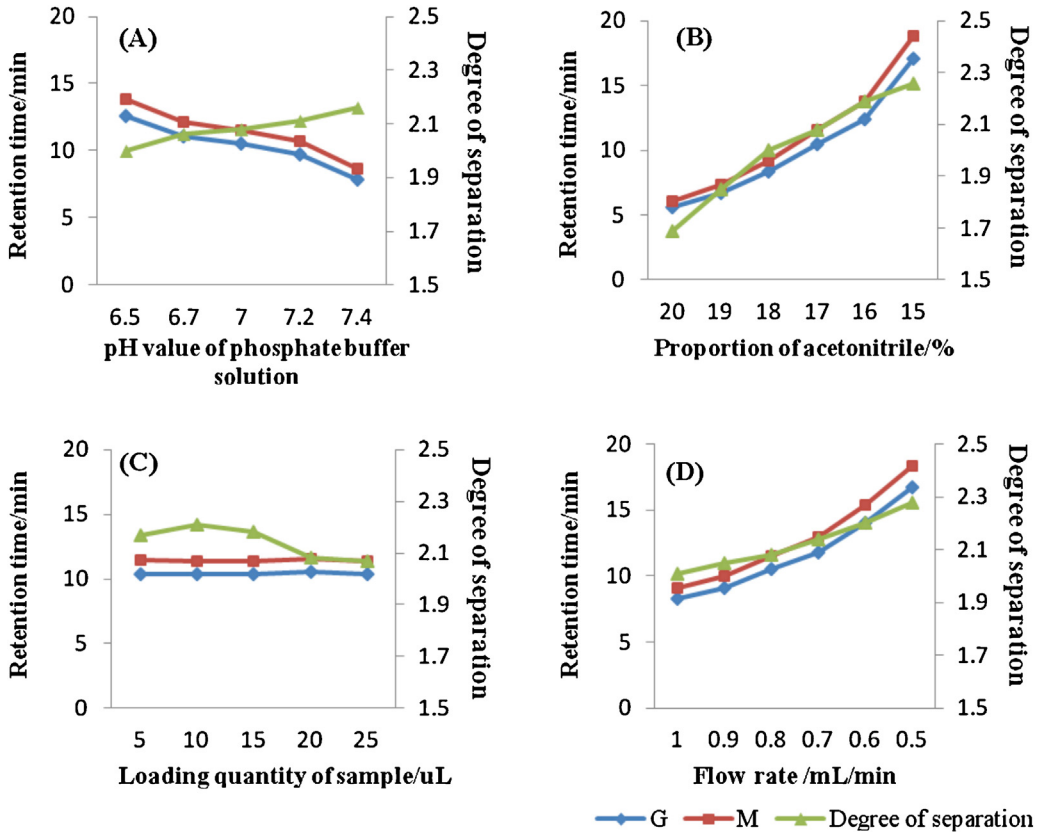

Fig. 2. The influences of chromatographic separation conditions on the retention behavior and resolution of *M* and *G* standard PMP derivatives. (A) Influence of the pH value of phosphate buffer (acetonitrile proportion 17%). (B) Influence of the acetonitrile proportion (phosphate buffer pH 7.0). (C) Influence of the loading quantity of sample (phosphate buffer pH 7.0 and acetonitrile proportion 17%). (D) Influence of the flow rate (phosphate buffer pH 7.0 and acetonitrile proportion 17%).

phosphate buffer (pH 7.0) and acetonitrile in a ratio of 83:17 (v/v, %) at a flow rate of 0.8 mL/min.

3.2. The choice of degradation methods

Uronic acids are liable to destruct during acid hydrolysis and the losses of *M* and *G* are different using different degradation methods, which cause a significant deviation in the *M*/*G* ratio determined even in the same sample. To choose a suitable hydrolysis method for PSS, four commonly used degradation methods for polysaccharides were evaluated in our experiments. Comparing the peak areas of *M* and *G* derivatives degraded by four different methods, which were determined at the same chromatographic separation conditions (as shown in Fig. 3), the destruction degrees of *M* and *G* by the microwave degradation method were the lowest (peak area, *M*: 260.07; *G*: 146.04, mAu × min, Fig. 3D) and were approximately equal to those of the dilute sulfuric acid (0.1 mol/L) hydrolysis method (peak area, *M*: 235.40; *G*: 133.69, mAu × min, Fig. 3C). The losses of *M* and *G* were the highest using the TFA hydrolysis method (peak area, *M*: 122.34; *G*: 18.11, mAu × min, Fig. 3B) and losses were next highest using the traditional acid degradation method (peak area, *M*: 173.46; *G*: 54.57, mAu × min, Fig. 3A). The *M*/*G* ratios of PSS degraded by the microwave degradation method or dilute sulfuric acid hydrolysis method were approximate to the *M*/*G* ratio determined by the ^{1}H NMR method, which suggested that the two methods had less uronic acid loss. Although the microwave degradation method was rapid and there was a low loss of uronic acid, it showed the poorest repeatability (RSD = 3.78%, $n = 3$). Therefore, compared with the other three methods, dilute sulfuric acid hydrolysis was the best method for the hydrolysis of PSS, with low uronic acid loss and good repeatability (RSD = 0.57%, $n = 3$).

To further explore the optimal conditions for dilute sulfuric acid hydrolysis of PSS, an orthogonal scheme of three factors and three levels, namely, the degradation temperature (110 °C, 120 °C, 130 °C; factor A), degradation time (3 h, 4 h, 5 h; factor B) and concentration of sulfuric acid (1.0 mol/L, 0.1 mol/L, 0.01 mol/L; factor C), was carried out as shown in Table 1. Results analyzed by orthogonal design assistant II V3.1 software showed that the concentration of sulfuric acid and degradation temperature were the main factors that influenced the peak areas of *M* and *G* derivatives, and the degradation time had the least influence among three factors ($R_C > R_A > R_B$). The optimal conditions for hydrolysis of PSS were determined to be the following: the concentration of sulfuric acid should be 0.1 mol/L, the degradation time should be 4 h and the degradation temperature should be 120 °C.

3.3. Method validation

The proposed HPLC method was validated in terms of specificity, linearity, detection limit, precision, repeatability and stability. As shown in Fig. 4, both *M* and *G* monosaccharide standards gave a single chromatographic peak because of the alkaline environment in the PMP derivatization reaction, indicating that the lactone forms of uronic acids did not interfere with the separation and analysis of *M* and *G*. The retention times of two peaks in PSS were consistent with *M* and *G* monosaccharide standards. The linear ranges of *M* and *G* were evaluated using their monosaccharide standards at nine different concentrations (0.125 mg/mL, 0.25 mg/mL, 0.50 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.25 mg/mL, 1.50 mg/mL, 2.0 mg/mL and 2.5 mg/mL). The linear correlations between the peak area (*Y*, mAu × min) and the concentration (*X*, mg/mL) of *M* or *G* were excellent, and the

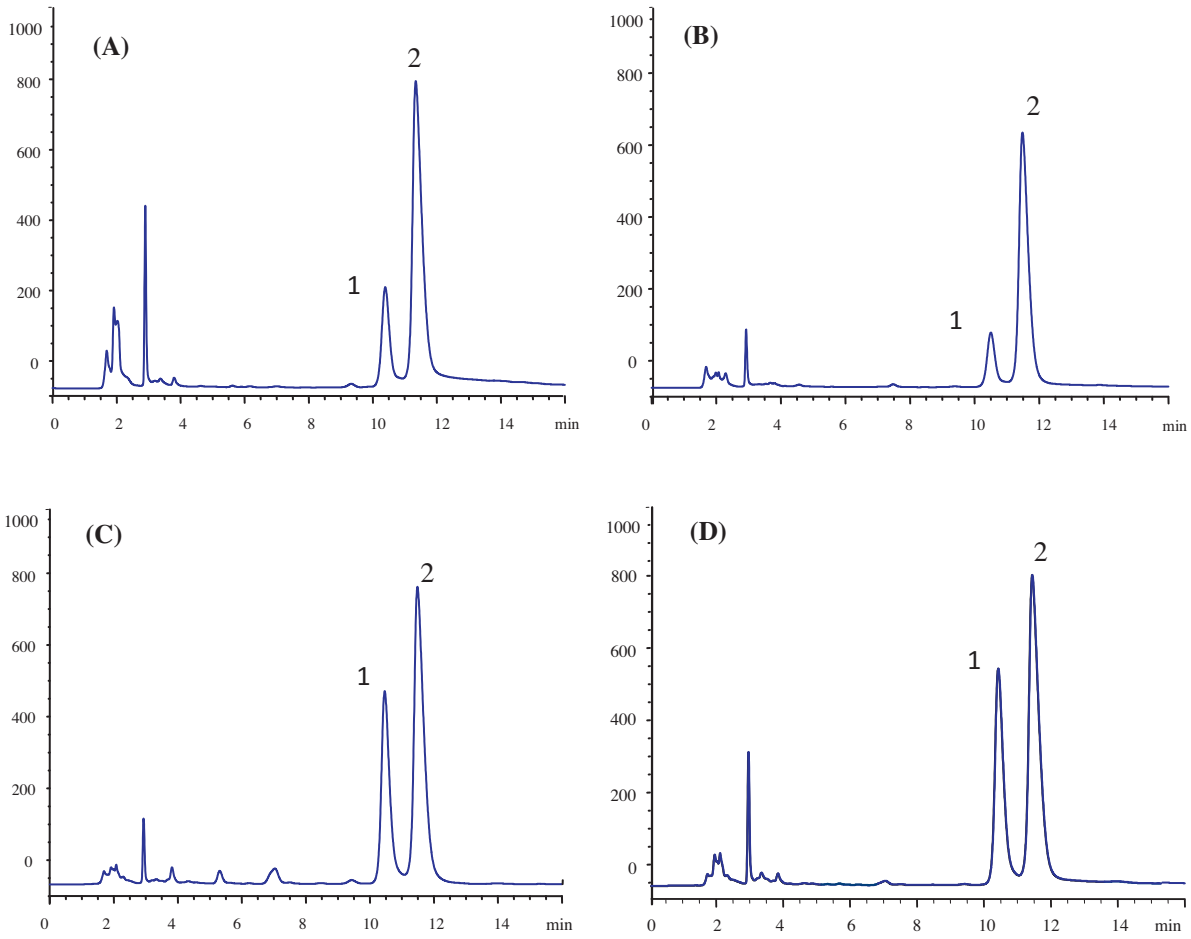

Fig. 3. Chromatograms of PSS degraded by four different methods: (A) conventional acid hydrolysis method with 80% H_2SO_4 at 20 °C for 18 h and then 2 mol/L H_2SO_4 at 100 °C for 6 h; (B) 2 mol/L TFA hydrolysis hydrolyzed at 110 °C for 6 h; (C) 0.1 mol/L sulfuric acid hydrolyzed at 120 °C for 4 h; (D) microwave irradiation (1600 W) at 130 °C for 15 min (1-guluronic acid, *G*; 2-mannuronic acid, *M*).

calibration curves were $Y = 248.94X - 9.2524$ ($R^2 = 0.9955$, *M*) and $Y = 131.75X + 4.1182$ ($R^2 = 0.9954$, *G*). The detector responses were different for the two uronic acids, and the relative response of *G* was 52.9% when *M* was 100%. The limit of detection (LOD) at the signal–noise ratio of 3 was determined to be 0.25 μg/mL for *M* and 0.40 μg/mL for *G* by successive dilutions of PMP-labeled monosaccharide standards. The limit of quantification (LOQ) at the signal–noise ratio of 10 was found to be 1.0 μg/mL for *M* and 1.5 μg/mL for *G*.

The intra-day relative standard deviation (RSD) for three PSS concentrations (4 mg/mL, 5 mg/mL and 6 mg/mL) were 0.79%, 0.49% and 0.23% ($n = 6$), respectively, and the inter-day RSD for the three PSS concentrations were 2.23%, 1.64% and 1.20% ($n = 5$), respectively, indicating that the method precision and reproducibility were satisfactory. The stability of the PSS derivative stored at room temperature was determined at 0 h, 1 h, 3 h, 5 h, 8 h and 24 h, and the RSD was 1.75% ($n = 6$), indicating that the PMP derivative was stable within 24 h.

Table 1
Results of L_9 (3^4) orthogonal test of PSS hydrolyzed by dilute sulfuric acid method.

No.	Temperature (factor A, °C)	Reaction time (factor B, h)	Acid concentration (factor C, mol/L)	Peak area of *M* and *G* (mAu × min)
1	130	5	1	32.82
2	130	4	0.1	225.43
3	130	3	0.01	125.57
4	120	5	0.1	329.16
5	120	4	0.01	113.69
6	120	3	1	279.47
7	110	5	0.01	45.95
8	110	4	1	343.76
9	110	3	0.1	116.51
K[a]1	127.94	135.98	87.67	
K2	240.77	227.63	223.70	
K3	168.74	173.85	95.07	
R[b]	112.83	91.65	128.63	

[a] K: mean value.
[b] R: range analysis.

Table 2
Comparison of *M*/*G* ratios of PSS, PMS, PGS and their desulfurization products, with the intermediates determined by the ^{1}H NMR method and HPLC methods, respectively[a].

HPLC method			^{1}H NMR method		
Sample	*M*/*G* ratio	Intermediate	*M*/*G* ratio	Desulfurization sample	*M*/*G* ratio
PSS	0.94	LMWA	0.98	DPSS	0.88
PMS	4.04	PM	4.01	DPMS	3.97
PGS	0.12	PG	0.11	DPGS	0.13

[a] (LMWA, low-molecular-weight alginate; PM, polymannuronate; PG, polyguluronate; DPSS, desulfurization product of PSS; DPMS, desulfurization product of PMS; DPGS, desulfurization product of PGS).

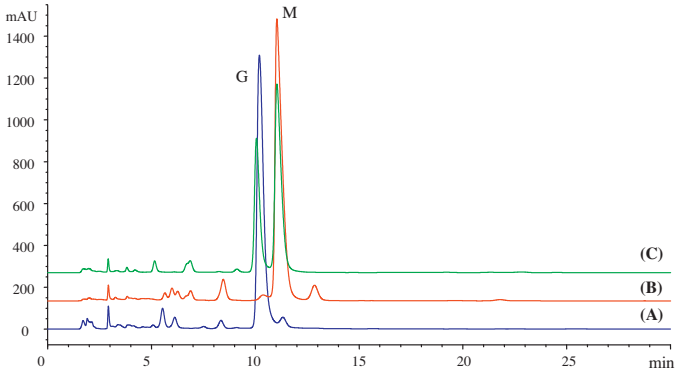

Fig. 4. Chromatograms of α-L-guluronic acid (A), β-D-mannuronic acid (B) and PSS(C).

Table 3
M/*G* ratios of PSS samples that were collected from different manufacturers in China.

Sample	Peak area (average, mAu × min)		Peak area ratio	*M*/*G* ratio	RSD (%) (*n* = 3)
	G	*M*			
1	76.49	181.91	2.38	1.26	1.62
2	68.90	164.98	2.39	1.26	0.83
3	72.71	165.44	2.28	1.21	1.60
4	128.18	224.39	1.75	0.93	1.81
5	127.01	240.08	1.89	1.00	1.34
6	132.81	238.29	1.79	0.95	1.10
7	112.84	233.91	2.07	1.10	0.74
8	124.35	204.55	1.65	0.87	1.54
9	106.58	227.59	2.14	1.13	0.63
10	89.94	174.61	1.94	1.03	0.53

3.4. *M*/*G* ratios of PSS from different manufacturers

To further demonstrate that the proposed HPLC method is optimal, we employed a desulfurization strategy to overcome the influence of the sulfate group on the resolution of anomeric proton signals. We determined the *M*/*G* ratio of PSS by the NMR technique using its intermediate (low-molecular-weight alginate) and desulfurization product (as shown in Fig. 5). In addition, the *M*/*G* ratios of two other alginate sulfated derivatives; i.e., polymannuronate sulfate (PMS) and polyguluronate sulfate (PGS), and their desulfurization product, intermediates (i.e., polymannuronate and polyguluronate, respectively) were also detected by NMR and HPLC methods, respectively, to demonstrate the feasibility of the desulfurization tactics. Results showed that the *M*/*G* ratios of PSS, PMS and PGS determined by the HPLC method were in good accordance with those obtained by the traditional non-destructive ^{1}H NMR method (as shown in Table 2) using their desulfurization products.

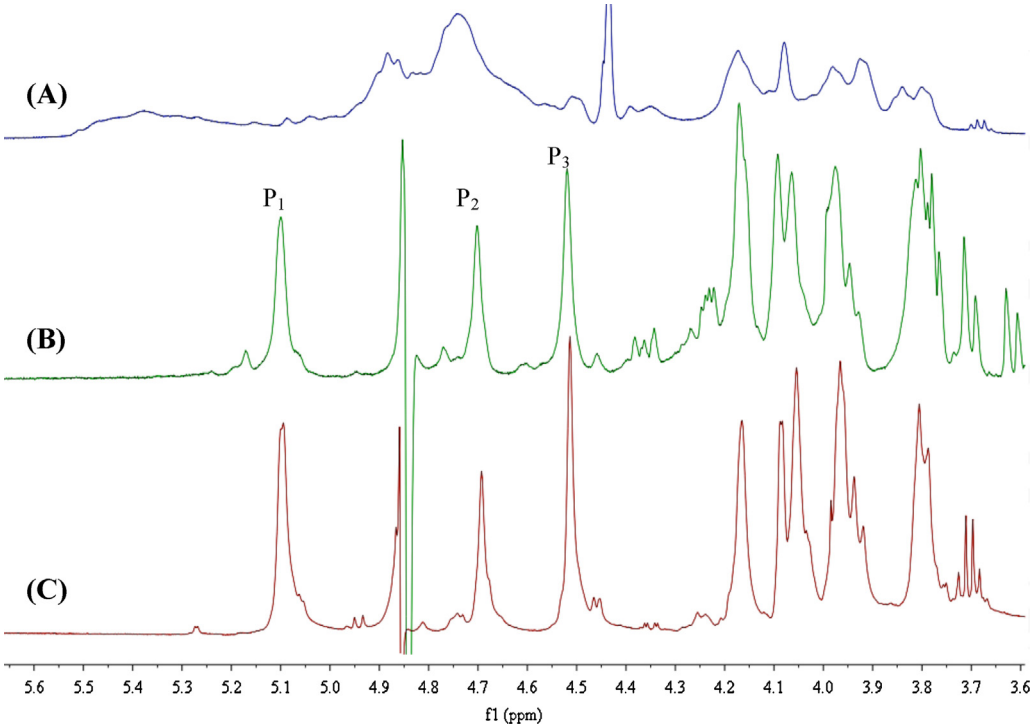

Fig. 5. ^{1}H NMR spectra of PSS (A), the intermediate (low-molecular-weight alginate) of PSS (B) and the desulfurization product of PSS (C) recorded at 20 °C on a Bruker Avance 500 MHz spectrometer (P_1, signal at 5.1 ppm, H_1-G; P_2, signal at 4.7 ppm, H_1-M and H_5-GM; P_3, signal at 4.5 ppm, H_5-GG).

This showed that the HPLC method was accurate in determining the *M*/*G* ratio of PSS. The *M*/*G* ratio results for PSS samples that were collected from different manufacturers in China are shown in Table 3. There were obvious differences in the *M*/*G* ratios for the ten PSS samples, which likely result from the differences in raw material alginates. This means it is necessary to strengthen the quality control of PSS.

4. Conclusion

A rapid and accurate HPLC with pre-column derivatization method was developed for determination of the *M*/*G* ratio of PSS. This method was validated by comparison with the non-destructive ^{1}H NMR method. The HPLC separation conditions of *M* and *G* derivatives were optimized and the pH value of the phosphate buffer and the proportion of acetonitriles obviously affected the resolution of the two derivatives. The dilute sulfuric acid (0.1 mol/L) degradation method is suitable for the hydrolysis of PSS with low uronic acid loss, has good repeatability and is simple to operate, while high losses of *M* and *G* are found in commonly used 2 mol/L TFA hydrolysis and traditional sulfuric acid degradation methods. There were significant differences in the *M*/*G* ratios of PSS samples that were collected from different manufacturers in China. It is necessary to strengthen the quality control of PSS to ensure its clinical efficacy and reduce the incidence of adverse drug reactions.

Acknowledgements

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