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Conductivity detection for molecular mass estimation of per-*O*-sulfonated glycosaminoglycans separated by high-performance size-exclusion chromatography

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Abstract

Chemically per-*O*-sulfonated polysaccharides, including glycosaminoglycans (GAGs) and hyaluronan oligosaccharides were analyzed using high-performance size-exclusion chromatography (HPSEC) with suppressed conductivity detection. The results were compared to those obtained by gel filtration HPLC using UV detection or fluorescence detection after the post-column reaction with 2-cyanoacetamide in strong alkaline solution. Analysis was performed on a TSKgel G3000SWXL HPSEC column in 5 mM boric acid (pH 7.0 adjusted by 10 mM NaOH). The use of conductivity detection, in the absence of any derivatization and under isocratic conditions gave a limit of detection in the picogram range. Preliminary studies suggest that this approach may be particularly useful in examining sulfonated polysaccharides and oligosaccharides having no UV chromophore, such as those prepared from *O*-sulfonated fucans and galactans isolated from algae. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

We have reported the preparation and characterization of chemically per-*O*-sulfonated glycosaminoglycan (GAG) chains in several papers and have also shown that these modified GAG chains exhibit interesting biological activities, such as anticoagulant and anti-proliferative actions [1–5]. Investigation of

molecular size and conformational structure of these chemically modified GAGs may be important for better understanding the mechanisms of the biological activities of GAGs prepared for medicinal applications [6]. The behavior of each of these chemically modified GAG chains under electrophoresis suggests that they maintain their original size (i.e., no breakdown of glycosidic linkages is observed) following *O*-sulfonation [1–3]. However, the relationship between the biological activity and the molecular size of these chemically modified GAG chains is still unclear.

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High-performance liquid chromatography has been widely used for the structural and compositional analysis of complex GAGs [7–10]. Enzymatic depolymerization of uronic acid containing GAGs, using polysaccharide lyases, affords oligosaccharides having an unsaturated uronic acid residue at their non-reducing terminus, which can be conveniently detected by measuring absorbance at 232 nm [7]. Furthermore, the post-column derivatization of unsaturated disaccharides produced from GAGs by enzymatic digestion, using 2-cyanoacetamide facilitates fluorescence detection and makes it possible to determine the unsaturated disaccharides at the femtomole level, even though the structure(s) of fluorophore(s) and the reaction mechanism are still unclear [11,12]. High-performance size-exclusion chromatography (HPSEC) is increasingly used to evaluate molecular sizes of polysaccharides such as GAGs and polygalacturonic acid [13–19]. However, some secondary effects, such as ionic interactions between the stationary phase and the polysaccharide can distort the actual size distribution of polymer molecules. These effects are especially pronounced in the case of polyelectrolyte molecules due to the electrostatic field surrounding polyanions, such as chemically persulfonated GAGs. Additionally, it is often impossible to detect GAG chains at nanomole level due to a lack of chromophore in their structures.

Conductivity detection is well established for the analysis of inorganic ions [20,21] and should be applicable to the analysis of sulfonated esters common to many acidic polysaccharides [22]. However, to obtain high sensitivity, all salts, such as sodium chloride, must be removed from an eluant. In contrast, borate solution at neutral pH shows no significant conductivity [23]. Borate ion also interferes with ionic interactions, including those between polysaccharides and the hydroxyl groups of a stationary phase, such as silica gel, that limit resolution in the HPSEC separation of polyanions. This report examines the use of size-exclusion HPLC with suppressed conductivity detection to analyze chemically per-*O*-sulfonated GAGs and hyaluronan oligosaccharides (HAoligos). The results are compared to those obtained using UV detection and fluorescence detection following derivatization with 2-cyanoacetamide in strong alkaline solution.

2. Materials and methods

2.1. Chemicals

Chondroitin sulfate (\overline{M}_r , 15 000) from bovine tracheal cartilage, and dermatan sulfate (\overline{M}_r , 16 000) from porcine skin were a kind gift from Shin-Nippon Yakugyo (Tokyo, Japan). Hyaluronan (HA) standards (\overline{M}_r 20 000 and 100 000, and \overline{M}_r , 790 000), from *Streptococcus zooepidemicus* and from human umbilical cord, respectively, were purchased from Kibun Food Chemipha (Tokyo, Japan) and Seikagaku (Tokyo, Japan). Heparin (\overline{M}_r , 16 000) and heparan sulfate (\overline{M}_r , 14 800) from porcine intestinal mucosa were purchased from Celsus (OH, USA). Hyaluronidase from *Streptomyces hyalurolyticus* (lyase, E.C.4.2.2.1) was purchased from Seikagaku Kogyo, Tokyo, Japan. Dextran sulfate sodium salts (\overline{M}_r , 5000, 8000 and 10 000) used as standards, were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Sephadex G-50 (superfine) and Hi-Trap desalting columns were purchased from Pharmacia Biotech Japan (Tokyo, Japan). Dialysis tubing (molecular mass cut-offs, MWCO, 500) was purchased from Wako (Osaka, Japan). All other chemicals used were the analytical grade unless otherwise stated.

2.2. Instruments

The HPSEC system consisted of a Jasco 980-PU pump (Nihonbunko, Tokyo, Japan) and Rheodyne 7725i loop injector (USA), and a conductivity detector (CM-8, Tosoh, Tokyo, Japan), equipped with platinum needle electrodes (0.1 mm diameter \times 1 mm long, 0.393 mm² active surface for each) spaced 1 mm with a columnar cell volume of 20 μ l. An TSK-GEL G3000SWXL (6 μ m, 300 mm \times 7.8 mm I.D., Tosoh) column connected with a Dowex 50W-X8 suppressor column (200–400 mesh, H⁺ form, 200 mm \times 5.0 mm I.D.) was used at 30 °C. The mobile phase, comprised of 5 mM borate (pH 7.0 adjusted by 10 mM NaOH), was delivered at a flow-rate of 0.5 ml/min. A Hitachi integrator D-2500 was used to acquire and analyze the data.

The gradient HPLC system to demonstrate the purity of HAoligos was assembled with gradient pumps (Jasco 980-PU, intelligent HPLC pumps), an

eluent mixer (Jasco HG-980-3, solvent mixing module) and a fluorescence detector (Jasco FP-1520S intelligent fluorescence detector) from Nihon Bunko, Japan. A variable sample injector (VMD-350) was from Shimamura, Japan. A UV detector (L-4000) was from Hitachi Seisakusho, Japan. The CE system was assembled with Beckman capillary electrophoresis system (P/ACE 5010) equipped with a UV detector and an operation system using version 0.4P/ACE station on an IBM-compatible personal computer, from Beckman, USA. JEOL ECP400 and 600 NMR instruments, equipped with a 5-mm field gradient tuneable probe with standard JEOL software, were used for one- and two-dimensional ^1H NMR experiments at 30 °C on 500 μl of each sample.

2.3. Preparation of chemically oversulfated glycosaminoglycans

Chemical *O*-sulfonation to obtain per-*O*-sulfonated GAGs was carried out under mild conditions with adducts of sulfur trioxide (SO_3) in aprotic solvents [1]. Briefly, the tributylammonium (TBA) purchased from Aldrich (Milwaukee, WI, USA) salt was prepared from 100 mg of the sodium salt of each GAG by strong cation-exchange chromatography with a Dowex 50W-X8 (H^+ form) column (5 cm \times 2 cm I.D.) and recovered by lyophilization. A portion (10 mg) of the resulting TBA salt was dissolved in 0.8 ml of *N,N*-dimethylformamide (DMF, Aldrich) to which a required excess (15 mol/equivalent of available hydroxyl group) of pyridine–sulfur trioxide complex had been added. After 1 h at 40 °C, the reaction was interrupted by addition of 1.6 ml of water and the crude product was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate obtained from Aldrich, and then collected by centrifugation at 1500 *g* for 15 min. Each resulting per-*O*-sulfonated GAG was dissolved in 5 ml water, dialyzed against water to remove salts for 16 h at 4 °C, and lyophilized. In the case of *O*-sulfonation of heparin and heparan sulfate, partial loss of the *N*-sulfonate group from GlcpNS residues occurred during *O*-sulfonation. These derivatives were re-*N*-sulfonated according to previously described methods [2].

2.4. Preparation of HA oligosaccharides [3]

The large-scale, partial depolymerization by bacterial hyaluronidase was carried out on 1.0 g of HA. To a solution containing 1.0 g of HA in 200 ml buffer, 2 ml of hyaluronidase solution (400 TRU/ml) was added, and the enzymatic digestion was performed in a glass flask at 60 °C. When the reaction reached to 40% digestion based on the absorbance at 232 nm, the reaction was stopped by immersing the flask for 3 min in a boiling water bath. The sample was cooled in an ice bath and dialyzed for several days at 4 °C in 500 MWCO dialysis tubing against deionized and distilled water, and then the sample was freeze-dried. The resulting white powder was dissolved in 20 ml water and was directly applied onto a low-pressure size-exclusion chromatography (LPSEC) column.

The 4- to 20-mer HAoligos were fractionated on a Sephadex G-50 (superfine) column (1 m \times 4.4 cm) eluted with 0.2 *M* sodium chloride at an optimum flow-rate defined by the Dracy's law. Freeze-dried HAoligo mixture sample (~100 mg) was dissolved in 20 ml water and applied to the column, 300 fractions were collected (5 ml/tube each) and absorbance at 232 nm of each fraction was measured. Each unified HAoligo fraction was collected and concentrated by evaporation. If necessary, this chromatographic separation was performed repeatedly.

Each size-uniformed HAoligo fraction obtained from second LPSEC was desalted using a Hi-Trap desalting column eluted with water at 1.0 ml/min. The eluent was collected and the fractions containing HAoligos were combined. The HAoligo samples were freeze-dried. Preparation of per-*O*-sulfonated HAoligos was based on a modification of previously described methods [1]. Tributylamine (50 μl) was added to size-uniformed HAoligos (1.0 mg) in 1.0 ml of distilled water adjusted to pH 2.80 with 0.1 *M* hydrochloric acid to obtain the TBA salt of HAoligos. The reaction mixtures were mixed vigorously and then freeze-dried. The resulting TBA salt was dissolved 0.2 ml of DMF to which a required excess (15 mol/equivalent of available hydroxyl group) of pyridine–sulfur trioxide complex had been added. After 3 h at 40 °C, the reaction was interrupted by the addition of 0.5 ml of water and the crude product

was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate, and then collected by centrifugation. The resulting per-*O*-sulfonated HAoligos were dissolved in 500 μ l of water, centrifuged and the supernatant was diluted with water and concentrated repetitively using a MW 3000 cut-off filtration membrane (Microcon YM-3) until all contaminating reagents had been removed.

2.5. Chemical compositional analysis

Unmodified and chemically modified GAGs and

oligosaccharides were prepared for the determination of sulfate and hexosamine by exhaustive dialysis (MWCO 3500) against distilled water, lyophilization, and drying for 2 days in a desiccator over P_2O_5 . Determination of sulfate group was performed by anion-exchange HPLC after acid hydrolysis of the sample in 2.5 *M* trifluoroacetic acid (TFA) at 100 °C for 2.5 h using conductivity detection (Tosoh model CM-8). Hexosamines were analyzed by the post-column HPLC derivatization method [24] after acid hydrolysis under identical conditions as described for sulfate analysis.

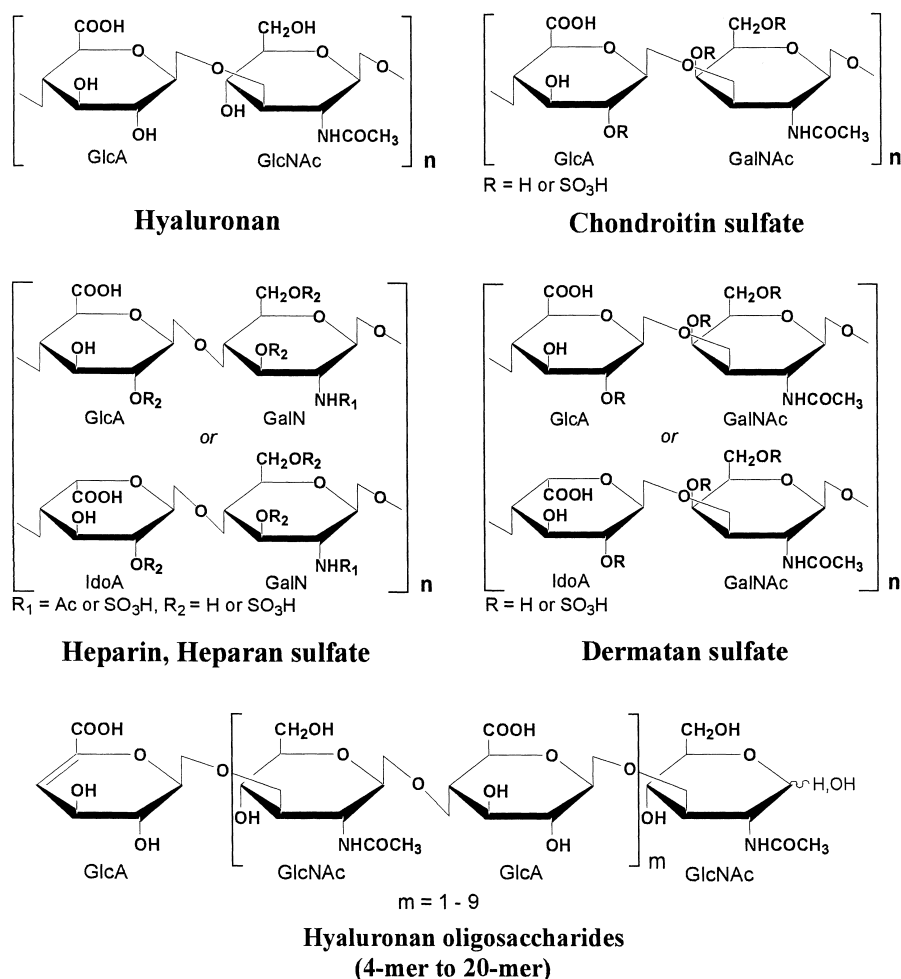


Fig. 1. Structure of unmodified GAGs and purified hyaluronan oligosaccharides produced by hyaluronidase digestion.

3. Results and discussion

3.1. Preparation and characterization of per-*O*-sulfonated GAGs

The polyanions examined in this study are currently used as medicinal agents and excipients. Poly-anionic polysaccharides natural products, known as glycosaminoglycans including heparin (an anticoagulant/antithrombotic), heparan sulfate, chondroitin sulfate and dermatan sulfate (all components of the antithrombotic drug ORG10172) and hyaluronan (an ophthalmic and anti-arthritis agent) were also evaluated. With the exception of hyaluronan, these GAGs show substantial sequence heterogeneity (Fig. 1). In addition, all GAGs are polydisperse mixtures that are characterized by an average molecular mass (\overline{M}_r). Each GAG was subjected to persulfonation with pyridine–sulfur trioxide complex to both increase their negative charge and to decrease (or eliminate in the case of chondroitin and dermatan sulfate) their sequence heterogeneity (Fig. 1). Since we had prepared a collection of highly sulfonated HA-derived oligosaccharides ranging in size from a tetrasaccharide [degree of polymerization (DP) 4] to an

Table 1
Sulfate contents of unmodified and chemically *O*-sulfonated oligo- and poly-saccharides

Sample ^a	Sulfo group/disaccharide, mol/mol	
	Unmodified	Persulfonated
HAoligo×10	0.00	4.04
HAoligo×12	0.00	4.23
HAoligo×14	0.00	4.09
HAoligo×16	0.00	4.21
HAoligo×18	0.00	4.19
HAoligo×20	0.00	4.03
HA 20K	0.00	3.90
HA 100K	0.00	3.97
ChS 12K	1.06	3.96
ChS 15K	0.93	3.81
ChS 31K	1.05	3.89
DS 16K	1.01	3.95
HP 16K	2.24	4.27
HS 14.8K	0.92	4.31

^a HA, hyaluronan; ChS, chondroitin sulfate; DS, dermatan sulfate; HP, heparin; HS, heparan sulfate. HAoligo×10 to 20 refer to hyaluronan decasaccharide to 20mer. HA 20K refers to hyaluronan polysaccharide of molecular mass 20 000.

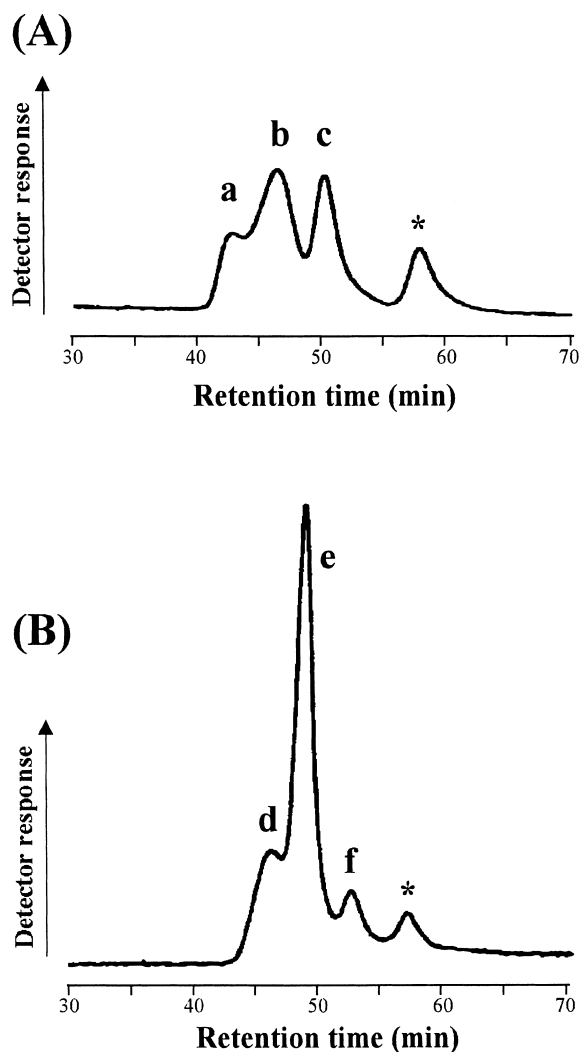


Fig. 2. Chromatograms of unmodified and *O*-sulfonated hyaluronans. (A) Intact hyaluronan polysaccharides and oligosaccharides; (B) chemically per-*O*-sulfonated hyaluronan polysaccharides and oligosaccharides. Peaks: a=hyaluronan standard from human umbilical cord (\overline{M}_r 790 000); b=hyaluronan (\overline{M}_r 20 000); c=hyaluronan decasaccharide; d=chemically per-*O*-sulfonated hyaluronan obtained from hyaluronan standard (\overline{M}_r 20 000); e=per-*O*-sulfonated hyaluronan decasaccharide; f=sulfate ion; *, system peak. Sample size, 0.2 $\mu\text{g}/2 \mu\text{l}$; flow-rate, 0.5 ml/min. These chromatograms were obtained using two columns of G3000SWXL connected in series with 5 mM borate buffer (pH 7.0). Conductivity detector response depends on analyte concentration, charge and molecular mass, thus, different analytes give different peak areas for identical sample size.

ecosaccharide (DP 20), we used these hyaluro-oligosaccharides as the standards for molecular mass calculation [3]. These were determined to be analytically pure by capillary electrophoresis (CE) and had the structure shown in Fig. 1. Exhaustive treatment of HAoligos and GAG-polysaccharides with pyridine–sulfur trioxide complex afforded the per-*O*-sulfonated products. The degree of sulfate substitution of each unmodified and per-*O*-sulfonated GAG is shown in Table 1 as the molar ratio of sulfo group to disaccharide.

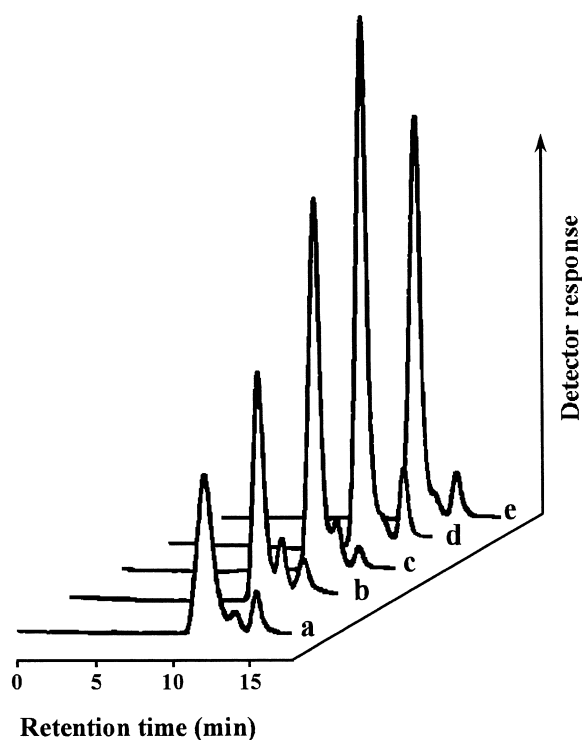


Fig. 3. Chromatograms of unmodified and *O*-sulfonated GAGs. (a) Per-*O*-sulfonated hyaluronan obtained from *Streptococcus zooepidemicus* (\bar{M}_r 100 000); (b) per-*O*-sulfonated dermatan sulfate obtained from porcine skin (\bar{M}_r 16 000); (c) per-*O*-sulfonated chondroitin sulfate obtained from chondroitin sulfate standard from bovine trachea (\bar{M}_r 15 000); (d) per-*O*-sulfonated heparin obtained from porcine intestine (\bar{M}_r 21 000); (e) per-*O*-sulfonated heparan sulfate obtained from porcine intestine (\bar{M}_r 14 800). These chromatograms were obtained using a column of G3000SWXL with the same chromatographic condition as shown in Fig. 2.

3.2. Detection of per-*O*-sulfonated GAGs

Table 2 shows the detection limit for each unmodified and persulfonated GAG sample under the flow injection system with the different detectors. Owing to the formation of a borate complex, hyaluronan, containing no *O*-sulfonate groups, was detected with a conductivity detector. It is well known that a borate complex of polyalcohol behaves as a strong acid and thus, it is highly negatively charged in aqueous solutions. The most sensitive detection of intact GAGs previously reported relied on fluorescence detection [12]. Conductivity detection, used in the current study, shows higher sensitivity than fluorescence for the detection of per-*O*-sulfonated GAGs. The detector response is linear over sample concentrations ranging from 2 to 500 $\mu\text{g/ml}$ (injection volume, 2 μl). These results demonstrate that, in the absence of contaminating salts, conductivity may be more suitable for the detection of chemically per-*O*-sulfonated GAGs than either UV or fluorescence detection.

Since the molecular masses of unmodified GAGs vary throughout a wide range (from 5000 to several hundred thousand [6]), relatively long (30–60 cm) columns are required for the determination of \bar{M}_r of

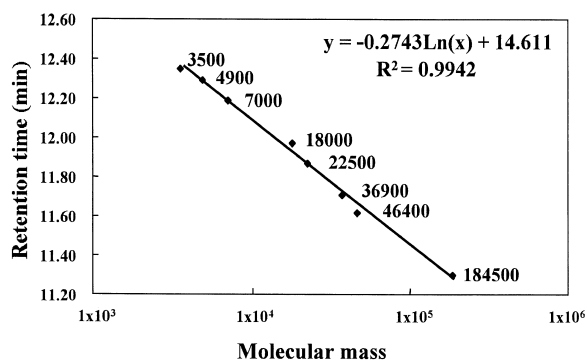


Fig. 4. Calibration curve of molecular mass of per-*O*-sulfonated GAGs. A calibration curve shows the retention time versus molecular mass of per-*O*-sulfonated HAoligo deca-saccharide (\bar{M}_r 3500) to per-*O*-sulfonated HA (\bar{M}_r 184 500). \bar{M}_r values: 4900, per-*O*-sulfonated HAoligo 14mer; 7000, per-*O*-sulfonated HAoligo 20mer; 36 900, per-*O*-sulfonated HA. (see Table 2 for other \bar{M}_r values). The retention times were obtained under the same chromatographic conditions as in Fig. 2.

GAG samples. In addition, no GAG molecular mass standards are commercially available. We have relied on per-*O*-sulfonated HA oligosaccharides and polysaccharides for calibration and estimation of the resolution by the separation column. The optimal conditions for separation have also been examined using variety of solvent systems and several types of columns, including polyhydroxy, polystyrene and silica matrices. The system described in the Experimental section was ultimately selected, based on the highly linear calibration curve obtained using per-*O*-sulfonated hyaluronan oligosaccharides and GAG polysaccharides standards. We should mention that potential interference by other negative charged oligo/polysaccharides might be possible. This conductivity detection method may be useful for the purified oligo/polysaccharides.

3.3. HPSEC profiles of per-*O*-sulfonated GAGs

Figs. 2 and 3 show the elution profiles of per-*O*-sulfonated HA (Fig. 2) and other GAGs (Fig. 3) using the current method. The detection limit for

each GAG was below 7 ng at a signal-to-noise (*S/N*) ratio of 3 (Table 2). These results demonstrate the practical application of conductivity for the detection of per-*O*-sulfonated GAGs. It should be emphasized that the presence of contaminating ions, such as salt, can limit the usefulness of this method of detection. The column used in this paper has shown the high-performance for the linearity between retention time and the log of molecular mass of each HAoligo standard. The small differences among molecular sizes of GAGs, determined using a single, column might be improved using two or three columns in series.

Since there are no commercially available GAG standards for molecular mass estimation, we prepared per-*O*-sulfonated HA oligosaccharides ranging from 4- to 20-mer for calibration of the separation column. Fig. 4 shows calibration curve of per-*O*-sulfonated HAoligo standards and the analytical results for the per-*O*-sulfonated GAG samples. The calibration curve is linear over a wide range of molecular mass values and the molecular mass of the per-*O*-sulfonated GAGs estimated by the present method are consistent with the molecular mass data

Table 2

Comparison of the detection limits for the analysis of unmodified and per-*O*-sulfonated oligosaccharides and polysaccharides using different detectors

Sample ^a	M_r	Detection limit (ng)		
		Conductivity	UV 200 nm	Fluorescence ^b
HA 20K	20 000	8.30	13.11	10.53
Sul HA 20K	36 900	4.22	21.13	36.89
HA 100K	100 000	6.78	14.91	17.79
Sul HA 100K	184 500	7.45	28.57	38.31
ChS 12K	12 000	2.90	13.41	8.46
Sul ChS 12K	18 000	2.11	15.52	10.83
ChS 15K	15 000	3.25	10.91	13.46
Sul ChS 15K	22 500	3.27	16.90	31.52
ChS 31K	31 000	3.08	13.48	18.38
Sul ChS 31K	46 400	4.65	21.24	26.63
DS 16K	16 000	3.07	12.67	181.09
Sul DS 16K	24 200	5.51	27.40	63.04
HS 14.8K	14 800	4.69	24.54	34.34
Sul HS 14.8K	22 000	3.21	29.93	36.89
HP 16K	16 000	2.39	35.61	34.34
Sul HP 16K	21 000	2.34	42.11	33.20

^a Abbreviations are the same as in Table 1. Sul indicates per-*O*-sulfonated.

^b Fluorescent detection of the cyanoacetamide derivative.

obtained from polyacrylamide gel electrophoresis analysis (data not shown) [3].

4. Conclusion

An HPSEC method with conductivity detection was developed for use as a rapid and simple molecular mass screening technique to determine the average molecular mass of both intact and chemically per-*O*-sulfonated polysaccharides. Application of the method noted distinct molecular mass distribution differences among various commercially available GAG samples. The method can be used as an aid in the selection of not only GAGs but also chemically *O*-sulfonated polysaccharides of a particular size and homogeneity for specific pharmaceutical applications.

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