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The use of high-performance size exclusion chromatography (HPSEC) as a molecular weight screening technique for polygalacturonic acid for use in pharmaceutical applications

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Abstract

Polygalacturonic acid is a linear carbohydrate polymer of monomeric galacturonic acid. It is commercially available as apple and citrus pectins comprised of a mixture of partially methoxylated and/or amidated polygalacturonic acids with molecular weights ranging from 25 000 to $>100\,000$ Da. Pectin can be chemically or enzymatically hydrolyzed to yield polygalacturonic acid fractions of diverse average molecular weight ranges and polydispersities for a variety of uses. Pectin and polygalacturonic acid are used extensively as gelling agents and stabilizers by the food industry, and have applications as therapeutic, and diagnostic pharmaceutical agents such as the magnetic resonance imaging agent LumenHance®. A simple high-performance size exclusion chromatography (HPSEC) method, employing commonly available non-specialized HPLC instrumentation, is described for use as a rapid molecular weight screening technique to determine the average molecular weight range and polydispersity of polygalacturonic acid intended for use in pharmaceutical formulations. A TosoHaas G3000PWXL HPLC column, 50 mM phosphate buffer (pH $\approx$ 6.9) mobile phase, and refractive index detection were used. A molecular weight calibration curve was linear for polysaccharide standards of 180–100 000 Da with a coefficient of correlation of 0.999. The method was employed to screen commercially available polygalacturonic acid raw materials for average molecular weight data (M_n, M_w, and M_p) and polydispersity (M_w/M_n).

Keywords: Polygalacturonic acid; Pectin; High-performance size exclusion chromatography; Average molecular weight; Polydispersity

1. Introduction

Polygalacturonic acid is a linear polysaccharide polymer comprised of α-(1 $\rightarrow$ 4)-linked monomeric galacturonic acid with the resulting formula $(C_6H_8O_6)_nH_2O$ (Fig. 1). Polygalacturonic acid is commercially available as apple and citrus pectins comprised chiefly of a mixture of partially methoxylated and/or amidated polygalacturonic acids (Fig. 2) with molecular weights ranging from 25 000 to $>100\,000$ Da [1]. Pectin can be chemically [2] or enzymatically [3,4] hydrolyzed,

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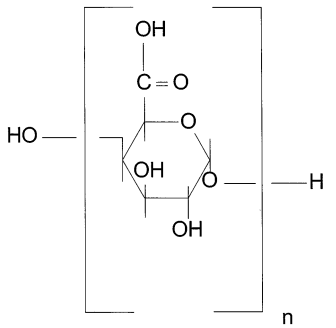

Fig. 1. Chemical structure of polygalacturonic acid.

the choice of which yields polygalacturonic acid of diverse average molecular weight ranges and polydispersities for a variety of uses in the food and, particularly, pharmaceutical industries [5,6]. Natural biocompatible polymers, such as polygalacturonic acid, have been used in admixture with paramagnetic, superparamagnetic or proton density contrast agents, such as LumenHance® [7], for use in diagnostic magnetic resonance imaging [8,9]. Polygalacturonic acid has also been used in combination with physiological compatible dispersions of stabilized superparamagnetic particles bound to tissue specific and pharmacologically active agents for NMR contrast media and other diagnostic purposes [10]. Antitumor effects have been demonstrated using a polygalacturonic acid ethylenimine derivative as a polymer carrier system for cytotoxic components [11]. Polygalacturonic acid has also been evaluated as a potential mucoadhesive carrier for ophthalmic drugs [12]. Sulfated polygalacturonic acid has been shown to inhibit the proliferation of cells in culture without producing cell damage [13]. Some allergy inhibiting compounds contain polygalacturonic acid as an active ingredient [14]. Polygalacturonic acid has been used in the treatment of hyperlipidemia, atherosclerosis [15,16], and in platelet aggregation inhibitors and antithrombotics [17]. Pharmaceutical preparations containing polygalacturonic acid have been used to block the attachment of pathogenic microorganisms to human cells [18] and as remitting agents for nephrotic syndrome and hepatopathy symptoms [19].

Numerous studies have been reported on the size exclusion chromatography analysis of pectin and polygalacturonic acid using modes of detection including refractive index, ultraviolet absorption, laser light scattering, and viscometry [20–24]. Our focus was to develop a simple, reproducible, and robust high-performance size exclusion chromatography (HPSEC) method which allowed rapid screening of commercially available polygalacturonic acid raw materials to be used in

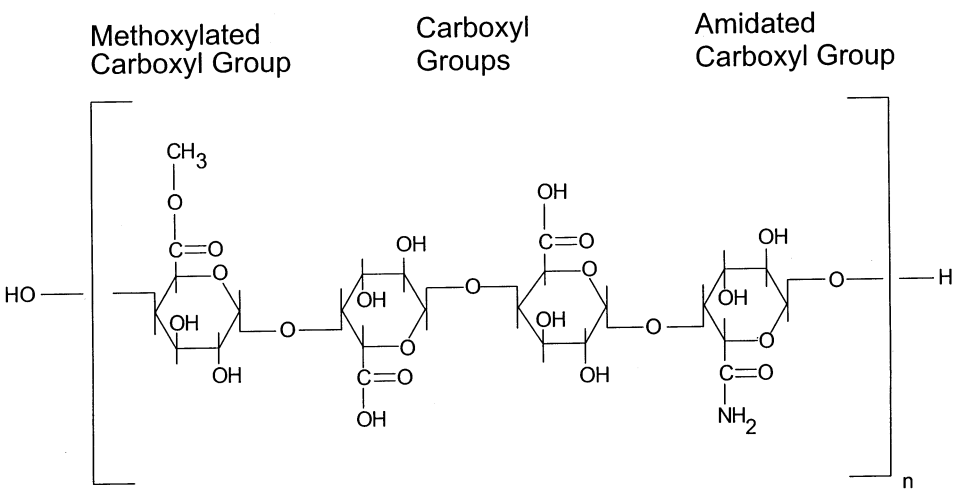

Fig. 2. Representative chemical structure of pectin showing typical repeating groups.

pharmaceutical agents by providing size (average molecular weight) and homogeneity (polydispersity) information. Within the working range of the method, narrow molecular weight distribution ($M_w/M_n = 1.1$) maltotriose polysaccharide standards were employed. These standards are comparable to polygalacturonic acid in terms of structure and appear to have similar hydrodynamic volumes (V_h) using pH 6.9, 50 mM buffer which suppresses electrostatic-base chain expansion of the polygalacturonic acids, ensuring the accuracy of the molecular weight data obtained.

2. Experimental

2.1. Materials

Polysaccharide molecular weight standards were supplied as a kit of ten standards, including glucose (180 Da), stachyose tetrahydrate (740 Da), and polymaltotrioses consisting of links of maltotriose from 6000 to 800 000 Da, measured by ultracentrifugal sedimentation equilibrium, from Polymer Labs (Amherst, MA, USA). Commercially available polygalacturonic acids were supplied from ICN (Costa Mesa, CA, USA) Cat. No. 102711, Fluka (Milwaukee, WI, USA) Cat. No. 81325, Pfaltz & Bauer (Waterbury, CT, USA) Cat. No. P21750, Selectchemie AG (Zurich, Switzerland), Sigma (St. Louis, MO, USA) Cat. No. P-3889, Lancaster Synthesis (Windham, NH, USA) Cat. No. 4206, United States Biochemical (Cleveland, OH, USA) Cat. No. 20595, R.W. Greeff Howard Hall (Greenwich, CT, USA) Cat. No. 201, and Scientific Polymer Products (Ontario, NY, USA) Cat. No. 153. De-ionized water was organic free, Type 1, 18-MΩ resistance, supplied from a Barnstead (Dubuque, IA, USA) Nanopure II system. Potassium phosphate monobasic anhydrous was analytical reagent grade supplied by Fisher (Pittsburgh, PA, USA) with declared purity of 99.5%. Potassium phosphate dibasic anhydrous was analytical reagent grade supplied by Mallinckrodt (Paris, KY, USA).

2.2. HPSEC system

The HPSEC system consisted of a Spectra-Physics 8800 pump and 8880 autosampler with a 50-μl, fixed loop injector (Thermo Separation Products, Piscataway, NJ, USA), and a Hewlett-Packard 1047A refractive index detector set at 40°C (Hewlett-Packard, Wilmington, DE, USA). A TSK-GEL® G3000PWXL, 6-μm, 300×7.8 mm, 200-Å column (molecular weight range up to 60 000 Da for dextrans; typical theoretical plates, 21000) with a PWXL, 40×6.0-mm guard column (TosoHaas, Montgomeryville, PA, USA), and a CH-30 column heater maintained at a temperature of 40 ± 1°C (Fiatron, Oconomowoc, WI, USA) were used. The mobile phase, comprised of aqueous potassium phosphate monobasic and dibasic (50 mM), delivered at a flow rate of 0.7 ml min^{-1}, resulted in typical operating pressures of approximately 300 lb in^{-2} and a pH of approximately 6.9 with no adjustments made. Multichrom® with GPC software (Thermo LabSystems, Shrewsbury, MA, USA) was used to acquire and analyze the molecular weight data.

2.3. Standard and sample preparation

Individual molecular weight standard solutions were prepared fresh daily by dissolving 25 mg each of seven polysaccharide standards from 180 to 100 000 Da in 50 ml of 50 mM phosphate mobile phase buffer. Polygalacturonic acid sample solutions were prepared in a similar fashion.

2.4. Procedure

The seven polysaccharide standard solutions were injected in duplicate in order of decreasing molecular weight and the GPC software was used to perform a narrow band linear regression standard calibration curve of log molecular weight versus HPSEC retention time. The sample preparations were then injected in duplicate. Once the calibration curve was established, the 24 000 molecular weight standard was used daily to verify system suitability. The entire standard calibration was rerun if the retention time of the 24 000 molecular weight standard changed by $> 2.0\%$.

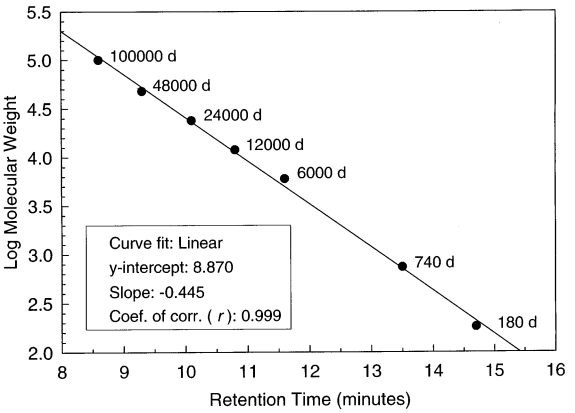

Fig. 3. Polysaccharide molecular weight (180–100 000 Da) standard curve.

Using the GPC software, the sample areas were integrated to include the entire polygalacturonic acid molecular weight distributions. Sample average molecular weight results (M_n, M_w, M_p, and M_w/M_n or polydispersity, a measure of the width of the molecular weight distribution, the higher the ratio, the greater the width of the distribution) were generated based on the standard curve. M_n = number average molecular weight = $ta/\text{sum}(a/m)$, M_w = weight average molecular weight = $\text{sum}(a*m)/ta$, where: ta, total area of the distribution; a, area of the slice; m, molecular weight of the slice; sum, sum of all. M_p = peak average molecular weight (average molecular weight at peak apex); M_w/M_n = polydispersity. M_n, M_w, and M_p results were rounded to the nearest 1000 Da, and polydispersity to the nearest 0.1.

3. Results and discussion

3.1. Method optimization

Since polygalacturonic acid is insoluble in acidic aqueous solutions, the procedure optimized the solvent system to be a neutral, aqueous, buffered solution. Doubling the mobile phase buffer strength from 50 to 100 mM phosphate buffer (pH $\approx$ 6.9) resulted in approximately a 1% increase in retention time for the 24 000 molecular weight standard, within the method's experimental variation, indicating that size exclusion was the only mode of separation taking place. To keep the salt concentration at a minimum while still maintaining adequate standard and sample solubility, 50mM phosphate buffer (pH $\approx$ 6.9) was chosen as both HPSEC mobile phase and standard and sample diluent. Since an HPSEC column separates molecules by size or hydrodynamic volume (V_h), not their molecular weight, it was important to choose molecular weight standards (maltotriose polysaccharides) which were very close in structure to polygalacturonic acid. With the application of this method as a screening technique, detection limit and sensitivity were not concerns, therefore, refractive index detection was chosen over UV absorbance due to the low UV extinction coefficients of carbohydrates.

3.2. Method validation

3.2.1. Linearity

The linearity of the method was evaluated over a >2 order of magnitude molecular weight range. A seven level molecular weight calibration curve showed linearity (coefficient of correlation, $r =$ 0.999) with polysaccharide standards (0.5 mg ml^{-1}) in the range of 180–100 000 Da when log molecular weight versus retention time was plotted (Fig. 3). The inclusion volumes obtained for the 180 (10.29 ml) and 100 000 (6.02 ml) polysaccharide standards fell within the total inclusion (11.46 ml) and exclusion (5.73 ml) volume limits provided by the column manufacturer. Although the total molecular weight exclusion limits are listed as up to 60 000 for dextrans, the 100 000 standard did not adversely affect the linearity of the calibration curve. Polysaccharide molecular weight standard linearity and peak distribution data are provided in Table 1.

3.2.2. Precision

The method's precision was demonstrated by evaluating molecular weight linearity of the calibration curve, on the same column, for 3 days. Coefficients of correlation (r) were ≥ 0.999 for the three linear regression analyses. Also, RSDs of 0.4, 0.5, and 0.6% ($n = 6$), for peak retention

Table 1
Polysaccharide molecular weight standard linearity and peak distribution data

Polysaccharide	Molecular weight (Da)	Retention time (min)	Polydispersity (M_w/M_n)
Glucose	180	14.7	1.0
Stachyose tetrahydrate	740	13.5	1.0
Polymaltotriose ($n \cong 12$)	6000	11.6	1.1
Polymaltotriose ($n \cong 25$)	12 000	10.8	1.1
Polymaltotriose ($n \cong 49$)	24 000	10.1	1.1
Polymaltotriose ($n \cong 99$)	48 000	9.3	1.1
Polymaltotriose ($n \cong 206$)	100 000	8.6	1.1
Slope	−0.445		
y-intercept	8.870		
Coefficient of correlation (r)	0.999		

Table 2
Precision data for molecular weight linearity of calibration curves and 180 (low end), 24 000 (mid-range), and 100 000 (high end) polysaccharide standards on one column for 3 days

Day	Coefficient of correlation (r)	Injection	Polysaccharide standard retention time (min)		
			180	24 000	100 000
1	0.999	1	14.7	10.2	8.7
		2	14.7	10.2	8.7
2	0.999	1	14.6	10.1	8.6
		2	14.6	10.1	8.6
3	0.999	1	14.7	10.1	8.6
		2	14.7	10.1	8.6
Mean			14.7	10.1	8.6
S.D.			0.1	0.1	0.1
RSD (%)			0.4	0.5	0.6

times of the 180 (low end), 24 000 (mid-range), and 100 000 (high end) polysaccharide molecular weight standards were obtained, respectively (Table 2).

3.2.3. Accuracy

The molecular weight results, generated by this HPSEC method for one of the polygalacturonic acid samples (Lancaster Synthesis), were confirmed by the alternate technique of laser light scattering (Polymer Laboratories, Church Stretton, Shropshire, UK) using a PL-LSP multi-angle light scattering photometer, with 50 mM phosphate buffer pH 6.9 as solvent, specific refractive index increment (dn/dc) of 0.1144 ml g^{-1}, and wavelength of 632.8 nm. All samples were filtered through 0.2-μm Millex syringe filters before measurement. The molecular weight data obtained verified the accuracy of the HPSEC method (Table 3). In addition, within the experimental errors of the light scattering and HPSEC methods, the light scattering data confirmed both the mode of separation in the HPSEC method as being size exclusion with little stationary phase

Table 3
Comparative molecular weight analysis of polygalacturonic acid (Lancaster Synthesis) by HPSEC and laser light scattering

Analytical technique	Molecular weight (Da)
HPSEC	52 000
Laser light scattering	60 000

Table 4
Evaluation of method robustness based on molecular weight linearity of the calibration curves, and molecular weight and polydispersity of the 180 (low end), 24 000 (mid-range), and 100 000 (high end) molecular weight standards using multiple columns and analysts on 3 days

Column ID	Day	Coefficient of correlation (r)	Slope	y-intercept	M_p/Polydispersity		
					180 mol.wt.	24 000 mol.wt.	100 000 mol.wt.
A	1	0.999	−0.445	8.870	200/1.0	23 000/1.1	105 000/1.1
B	2	0.999	−0.456	8.977	200/1.0	23 000/1.1	105 000/1.2
C	3	0.998	−0.443	8.983	200/1.0	23 000/1.1	111 000/1.1
Mean			−0.448	8.943	200/1.0	23 000/1.1	107 000/1.1
S.D.			0.007	0.064			
RSD (%)			1.6	0.7			

interaction and the structural similarity between the polysaccharide standards and polygalacturonic acid.

3.2.4. Robustness

The robustness of the method was examined in terms of column-to-column, analyst-to-analyst, and day-to-day performance; three columns of differing lots compared favorably when results obtained by multiple analysts on 3 days were evaluated for molecular weight linearity of the calibration curves, and molecular weight and polydispersity of the 180 (low end), 24 000 (mid-range), and 100 000 (high end) molecular weight standards (Table 4). As further evidence of robustness, injections ($n = 6$) of the 24 000 mid-range molecular weight standard produced an RSD of 0.4%, when the mobile phase buffer concentration was varied from 40 to 50 to 60 mM, indicating that mobile phase buffer concentration changes up to 20% had insignificant effects on the method's performance. These buffer concentration changes had no effect on pH (≈ 6.9). In addition, column durability was established with approximately 2000 injections made over 5 years on a single column, during which period the retention time of the 24 000 mid-range molecular weight standard did not vary by more than 2% and the same peak shape was maintained (indications that theoretical plate count was maintained), and column pressure increased by only 30 lb in^{-2}.

3.2.5. System suitability

Fig. 4 demonstrates the suitability of the method in the ability to resolve polysaccharide standards of 740, 6000, 24 000 and 100 000 Da. The 24 000 mid-range standard was used as a daily system suitability check. Restandardization was performed if the observed retention time of the 24 000 mid-range standard varied by more than 2% from the calibration curve retention time.

3.3. Method application

The application of the method to demonstrate the diversity and denote distinct differences in the molecular weight averages and polydispersities of

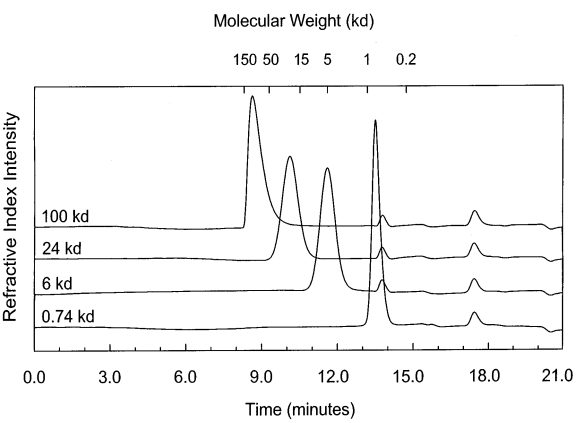

Fig. 4. Chromatograms of four polysaccharide standards: 740, 6000, 24 000, and 100 000 Da.

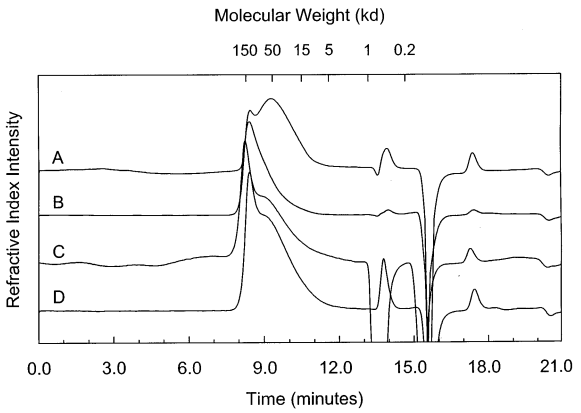

Fig. 5. Representative chromatograms of four commercially available polygalacturonic acids, A: Lancaster Synthesis, B: Selectchemie AG, C: Scientific Polymer Products, D: Fluka.

polygalacturonic acid from varying commercial suppliers is emphasized in the chromatographic molecular weight distribution profile overlays in Fig. 5. These varying profiles also show the presence or absence of lower molecular weight components (< 1000 Da). Average molecular weight data for nine representative commercially available polygalacturonic acid raw materials are summarized in Table 5. These results show that polygalacturonic acids can be supplied as high molecular weight polymers ($M_p \cong 150\,000$) with broad ($M_w/M_n \cong 2.5$) distributions, intermediate molecular weight ($M_p \cong 130\,000$) with intermediate ($M_w/M_n \cong 2.0$) or narrow ($M_w/M_n \cong 1.3$) distributions, or lower molecular weight ($M_p \cong 50\,000$) with intermediate ($M_w/M_n \cong 1.8$) distributions. These differences are reflective of the manufacturing process (hydrolysis type, time, temperature, etc.) and purification used by the supplier. As can be seen, experimentally obtained molecular weight values can be quite different than the vendor supplied values likely to have been determined by a variety of methods. Since the inclusion volume (6.02 ml) of the 100 000 molecular weight standard approaches the total exclusion volume limit (5.73 ml) of the column, the use of a larger pore-size column calibrated with higher molecular weight standards would be recommended for polygalacturonic acid samples of significantly higher molecular weight beyond the working range of this method.

4. Conclusions

An HPSEC method with refractive index detection was developed for use as a rapid and simple molecular weight screening technique to determine the average molecular weights and polydispersities of polygalacturonic acid. Application of the method noted distinct molecular weight distribution differences among various commercial sources of polygalacturonic acid. The method can be used as an aid in the selection of polygalacturonic acid of a particular size and homogeneity for specific pharmaceutical applications.

Table 5
Average molecular weight data for representative commercially available polygalacturonic acid raw materials

Vendor	Vendor description (Da)	Molecular weight (Da)			Polydispersity
		M_n	M_w	M_p	(M_w/M_n)
ICN	25 000–70 000	34 000	74 000	130 000	2.2
Fluka	25 000–50 000	36 000	73 000	126 000	2.0
Pfaltz & Bauer	ND[a]	39 000	86 000	131 000	2.2
Selectchemie AG	ND[a]	77 000	96 000	129 000	1.3
Sigma	15 000–40 000	43 000	86 000	134 000	2.0
Lancaster Synthesis	25 000–50 000	32 000	59 000	52 000	1.8
United States Biochemical	ND[a]	42 000	91 000	137 000	2.1
R.W. Greeff Howard Hall	25 000–70 000	56 000	100 000	147 000	1.9
Scientific Polymer Products	50 000	36 000	90 000	151 000	2.5

[a] ND, not described.

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