ANALYTICAL BIOCHEMISTRY 181, 288–296 (1989)

Analysis of Glycosaminoglycan-Derived Oligosaccharides Using Reversed-Phase Ion-Pairing and Ion-Exchange Chromatography with Suppressed Conductivity Detection

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Received April 3, 1989

Oligosaccharides prepared from glycosaminoglycans (GAGs) including heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate, and keratan sulfate were analyzed using reverse-phase ion-pairing HPLC and ion-exchange HPLC with suppressed conductivity detection. The results were compared with those obtained by strong anion-exchange HPLC using uv detection. These oligosaccharides were first prepared by enzymatically depolymerizing the GAGs with enzymes including heparin lyase (EC 4.2.2.7), heparan sulfate lyase (EC 4.2.2.8), chondroitin ABC lyase (EC 4.2.2.4), and keratan sulfate hydrolase (EC 3.2.1.103). Analysis was then performed without derivitization under isocratic conditions with a limit of sensitivity in the picomole range. Preliminary studies suggest that this approach may be particularly useful in examining oligosaccharides having no uv chromophore such as those prepared from keratan sulfate. © 1989 Academic Press, Inc.

High-performance liquid chromatography has been widely used for the structural determination and analysis of complex glycosaminoglycans (GAGs).[2] Enzymatic depolymerization of uronic acid containing GAGs, using polysaccharide lyases, affords oligosaccharides having an unsaturated uronic acid residue, at their nonreducing terminus which can be conveniently detected by measuring absorbance at 232 nm (1,2). Deaminative cleavage of N-sulfated GAGs results in ring contraction and breakage of the adjacent glycosidic linkage affording an anhydromannose residue which can be radiolabeled using NaB^3H_4 (3–7) or tagged with a fluorescent label (8–10) to obtain detectable derivatives. Keratan sulfate contains neither uronic acid nor N-sulfation. It can be hydrolyzed chemically using acid, de-N-acetylated, and then deaminatively cleaved (11), or it can be enzymatically cleaved using keratan hydrolase (12), but the oligosaccharides formed by any of these methods are not easily detected.

Both strong-anion exchange (SAX)-HPLC (1,2,11) and reverse-phase ion-pairing (RPIP)-HPLC (5,10,13–15) have demonstrated utility in separating highly polar molecules such as GAG-derived acidic oligosaccharides. A simple HPLC-based analysis capable of both separating and detecting any GAG-derived oligosaccharide without derivitization would therefore be extremely useful.

Conductivity detection has been established in the analysis of inorganic and organic ions and hence should be applicable to the analysis of the carboxylate and sulfate salts common to all GAGs (16). Suppressed conductivity detection represents a relatively recent technological improvement making it possible to substantially increase detector sensitivity by reducing eluant conductivity by the on-line removal (prior to detection) of ion-pairing reagents such as tetrapropyl ammonium hydroxide (TPAOH) (J. Stillian, Dionex Corp., personal communication). A micromembrane suppressor, placed between the separating column and the conductivity detector, comprised of a strong-acid ion-exchange membrane having permeability to cations with exclusion to anions, is used. The separating column eluant, containing TPAOH, flows along one side of this membrane while a countercurrent flow of aqueous sulfuric acid regenerant flows along the opposite side of the membrane.

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[2] Abbreviations used: GAGs, glycosaminoglycans; RPIP, reverse-phase ion-pairing; TPAOH, tetrapropyl ammonium hydroxide; IC, ion-exchange chromatography; SAX, strong-anion exchange; AUFS, absorbance units full scale; μSFS, microSiemans full scale; EDM, eluant degas module.

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TPA^+ ions move across the membrane and are carried as waste from the suppressor. To maintain electrical neutrality H^+ ions move stoichiometrically in the opposite direction replacing TPA^+ ions. Water (in place of TPAOH) flows into the conductivity detector reducing the background conductance.

This report examines the use of RPIP-HPLC and ion-exchange chromatography (IC-HPLC) with suppressed conductivity detection to analyze oligosaccharides derived from heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate, and keratan sulfate. The resulting separations are compared to those obtained using SAX-HPLC using uv detection.

EXPERIMENTAL

Materials

Porcine mucosal heparin, sodium salt, was from Hepar Industries (Franklin, OH). Porcine mucosal heparan sulfate (Org 553) sodium salt was a gift from Organon (Oss, The Netherlands). Chondroitin sulfates A (whale cartilage) and C (shark cartilage) were obtained as the sodium salts from Sigma Chemical Co. (St. Louis, MO). Porcine skin dermatan sulfate sodium salt and bovine corneal keratan sulfate sodium salt were from Sigma Chemical Co. Heparin lyase (EC 4.2.2.7), heparan sulfate lyase (EC 4.2.2.8), chondroitin ABC lyase (EC 4.2.2.4), keratanase (EC 3.2.1.103), and chondroitin disaccharide standards were from ICN (Costa Mesa, CA). Sephadex G-10 was from Pharmacia. Tetrapropylammonium hydroxide (1 M solution) was from Dionex Corp. (Sunnyvale, CA). ^2H_2O (99.96% D) and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt was obtained from Aldrich. HPLC-grade acetonitrile and methanol were from Mallinkrodt (Paris, KY). RPIP-HPLC was performed using a GPM-1 gradient pump connected to a microinjector, an IonPac NS1 5-μm polystyrene pellicular resin-based column, an Anion (MPIC) Micro membrane suppressor (AMMS-MPIC), and a QIC conductivity detector, all from Dionex. The eluant and suppressor regenerant were degassed with helium and introduced into the chromatography system using an eluant degas module (EDM-1) degasser from Dionex. The data was processed using a Dionex 4270 dual channel integrating recorder. IC-HPLC was performed on a Dionex Bio L.C. consisting of EDM degasser, GPM pump, liquid chromatography module, conductivity detector (CDM-1), AMMS suppressor, and variable wavelength uv detector (all from Dionex). Data were processed using a Dionex A.I.450 data station with a Dionex Model II advanced computer interface (Model II.A.C.I.) and an IBM PS-2 Model 50 computer. A Dionex Ion Pac AS5A (4 × 150 mm, 5 μm) polystyrene pellicular resin-based anion-exchange column was used to analyze monosulfated chondroitin disaccharides and keratan sulfate oligosaccharides and a Dionex Carbopac PA-1 (4 × 250 mm, 10 μm) column was used to analyze unsulfated chondroitin disaccharide. SAX-HPLC was performed using two Shimadzu LC-7A HPLC pumps with gradient control by a D/A interface with an Apple IIe computer equipped with Chromatochart software from Interactive Microware, Inc., Pennsylvania, an LDC low volume mixing chamber connected to a Rheodyne 7125 injector, a Spherisorb 25 cm × 4.6 mm 5-μm SAX column from Phase Separations, Inc. (Norwalk, CT). An ISCO Model 1840 variable wavelength uv detector connected through an A/D convertor to an Apple IIe computer was used for continuous monitoring and integration report. Freeze-drying was performed on a Virtis lyophilizer. Spectrometric measurements were performed on a Shimadzu UV-160 and conductimetric measurements were performed on a 4503A solution analyzer from Amber Science (San Diego, CA).

Methods

Depolymerization of GAGs. Heparin (2 mg in 1 ml) was treated for 9 h at 30°C with 15 mIU (IU = 1 μmol bonds cleaved/min) of heparin lyase in 5 mM sodium phosphate buffer, pH 7.0, containing 200 mM sodium chloride. Heparan sulfate (120 mg in 2 ml) was first treated for 24 h at 37°C with 5 IU of chondroitin ABC lyase in 250 mM sodium acetate, 2.5 mM calcium acetate, pH 6.8, and dialyzed (3000 M_r cutoff membrane) against 1000 vol of water to remove contaminating dermatan and chondroitin sulfates and freeze-dried. This heparan sulfate (2 mg/ml) was depolymerized using 20 mIU/ml heparan sulfate lyase for 13 h at 43°C in 5 mM sodium phosphate, pH 7, buffer containing 200 mM sodium chloride. Chondroitin sulfates and dermatan sulfate were each prepared at 16 mg/ml in 10 mM sodium phosphate, pH 7.0, buffer and depolymerized with chondroitin ABC lyase (46 mIU/ml) for 10 h at 37°C. The lyase-catalyzed depolymerization reactions were monitored by removing aliquots throughout the reaction time course, diluting these in 0.03 N hydrochloric acid, and measuring their absorbance at 232 nm. Keratan sulfate was prepared at 1.0 mg/ml in 5 mM phosphate buffer and treated for 2 h at 37°C with 20 units of keratan hydrolase. This hydrolysis reaction was followed using azure A dye-binding assay (17) on aliquots collected throughout the reaction's time course. Each of the depolymerized GAG preparation was analyzed by SAX-HPLC directly or was freeze-dried and desalted using a 40 × 5-cm Sephadex-G10 column eluted with water at 2 ml/min. Each desalted, depolymerized GAG sample was freeze-dried and reconstituted to 1 mg/ml and stored at −20°C for RPIP-HPLC analysis.

Average molecular weight and specific conductivity of oligosaccharide samples. The average molecular weights of oligosaccharides prepared from chondroitin sulfates and dermatan sulfate were determined by low pressure gel permeation chromatography (GPC) on a Fractogel TSK HW40 column (18). The average molecu-

TABLE 1

Properties of the Depolymerized GAGs

	Estimated M_r (av) of product	Specific conductivity S/M product[a]
Chondroitin sulfate A	590[b]	0.592
Chondroitin sulfate C	550[b]	0.428
Dermatan sulfate	570[b]	0.562
Heparin	890[c]	2.87
Heparan sulfate	570[d]	1.11
Keratan sulfate	2000[e]	3.28[e]

[a] On the basis of a molar absorptivity of 5000 M^{-1} cm^{-1} at 232 nm in 0.03 N hydrochloric acid.

[b] Disaccharides represent 85–95 mol% of the product mixture as determined by low pressure gel permeation chromatography using uv detection at 232 nm.

[c] A product distribution of 56 mol% disaccharides, 26 mol% tetrasaccharides, 9 mol% hexasaccharides, 4 mol% decasaccharides, and 2 mol% higher oligosaccharides has been measured using low pressure gel permeation chromatography (18).

[d] On the basis of the absorbance at 232 nm of the oligosaccharide products about 80–85 mol% disaccharide and 15–20 mol% tetrasaccharides (or higher oligosaccharides) were expected.

[e] On the basis of the gradient polyacrylamide gel electrophoresis banding pattern of the oligosaccharide mixture (19) using silver staining (20) and by increase in reducing ends (21).

lar weights of heparin and heparan sulfate oligosaccharide products were determined using uv absorbance at 232 nm (2). Keratan sulfate oligosaccharides were analyzed by electrophoresis (19) (using both Alcian blue dye and silver staining (20) for visualization) and by reducing sugar assay (21) to estimate their average molecular weight. Heparin oligosaccharide standards, heparan sulfate disaccharide standards, and chondroitin sulfate disaccharide standards stored at 2 mg/ml in water were similarly quantitated in 0.03 N hydrochloric acid and a molar absorptivity listed in Table 2 or of 5000 M^{-1} cm^{-1} (2) was used to calculate their concentrations. The specific conductivities of the disaccharide and oligosaccharide standards were determined from the slope (S/M) obtained from a series of dilutions of each sample in water, ranging from 50 to 3000 μM. The concentration of the hydrolase-depolymerized keratan sulfate was determined from the sample's original mass assuming no mass loss on desalting.

RPIP-HPLC analysis. Desalted samples, prepared at a variety of dilutions, were injected (10 μl) through a 50-μl loop onto an IonPac NS1 5-μm column and eluted under conditions described in the text. Regenerant, 28 mN sulfuric acid, was passed through the micromembrane suppressor, countercurrent to the eluant flow, by applying 100 psi constant pressure of helium gas resulting in a flow rate of approximately 4 ml/min. The system was run for 0.5 h to stabilize the detector before sample analysis was begun. The separation was performed at ambient temperature using detector sensitivities ranging from 3 to 30 microSiemans full scale (μSFS). The peaks observed on fractionating the depolymerized heparin, heparan sulfate, chondroitin, and dermatan sulfate samples were identified by coinjection of disaccharide and oligosaccharide standards of known structure.

IC-HPLC analysis. Desalted samples were prepared and applied to the column, as previously described for RPIP-HPLC, and eluted using a mixture of sodium carbonate and sodium bicarbonate (see text) at a flow rate of 1 ml/min. Regenerant, 25 mN sulfuric acid, was passed through the micromembrane suppressor at 5 ml/min. Sample detection was performed simultaneously by suppressed conductivity at 0.3 to 3 μSFS and by uv at 232 nm and 0.02 absorbance units full scale (AUFS). Peaks observed in the lyase-depolymerized samples were identified by coinjection of authentic standards.

SAX-HPLC analysis. Samples were applied at various concentrations ranging from 1 to 9 μg in 10 to 25 μl onto a 200-μl fixed injection loop. The samples were eluted using a linear gradient (see text) of sodium chloride in water adjusted to pH 3.5 at a flow rate of 1.5 ml/min. Sample detection was by absorbance at 232 nm with a sensitivity of 0.02 AUFS. Peaks observed in the lyase-depolymerized samples were identified by coinjection of authentic standards.

Spectral characterization of heparan sulfate disaccharides. Disaccharides **7** (0.2 mg) and **8** (0.3 mg) were each thoroughly exchanged by freeze-drying three times from ^2H_2O. The exchanged samples were each reconstituted in 0.4 ml of ^2H_2O containing 0.1 μg of 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt. Proton NMR was obtained at 360 MHz and the signals observed were assigned based on shift values and literature precedent (22,23). **7,** ΔUA (1 → 4)-D-GlcNAc, ΔUA: 5.23 (H1), 4.26 (H2), 5.84 (H4), 3.80–3.90 (H3) ppm, D-GlcNAc: 2.05 (-$COCH_3$), 5.17 (H1), 3.60–4.15 (H2, H3, H4, H5, and HH′6) ppm. **8,** ΔUA (1 → 4)-D-GlcN2S, ΔUA: 5.47 (H1), 4.26 (H2), 5.83 (H4), 3.80–3.90 (H3), D-GlcN2S: 5.17 (H1), 3.28 (H2), 3.70–4.15 (H3, H4, H5, and HH′6) ppm.

RESULTS AND DISCUSSION

Depolymerization of GAGs and Preparation of Oligosaccharides for Analysis

Heparin, heparan sulfate, chondroitin sulfates, and dermatan sulfate were depolymerized using the appropriate lyase. Quantitation of the products of these reactions by uv absorbance at 232 nm (18) suggests that the chondroitin sulfates, dermatan sulfate, and heparan sulfate samples are depolymerized to the greatest extent resulting primarily in disaccharide products while heparin is depolymerized to a lesser extent (Table 1). A set of heparin oligosaccharide standards had been prepared previously and their structures (Fig. 1) characterized (2,21,24). The two major disaccharide products of the ly-

1-2

3-5

6

7-8

9-11

12

FIG. 1. Structure of glycosaminoglycan-derived oligosaccharides. For heparin, oligosaccharides **1–6** are shown where (**1**) X = SO_3^-; **2** X = H; (**3**) X (glucosamine) = H, X (iduronic acid) = SO_3^-; (**4**) X (glucosamine) = SO_3^-, X (glucuronic acid) = H; and (**5**) X (glucosamine) = SO_3^-, X (iduronic acid) = SO_3^-. For heparan sulfate, oligosaccharides **7** and **8** are shown where (**7**) Y = $COCH_3$; and (**8**) Y = SO_3^-. For chondroitin and dermatan sulfates, oligosaccharides **9–11** are shown where (**9**) X = H, X′ = H; (**10**) X = H, X′ = SO_3^-; and (**11**) X = SO_3^-, X′ = H. For keratan sulfate oligosaccharide **12** is shown where X = SO_3^- or H and $n = 0, 1, 2, 3 \cdots$.

ase-catalyzed depolymerization of heparan sulfate were prepared by preparative SAX-HPLC as previously described (25) and their structure was determined by proton NMR analysis to be ΔUA (1 → 4)-D-GlcNAc and ΔUA (1 → 4)-D-GlcN2S where ΔUA is 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid (Fig. 1). The specific conductivities of the oligosaccharide standards including three commercially available chondroitin sulfate disaccharide standards (Fig. 1) were determined and are reported in Table 2.

Samples obtained directly from the depolymerization reaction were analyzed by SAX-HPLC using uv detection. Although these samples could also be analyzed by RPIP-HPLC and IC-HPLC using suppressed conductivity detection, additional peaks resulting from inorganic buffer salts were observed. These extraneous peaks could be eliminated and sensitivity of detection enhanced if the depolymerized GAG samples were desalted prior to HPLC analysis.

Separation of Oligosaccharide Mixtures

HPLC separation of enzymatically and chemically depolymerized GAGs have been widely used in their analysis. The columns used include amino columns (7–9,14,26), reverse-phase columns, with (14–16) and without (10,27) ion-pairing reagents, strong-anion exchange columns (1–4,6,11,28,29), and others (27,30–34). SAX-

TABLE 2

Properties of the Purified Oligosaccharide Standards

Oligosaccharide standard[a]	No. of sulfates	RPIP-HPLC retention		ϵ_M (M^{-1} cm^{-1})	Specific conductivity (S/M)
		Time (min)[b]	%Methanol		
1	3	4.96	34	5063[c]	0.33
2	2	3.83	34	nd[d]	0.25
3	5	8.41	34	5331[c]	0.47
4	5	8.67	34	5066[c]	0.79
5	6	11.26	34	5657[c]	1.00
6	7	22.72	34	5275[c]	0.75
7	0	4.30	16	nd	nd
8	1	5.13	16	nd	nd
9	0	4.98	16	5700[e]	0.08
10	1	12.00	16	5100[e]	0.13
11	1	12.11	16	5500[e]	0.14

[a] See Fig. 1 for structures of the oligosaccharide standards.
[b] Solvent front runs at 3.5 min retention time.
[c] Ref. (2).
[d] Not determined.
[e] Ref. (38).

HPLC separations generally rely on gradient elution techniques reducing the reproducibility of the retention times obtained and thus diminishing their value in routine analysis. RP-HPLC generally requires the derivitization of hydrophilic GAG-derived oligosaccharides to impart sufficient hydrophobicity to retain these molecules on RP columns (8,9). Amino-cyano columns using isocratic elution have been applied only to GAG-derived disaccharides having a relatively low degree of sulfation (27). RPIP-HPLC, however, permits the efficient separation of a wide array of GAG-derived oligosaccharides using isocratic elution conditions (5,14–16).

SAX-HPLC separation of the heparin oligosaccharides has been previously described in some detail (1,2) and is shown in Fig. 2a. The heparan monosulfate oligosaccharides (Fig. 3a) and the chondroitin sulfate disaccharides (Fig. 4a) could be similarly separated by applying gradients of reduced slope because of the lower level of sulfation of these oligosaccharides. The analysis of oligosaccharide products of keratanase depolymerization of keratan sulfate was not possible due to the absence of an unsaturated uronic acid residue in these products and hence their low molar absorptivity at 232 nm.

Developing an RPIP separation for depolymerized GAGs began with examining the effect a variety of eluants on the separation of heparin-derived, trisulfated disaccharide, **1**, and heparin-derived hexasulfated tetrasaccharide, **5**. The goal was to optimize the separation of **1** from **5** while resolving **1** from the early peak due to salts of inorganic anions. Mixtures of water:acetonitrile (10:90 to 80:20) and water:methanol (50:50 to 85:15) using 10 mM TPAOH were first examined. While 25%

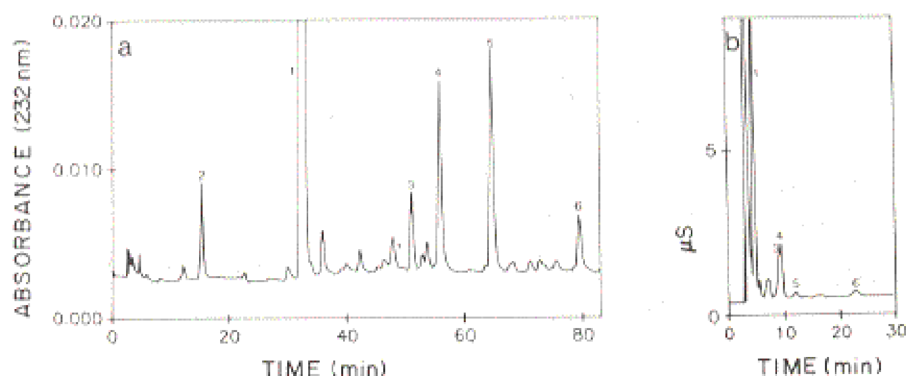

FIG. 2. Analysis of heparin lyase-depolymerized heparin. (a) SAX-HPLC performed on 4 μg of sample using a 0.2–1 M sodium chloride gradient over 100 min. (b) RPIP-HPLC performed on 10 μg desalted sample eluted isocratically using 34% methyl alcohol containing 5 mM TPAOH at 30 μSFS. Oligosaccharide **2** elutes earlier than oligosaccharide **1** as a shoulder on the peak at the injection front.

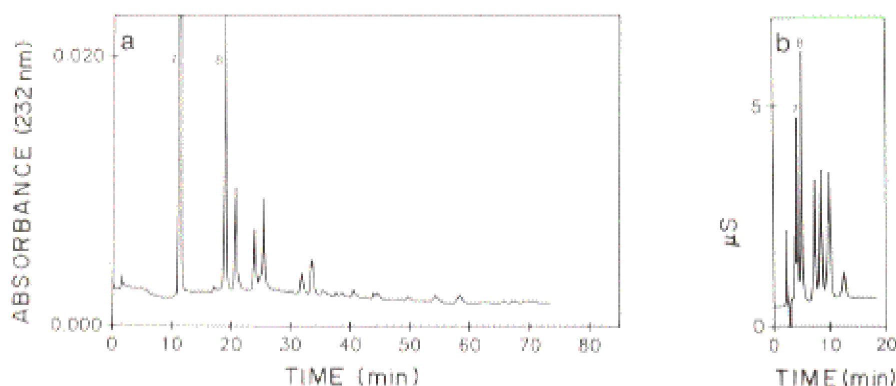

FIG. 3. Analysis of heparan sulfate lyase-depolymerized heparan sulfate. (a) SAX-HPLC performed on 9 μg of sample using a 0–1 M sodium chloride gradient over 180 min. (b) RPIP-HPLC performed on 4.5 μg desalted sample eluted isocratically at 0.5 ml/min using 16% aqueous methanol containing 5 mM TPAOH at 30 μSFS.

aqueous acetonitrile provided a slightly improved separation over 34% aqueous methanol the sensitivity was reduced by threefold due to the noisier baseline observed for aqueous acetonitrile. Ternary solvent mixtures such as acetonitrile:methanol:water gave no improvement in this separation and also resulted in reduced sensitivity when compared to aqueous methanol. Aqueous methanol, 34%, was selected for use in studies where the concentration of TPAOH ion-pairing reagent was varied between 5 and 15 mM. The best separation was observed at 5 mM TPAOH in this aqueous methanol solvent system. Increasing the concentration of ion-pairing reagent over the optimum level of 5 to 10 mM lengthens the retention time, lowers the sensitivity, and broadens the resulting peaks. Flow rates ranging from 0.5 to 1 ml/min resulted in optimum resolution at a reasonable, 1500-psi back pressure. Gradient elution results in a drifting baseline and thus its use was avoided.

The best separation of the heparin lyase-depolymerized heparin sample was obtained using 34% aqueous methanol containing 5 mM TPAOH at a flow rate of 1 ml/min. These conditions resulted in the separation of four of the five major heparin oligosaccharides (Fig. 2b). Pentasulfated tetrasaccharides **3** and **4**, differing only in the chirality at the 5-position of the internal uronic acid residue and the position of two sulfate groups, gave slightly different retention times (8.41 and 8.67 min, respectively) but could not be well separated under any of the conditions examined. Initial studies using 10 μm particle size SAX-HPLC also had failed to separate these isomers (23). SAX-HPLC on 5 μm particle size packing is the only method reported capable of separating these isomeric tetrasaccharides (2,25). Separation conditions were similarly optimized for heparan sulfate lyase-depolymerized heparan sulfate (Fig. 3b), chondroitin ABC lyase-depolymerized chondroitin sulfates, and dermatan sulfate. For example, RPIP-HPLC separated 5 μg of unsulfated chondroitin disaccharide **9** (retention time 4.98 min, 16% aqueous methanol, 5 mM TPAOH, at 0.5 ml/min) from 5 μg each of monosulfated chondroitin disaccharides **10** and **11** (retention times 12.00 and 12.11 min, respectively) (chromatogram not shown). In each separation the retention time at a given concentration of methanol increased with both the degree of sulfation and the degree of polymerization of the oligosaccharide being analyzed.

Failure to separate the isomeric 4-sulfated, **11**, and 6-sulfated, **10**, chondroitin disaccharides by RPIP-HPLC led us to investigate IC-HPLC as an alternative method of separation which would also permit detection by suppressed conductivity. IC-HPLC was performed on chondroitin ABC lyase-depolymerized chondroitin sulfates and dermatan sulfate (Fig. 4d–4f). Using an AS5A column (equipped with an AG5A guard column), a mixed eluant of 12 mM sodium carbonate and 6 mM sodium bicarbonate cleanly resolved monosulfated chondroitin disaccharides **10** and **11**. The elution order, **11** followed by **10**, was the reverse of that observed on SAX-HPLC. The chromatograms of the chondroitin and dermatan sulfates, obtained by measuring absorbance at 232 nm at 0.02 AUFS, were nearly identical to those shown in Figs. 4d–4f except the phosphate peak (from residual buffer salts) was only observed by suppressed conductivity detection. Unsulfated chondroitin disaccharide **9** eluted too early in the chromatogram for quantitation, so a CarboPac PA-1 column eluted at 1 ml/min with a mixture of 0.4 mM sodium carbonate and 0.2 mM sodium bicarbonate was used for its analysis. Under these conditions 50 nmol of **9** gave a full scale peak at 30 μSFS with a retention time of 6 min (chromatogram not shown). Coinjection of standard disaccharide **9** with the depolymerization mixtures obtained from chondroitin sulfates and dermatan sulfate (on IC-HPLC with simultaneous suppressed conductivity and uv detection and SAX-HPLC with uv detection) showed that none of the depolymerization mixtures contained unsulfated chondroitin disaccharide **9**. Chondroitin sulfate A contained a higher content of 4-sulfated disaccharide, **11**, than did chondroitin sulfate C as expected. However, chondroitin

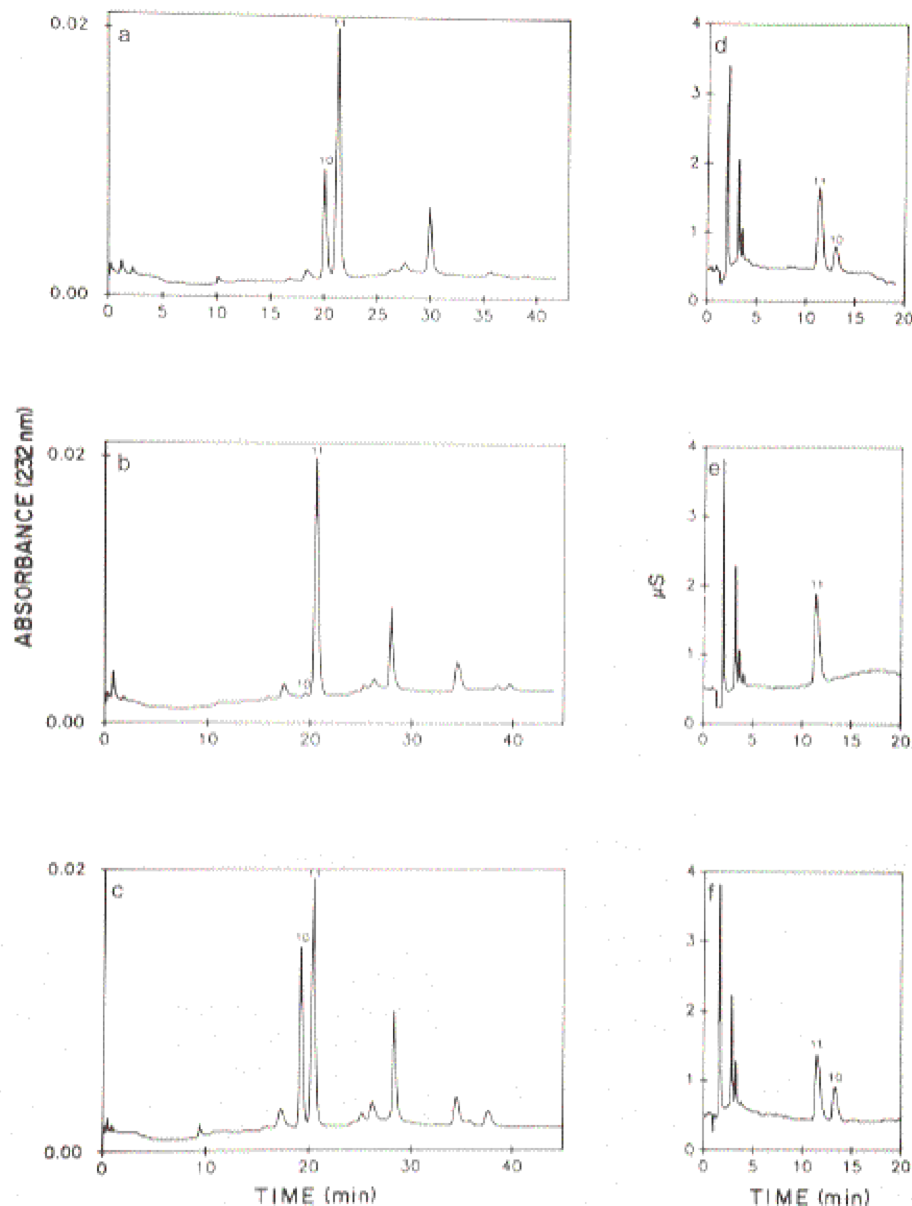

FIG. 4. Analysis of chondroitin ABC lyase-depolymerized chondroitin and dermatan sulfate. SAX-HPLC performed on 4 μg of depolymerized chondroitin sulfate A (a), dermatan sulfate (b), and chondroitin sulfate (c) C, using a 0–1 M sodium chloride gradient over 180 min. IC-HPLC performed on 20 μg of desalted, depolymerized chondroitin sulfate A (d), dermatan sulfate (e), and chondroitin sulfate C (f) eluted isocratically from an AS5A column using a mixture of 12 mM sodium carbonate and 6 mM sodium bicarbonate at 3 μSFS.

sulfate C unexpectedly contained more 4-sulfated disaccharide, **11**, than 6-sulfated disaccharide, **10**.

Depolymerized keratan sulfate **12** was analyzed by both RPIP-HPLC and IC-HPLC (Figs. 5a and 5b). In these preliminary studies, suppressed conductivity detection demonstrated the presence of only a single major peak which was not observed using uv detection. Gradient polyacrylamide gel electrophoresis (19) using either Alcian blue or silver staining (20) for visualization shows multiple bands corresponding to the presence of many keratan sulfate oligosaccharides. Because of the lack of appropriate keratan sufate standards, it is not possible to say whether the single major peak observed in Figs. 5a and 5b is due to a single oligosaccharide component or multiple components. However, the peak area observed correlated well to the amount of depolymerized keratan sulfate applied to the column.

Detection of Acidic Oligosaccharides

Detection methods used for acidic oligosaccharides fall into several categories including uv, radioisotope, and fluorescence. Ultraviolet detection has been used primarily with lyase-depolymerized GAGs which afford

products containing an unsaturated uronic acid residue in their nonreducing terminus (35). The molar absorptivity at 232 nm of these oligosaccharides range from 5000 to 6000 M^{-1} cm^{-1} (2) suggesting theoretical limits of detection of a few picomoles. Reported limits of detection range from 3 to 100 pmol with the highest sensitivities of 3–10 pmol observed in the separation of heparan sulfate using RPIP-HPLC (15). Our current studies using gradient elution SAX-HPLC and uv 232 nm detection result in limits of detection of 10 pmol. Radiochemical detection of nitrous acid-depolymerized, NaB^3H_4-reduced heparin and heparan sulfate oligosaccharides has been reported. On the basis of the highest specific activity NaB^3H_4 available (75 Ci/mmol) and the detector sensitivity of a standard radiochemical flow cell, the limit of detection of this method should be in the femtomole range although no sensitivity data have been reported using this approach. Two problems associated with this approach are that derivitization is required and that only GAGs which are *N*-sulfated, such as heparin and heparan sulfate, are easily depolymerized using nitrous acid (36). Fluorescent derivatives of lyase-produced oligosaccharides have been prepared and sensitivities as high as a few picomoles have been reported (8).

The sensitivity of suppressed conductivity detection was examined in this study. The specific conductivity of each oligosaccharide standard was measured and is given in Table 2. These values generally increased as the net charge of these oligosaccharide standards increased. However, the sensitivity of detection in the RPIP-HPLC experiments did not correlate well to the specific conductivity of each oligosaccharide standard. Further experiments involving oligosaccharides having a wider range of charge and size will be required in order to develop a method to accurately predict the detector sensitivity for a particular sample. An experiment was performed to establish the limit of detection of two oligosaccharides, **2** and **6**, using RPIP-HPLC with suppressed conductivity detection (Fig. 6). In each case the solvent composition was adjusted to obtain a sharp, symmetrical peak having a retention time of approximately 5 to 6 min. As little as 11 ng corresponding to 6 pmol of heptasulfated hexasaccharide **6** could be detected at 3 μSFS. Detector linearity (correlation of $r > 0.99$) was observed between 50 and 800 ng injected and between 1 and 20 μg injected for oligosaccharide standards **1–6.** A slightly different slope was observed over each range of concentrations.

Suppressed conductivity provides a highly sensitive method for detecting all GAG-derived oligosaccharides, without derivitization, prepared by virtually any depolymerization method. The sensitivity of this method of detection is high with limits of detectability ranging from 10 μg to 10 ng and results in good linearity in standard curves prepared using oligosaccharide standards within a given detector attenuation. The major advantage of this method is the large diversity of samples which can be detected. This is particularly important when analyzing acidic polysaccharides such as keratan sulfate which provide no other ready means of detection. Further studies are underway to examine the utility of RPIP-HPLC with suppressed conductivity detection for the analysis of GAGs present in biological fluids (37).

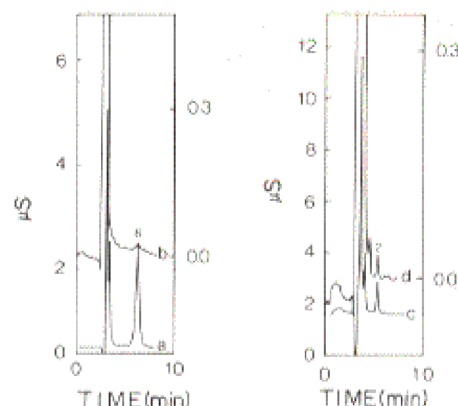

FIG. 6. Limit of sensitivity of RPIP-HPLC using suppressed conductivity detection. Analyses of **6** (9.8 nmol) at 30 μSFS (a) and (6 pmol) at 3 μSFS (b) and **2** (760 pmol) at 30 μSFS (c) and (7.6 pmol) at 3 μSFS (d) are shown. These separations were performed isocratically at a flow rate of 0.5 ml/min in aqueous methanol (38:62) and (28:72), respectively.

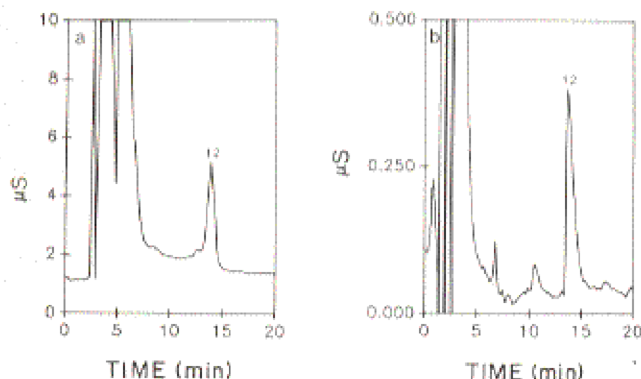

FIG. 5. Analysis of keratan hydrolase-depolymerized keratan sulfate. (a) RPIP-HPLC performed on a desalted 50 μg sample of depolymerized keratan sulfate eluted isocratically at a flow rate of 0.5 ml/min using 16% methyl alcohol containing 5 mM TPAOH at 3 μSFS. (b) IC-HPLC performed on 100 μg of desalted sample eluted isocratically from an AS5A column using a mixture of 20 mM sodium carbonate and 10 mM sodium bicarbonate at 0.3 μSFS.

ACKNOWLEDGMENT

This work was supported by NIH Grants HL29797 and GM38060.

REFERENCES

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