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Compositional Analysis of Glycosaminoglycans by Electrospray Mass Spectrometry

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The purpose of this work is to analyze glycosaminoglycans (GAGs) directly from complex mixtures without the need to purify individual components. Novel conditions for negative ion electrospray MS of chondroitin sulfate (CS) oligosaccharides are described in which sodium adduction and fragmentation are avoided. Differentiation between positional sulfation isomers is demonstrated for CS disaccharides, and a selected reaction monitoring scheme is used to quantify sulfation isomers in disaccharides liberated from decorin and biglycan. A size exclusion chromatography LC/MS method is shown to be effective for compositional analysis of longer CS oligosaccharides. The SEC step serves to simplify the composition of GAGs entering the mass spectrometer at any time, thus allowing the masses of the constituent molecules to be extracted. Mass spectrometric detection produces far more information than conventional UV or fluorescent detectors and allows the monosaccharide composition of individual components to be determined.

The structural diversity of glycosaminoglycans (GAGs) poses significant challenges in the field of glycobiology. These oligosaccharides modify proteoglycan core proteins and are responsible for many aspects of their biological activity. Proteoglycans, found on the surface of all higher animal cells, in the extracellular matrix of connective tissues, and in basement membranes, function as structural molecules in the extracellular matrix and as scaffold structures, binding a wide variety of protein ligands through GAG−protein and protein−protein interactions.[1] All GAGs consist of repeating disaccharides of uronic acid or galactose and hexosamine. Depending on GAG type, saccharides may be epimerized at the C-5 position of uronic acid residues and may be N- or O-sulfated and/or N-acetylated. While core proteins have fairly homogeneous compositions, the lengths and compositions of GAG chains are highly variable.

Purified sulfated GAG oligosaccharides have been analyzed using a number of mass spectrometric techniques. Plasma desorption mass spectrometry has been used to determine the molecular mass of heparin-like glycosaminoglycans (HLGAGs) up to hexasaccharide in size,[2] requiring more than 10 nmol of sample. HLGAG oligosaccharides have been analyzed using fast atom bombardment (FAB) mass spectrometry,[3,4] and up to octasaccharides have been detected at the 10-nmol level.[5,6] In conjunction with nuclear magnetic resonance spectrometry, FAB MS has been used to identify unusual sulfated disaccharide units in digests of chondroitin sulfate E from various sources.[7,8] Derivatization of sulfated GAG samples has also been investigated for FAB MS.[4,9] Using FAB tandem MS, it is possible to differentiate positional isomers of chondroitin sulfate (CS) disaccharides including 2-acetamido-2-deoxy-3-*O*-(*β*-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose (ΔDi4S) and 2-acetamido-2-deoxy-3-*O*-(*β*-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi6S).

Sulfated GAGs ionize poorly when mixed directly with any of the matrixes commonly used for UV MALDI-TOF MS.[10] MALDI analysis can be facilitated, however, by pairing the oligosaccharide with a basic polypeptide, to form a neutral complex, which has been observed to ionize readily.[10,11] The method has been applied in conjunction with capillary electrophoresis to investigate the mechanism of HLGAG depolymerization by heparinases.[12−14] UV MALDI-TOF MS detection of HLGAGs up to octasaccharides has been reported with sensitivity in the high-femtomole detection range. This type of experiment provides molecular weight profiles of short oligosaccharides and can be used to sequence pure HLGAGs when used in conjunction with heparin lyases and nitrous acid cleavage.[15]

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Sulfated GAGs ionize readily by negative ion electrospray ionization (ESI) mass spectrometry and are detected as alkali or ammonium salts.[16−18] The molecular mass distribution can be extracted from these spectra, and results have been reported for HLGAGs up to decasaccharide size[16] and CS up to tetradecasaccharide size.[17] ESI spectra of sulfated GAGs are very complex, due to several factors: (A) samples from biological sources represent a mixture of chain lengths; (B) in ESI-MS each molecule is detected with a range of charge states; (C) the sulfate groups are adducted with cations to varying degrees; and (D) the molecules are relatively fragile and may fragment to lose sulfate during ionization. Although all GAG molecules produce abundant ions by negative ESI MS, it is often difficult to extract molecular mass information from the spectra due to the presence of overlapping peaks.

This paper presents improved conditions for ESI MS analysis of GAGs. Negative ESI mass spectra of GAG oligosaccharides are studied in detail. Tandem mass spectra of chondroitin sulfate disaccharides are described and used as the basis for a selective reaction monitoring method for determination of sulfation patterns in chondroitin sulfate. The method is applied to CS disaccharides liberated from decorin and biglycan, small leucine-rich proteoglycans containing one and two CS chains, respectively. Size exclusion chromatography (SEC) LC/MS is shown to be effective for simplifying mass spectra produced from mixtures of CS oligosaccharides, thus facilitating extraction of neutral masses and oligosaccharide compositions.

EXPERIMENTAL SECTION

Electrospray mass spectrometry was conducted using a Micromass/Fisons Quattro II mass spectrometer in the negative ion mode. Solution compositions and infusion rates are given in the figure legends. Collisional-induced dissociation (CID) tandem mass spectra for CS disaccharides were collected using 30-V collision voltage and 3.0×10^{-4}-mbar (argon) gas cell pressure. For the selected reaction monitoring experiments, MS1 was set to transmit m/z 458.1 and MS2 to alternate between m/z 282.0 (Z_1) and 300.0 (Y_1) with a 0.1-s dwell time. Collision voltage was set to 25 V, and the gas cell pressure was 6.4×10^{-4} Torr. CS disaccharide standards ΔDi4S and ΔDi6S were purchased from Seikagaku, America (Falmouth, MA).

Enzymatic Digestion. Bovine articular cartilage decorin or biglycan (Sigma Chemical Co., St. Louis, MO) was dissolved in ammonium acetate and digested with chondroitinase ABC as described in the Figure 6 caption overnight at 37 °C. Chondroitin sulfate A (Sigma) was dissolved at 10 mg/mL in 0.1 M sodium acetate pH 5.0/0.15 M NaCl. An aliquot (100 μL) was taken and testicular hyaluronidase (4 wt %, Sigma) was added. The mixture was incubated at 37 °C for 60 h after which an aliquot (5 μL) was used for LC/MS analysis.

SEC LC/MS of CS Oligosaccharides. A high-performance SEC column (Amersham Pharmacia, Piscataway, NJ, Superdex Peptide PC 3.2/30) was equilibrated in 30% methanol/10 mM HCl at 100 μL/min. The column effluent was split with 10 μL entering the source of the Quattro ESI mass spectrometer. Injections were made with continuous MS detection for 20 min.

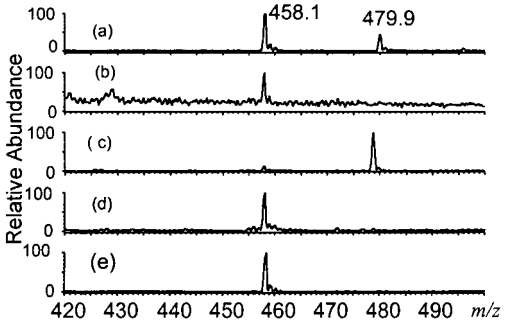

Figure 1. Negative polarity electrospray mass spectra of chondroitin sulfate disaccharides released by treatment of decorin with chondroitinase ABC (structures shown in Figure 4). Disaccharides were diluted to ~10 pmol/μL and infused at 2 μL/min. Disaccharides diluted in (a) 0% methanol; (b) 30% methanol/1% ammonium acetate; (c) 30% methanol/0.1% trifluoroacetic acid; (d) 30% methanol/10 mM HCl; (d) commercial ΔDi4S standard diluted in 30% methanol/10 mM HCl.

RESULTS

Electrospray MS of Chondroitin Sulfate Disaccharides. Chondroitinase ABC acts by eliminative cleavage to produce disaccharides with an unsaturated C4−C5 bond on the uronic acid residue (see Figure 4 for structures). The disaccharides are sulfated at either the 4S or the 6S position on the *N*-acetylgalactosamine residue. Decorin and biglycan were digested with chondroitinase ABC, and the resultant disaccharides were analyzed using negative ESI MS under several different solvent conditions (Figure 1). When diluted in 30% methanol, disaccharides released from decorin produced ions corresponding to $[M - H]^-$ at m/z 458.1 (calculated m/z 458.06) and to $[M - 2H + Na]^-$ at m/z 479.9 (calculated m/z 480.04) (Figure 1a). The sodiated adduct was observed at ~50% abundance relative to the $[M - H]^-$ ion. Dilution of the sample in 30% methanol/1% ammonium acetate resulted in reduced signal-to-noise ratio and detection of the $[M - H]^-$ ion only (Figure 1b). Dilution of the sample in 30% methanol/0.1% TFA resulted in a spectrum in which the $[M - 2H + Na]^-$ ion is severalfold more abundant than the $[M - H]^-$ ion (Figure 1c). Dilution of the sample in 30% methanol/10 mM HCl resulted in a spectrum characterized by an abundant $[M - H]^-$ ion and the absence of an ion corresponding to $[M - 2H + Na]^-$ (Figure 1d). The spectrum of purified ΔDi4S diluted in 30% methanol/10 mM HCl is characterized by an abundant $[M - H]^-$ and no ion corresponding to the sodium adduct (Figure 1e). All subsequent chondroitin sulfate disaccharide mass spectra were acquired from samples diluted in 30% methanol/10 mM HCl.

Solutions of ΔDi4S and ΔDi6S were stored at −20 °C in 30% methanol/10 mM HCl and used as sensitivity standards for electrospray MS. To test stability, aliquots of ΔDi4S and ΔDi6S were (a) dried in a centrifugal concentrator and reconstituted, (b) incubated at 37 °C for 24 h, and (c) incubated at 50 °C for 24 h. Spectra were acquired for these samples as well as freshly made disaccharides in 30% methanol/10 mM HCl. As shown in Figure 2, the abundance of the m/z 458.1 ion is nearly the same among the freshly diluted controls, the dried and reconstituted samples,

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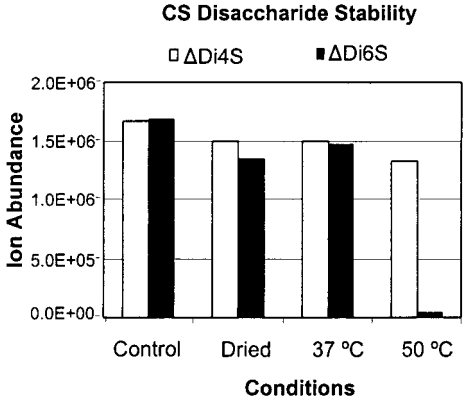

Figure 2. Comparison of CS disaccharide stability under various conditions. Disaccharides were dissolved in 30% methanol/10 mM HCl at 10 pmol/μL and infused at 2 μL/min. Conditions: Control, spectra acquired immediately; Dried, sample dried in a vacuum concentrator and reconstituted in the same solvent before acquisition; 37 °C, sample incubated at 37 °C for 24 h before spectra were acquired; 50 °C, sample incubated at 50 °C for 24 h before spectra were acquired

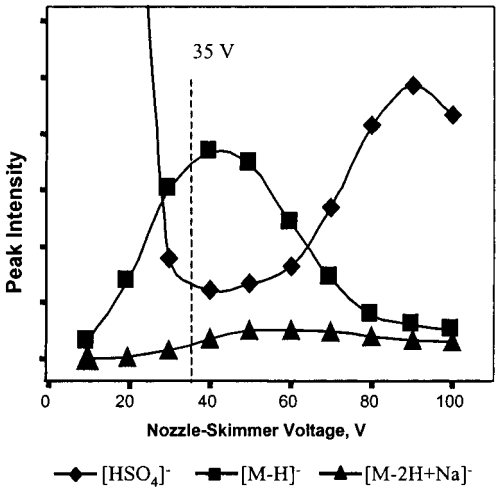

Figure 3. Peak intensity of ions corresponding to $[HSO_4]^-$, (*m*/*z* 96.9), $[M - H]^-$ (*m*/*z* 458.1), and $[M - 2H + Na]^-$ (*m*/*z* 479.9) for ΔDi4S as a function of nozzle−skimmer voltage. Sample (10 pmol/μL) was infused at 2 μL/min in 30% methanol/10 mM HCl, and the nozzle−skimmer voltage was changed in 1-min increments. Spectra were summed in each increment, and the peak intensity for each ion was taken to produce the plot.

and those incubated at 37 °C. While the abundance of the ion corresponding to ΔDi4S indicated that it is stable at 50 °C, that corresponding to ΔDi6S was substantially reduced, indicating degradation. These observations indicate that the disaccharides are stable in 30% methanol/10 mM HCl at temperatures of 37 °C and below. It is therefore unlikely that degradation of disaccharides occurs in solution before they are introduced into the mass spectrometer.

Figure 3 shows the plot of the signal intensity of ions corresponding to $[HSO_4]^-$, $[M - H]^-$, and $[M - 2H + Na]^-$ produced from a sample of purified ΔDi4S as a function of electrospray nozzle−skimmer voltage. The maximum signal intensity occurs at approximately 40 V for the $[M - H]^-$ ion, below which ionization is presumably inefficient. The $[M - H]^-$ ion fragments at nozzle−skimmer voltages greater than 40 V, as evidenced by the increasing strength of the signal corresponding

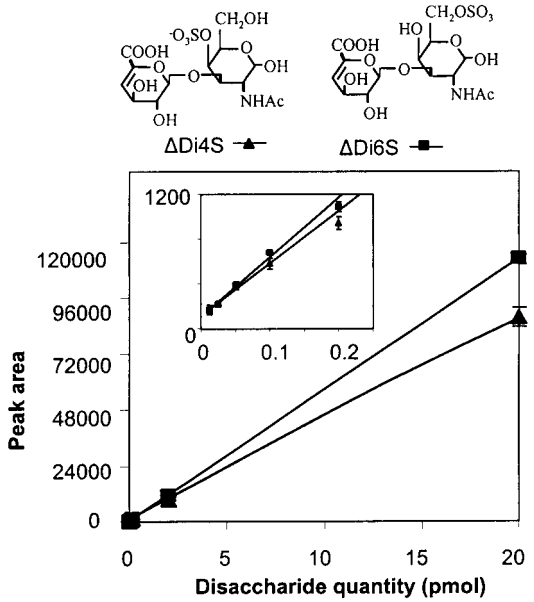

Figure 4. Negative polarity electrospray ionization response for standard chondroitin sulfate disaccharides ΔDi4S and ΔDi6S. Disaccharides were quantified using published molar absorbtivity values and diluted to make a standard series. Solvent (30% methanol/10 mM HCl) was infused at 2 μL/min, and triplicate 0.2 μL injections were made at each concentration for each disaccharide.

to $[HSO_4]^-$. At nozzle−skimmer voltage below 30 V, background ions at *m*/*z* 97 produce a very strong signal. Although sodium adducts produce weak signals at all nozzle−skimmer voltages, the signals are stronger relative to those of the $[M - H]^-$ ion at the higher values. A nearly identical plot was obtained using ΔDi6S (not shown). On the basis of Figure 3, a nozzle−skimmer voltage of 35 V was chosen to avoid fragmentation and minimize abundance of sodium adducts.

Purified disaccharides ΔDi4S and ΔDi6S were quantified using published molar absorbtivities[19,20] and used to make standard dilutions. Electrospray response curves were generated by injecting fixed volumes into the mass spectrometer with selected ion detection at *m*/*z* 458.1 (Figure 4). Disaccharide quantities from 0.0125 to 20 pmol were injected in triplicate, and the peak areas were found to fit second-degree polynomial curves with R^2 values of 1.000, respectively. Using these curves, quantitation can be accomplished over 3 orders of magnitude. The distinct curves produced by the two disaccharides may be due to different ionization responses for ΔDi4S vs ΔDi6S. Alternatively, a small error in the reported molar absorbtivities could result in the differences in slope observed.

CID MS/MS of CS Disaccharides. Figure 5 shows negative ion CID mass spectra for ΔDi4S (a) and ΔDi6S (b) acquired using a triple quadrupole instrument (for conditions see Experimental Section). The spectrum of ΔDi4S shows an abundant ion at *m*/*z* 96.9 corresponding to SO_4^- (calculated *m*/*z* 96.96). The ion at *m*/*z* 175.0 corresponds to C_1 (calculated *m*/*z* 175.02), that at *m*/*z* 299.9 to Y_1 (calculated *m*/*z* 300.04), and that at *m*/*z* 342.0 to $^{0,2}X_1$ (calculated *m*/*z* 342.05). The CID spectrum of ΔDi6S (Figure 5b) shows a remarkably different pattern of fragment ions. The C_1

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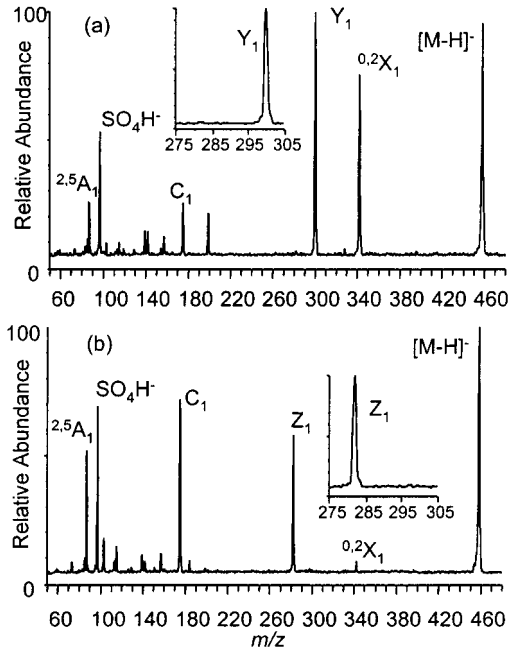

Figure 5. Negative polarity CID MS/MS spectra for chondroitin sulfate disaccharides. Disaccharides were dissolved at 10 pmol/μL in 30% methanol/10 mM HCl and infused at 2 μL/min. Collision voltage was set to 40 V, the gas cell pressure was set to 3.0×10^{-4} mbar, and spectra were acquired for 1 min. Insets show the expanded *m*/*z* 275−305 region. (a) ΔDi4S; (b) ΔDi6S

Table 1. Monoisotopic *m*/*z* Data for CS Disaccharide Negative ESI CID MS/MS Spectra

ion	composition	calcd *m*/*z*	ΔDi4S obsd *m*/*z*	ΔDi6S obsd *m*/*z*
$[SO_4H]^-$	SO_4H	96.96	96.9	96.8
$^{2,5}A_1$	$C_4H_7O_2$	87.04	87.0	87.0
C_1	$C_6H_7O_6$	175.02	175.0	174.9
Z_1	$C_8H_{12}NO_8S$	282.03	nd[a]	282.0
Y_1	$C_8H_{14}NO_9S$	300.04	299.9	nd[a]
$^{0,2}X_1$	$C_{10}H_{16}NO_{10}S$	342.05	342.0	342.1
$[M-H]^-$	$C_{14}H_{20}NO_{14}S$	458.06	458.1	458.2

[a] nd, not detected.

ion is more abundant relative to the SO_4H^- ion and the $^{0,2}X_1$ significantly less abundant than those ions observed in Figure 5a. Most significantly, the Y_1 ion is absent and an abundant Z_1 ion is observed at *m*/*z* 282.0 (calculated *m*/*z* 282.03). Fragment ion data for Figure 5 are summarized in Table 1. Comparison of expanded *m*/*z* regions (Figure 5 insets) shows that the Z_1 ion is not detected above the noise level in the CID mass spectrum of ΔDi4S and that the Y_1 ion is not detected above the noise in the spectrum of ΔDi6S. This result establishes the validity of a selected reaction monitoring (SRM) scheme for quantifying ΔDi4S and ΔDi6S in a mixture such as produced by digestion of a chondroitin sulfate proteoglycan with chondroitinase ABC.

Mixtures of ΔDi4S and ΔDi6S were prepared at ratios ranging from 90:10 ΔDi4S/ΔDi6S to 10:90. Aliquots corresponding to 10 pmol of total disaccharide were injected in 0.2-μL volumes. The first quadrupole was set to transmit ions at *m*/*z* 458.1 and the third set to monitor fragment ions at *m*/*z* 282.0 and 300.0 for Z_1 and Y_1, respectively. CID conditions were set to maximize abundance of the Z_1 and Y_1 ions resulting from infusion of a 1:1

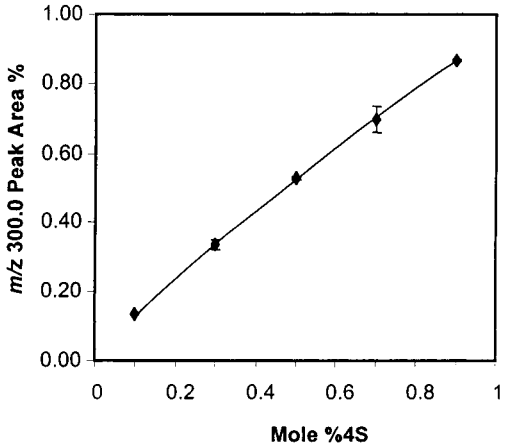

Figure 6. Selected reaction monitoring standard curve for ΔDi4S (*m*/*z* 300.0) and ΔDi6S (*m*/*z* 282.0). Mixtures containing 10 pmol of total disaccharide in ΔDi4S/ΔDi6S ratios of 90:10−10:90 were made, solvent (30% methanol/10 mM HCl) was infused at 2 μL/min, and triplicate 0.2-μL aliquots of each mixture were injected. Decorin (1 μg) and biglycan (0.2 μg) were digested overnight with chondroitinase ABC (0.05mU/μg) in ammonium acetate (5 μL, 0.1 M, pH 7.0) and dried. Samples were dissolved in solvent (30% methanol/10 mM HCl, 10 μL) and injected in triplicate. For each injection, the percent peak area of the *m*/*z* 300.0 peak relative to the total area for *m*/*z* 282.0 and 300.0 for each standard mixture was calculated. The average values from triplicate injections are plotted versus the mole percent ΔDi4S as shown. The percent ΔDi4S values for decorin and biglycan were determined using a second-order polynomial curve (R^2 = 0.9999).

mixture of ΔDi4S and ΔDi6S. The percent area of the *m*/*z* 300.0 peaks relative to the total area for the two peaks was calculated for each injection, and the average values from triplicate injections are plotted versus the ΔDi4S mole percent as shown in Figure 6. Decorin (1 μg) and biglycan (0.2 μg) were digested with chondroitinase ABC in ammonium acetate buffer and dried. The samples were dissolved in 30% methanol/10 mM HCl and injected in triplicate using the mass spectrometric conditions described above. The average *m*/z 300.0% abundance values were used to calculate the percent ΔDi4S values using the second-order polynomial curve calculated from the standard disaccharides. The results showed ΔDi4S values of 87 ± 0.02% for decorin and 81 ± 0.01% for biglycan.

SEC LC/MS of CS Oligosaccharides. Testicular hyaluronidase cleaves the GalNAc−HexA bond of CS to produce oligosaccharides ranging from 4- to 14-mers by hydrolytic cleavage. CS oligosaccharides generated with this enzyme may be fractionated using a 1−2-m SEC column[21] and analyzed off-line using fast atom bombardment[22] or ESI MS.[7] It is also possible to use a high-performance SEC column to rapidly fractionate GAG oligosaccharides with on-line ESI MS detection. ΔDi6S (100 pmol) was injected onto a SEC column that had been equilibrated in 30% methanol/10 mM HCl at 100 μL/min. The effluent was split and 10 μL/min was infused into the ion source. A peak corresponding to *m*/*z* 458 was observed at 17.5 min (data not shown).

Figure 7 shows the total ion chromatogram produced when testicular hyaluronidase-generated oligosaccharides were loaded

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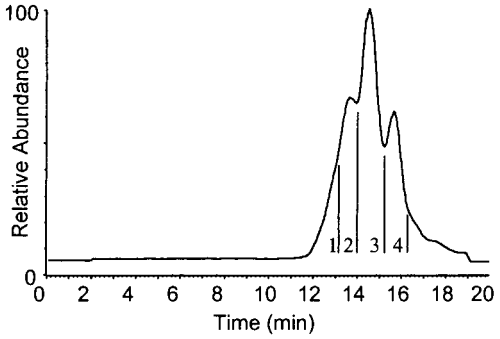

Figure 7. Total ion chromatogram for the SEC fractionation of a testicular hyaluronidase digestion of CS. The Superdex peptide column was equilibrated in 30% methanol/10 mM HCl. Spectra summed from the four indicated regions are shown in Figure 8.

on the column under the same conditions. Because of the presence of buffer salts in the digestion buffer, the eluent was diverted from the mass spectrometer at 19 min. The four chromatographic regions indicated were summed, and the spectra are shown in Figure 8. CS oligosaccharide compositions are given in parentheses, for example (5,5,5)4- refers to the composition $(GlcA)_5(GalNAc)_5(SO_4)_5$ with 4- charge state. In Figure 8a (region 1) the m/z of the most abundant ion (557.8) corresponds to (5,5,4)4- (calculated m/z 557.71). Ions corresponding to CS 12-mers (6,6,6)4- and 14-mers (7,7,7)4- are also present in the spectrum (observed and calculated m/z values are given in Table 2). Because ion source conditions were set to prevent gas-phase fragmentation (Figure 3), the oligosaccharide compositions with fewer sulfates than GalNAc residues (i.e., those with sulfate/GalNAc ratios of ≤ 1) are believed to be present in solution. SEC region 2 (Figure 7) is composed primarily of CS octamers, as can be seen from the summed spectrum in Figure 8b. Ions corresponding to decamers are also observed, and both fully and partially sulfated compositions are evident. Ions corresponding to hexamers are most abundant in Figure 8c (region 3), and ions corresponding to tetramers dominate Figure 8d (region 4).

DISCUSSION

The negative ESI mass spectra of chondroitin sulfate disaccharides diluted in 30% methanol are characterized by abundant sodium adducts (Figure 1a). Since the presence of these adducts would complicate the task of disaccharide quantitation, efforts were made to find solvent conditions that produce only the $[M - H]^-$ ion without compromising sensitivity. Of the solvent conditions tested in Figure 1, 30% methanol/10 mM HCl resulted in no sodium adduction with little reduction. This result is significant, in that the samples used were generated by chondroitinase ABC with no purification step. It is also important to note that no fragmentation of the chondroitin sulfate disaccharides is observed below electrospray nozzle−skimmer voltage of 50 V. This result has implications for the analysis of sulfated glycosaminoglycans by electrospray MS. It is likely that detection of the free acid will result in spectra that are not complicated by sodium adduction or nozzle−skimmer fragmentation. Stability studies (Figure 2) indicate that CS disaccharides are stable in 30% methanol/10 mM HCl at 37 °C or below. Since the primary concern during ESI MS is gas-phase desulfation, these results support use of this solvent mixture for sulfated CS oligosaccharides in general.

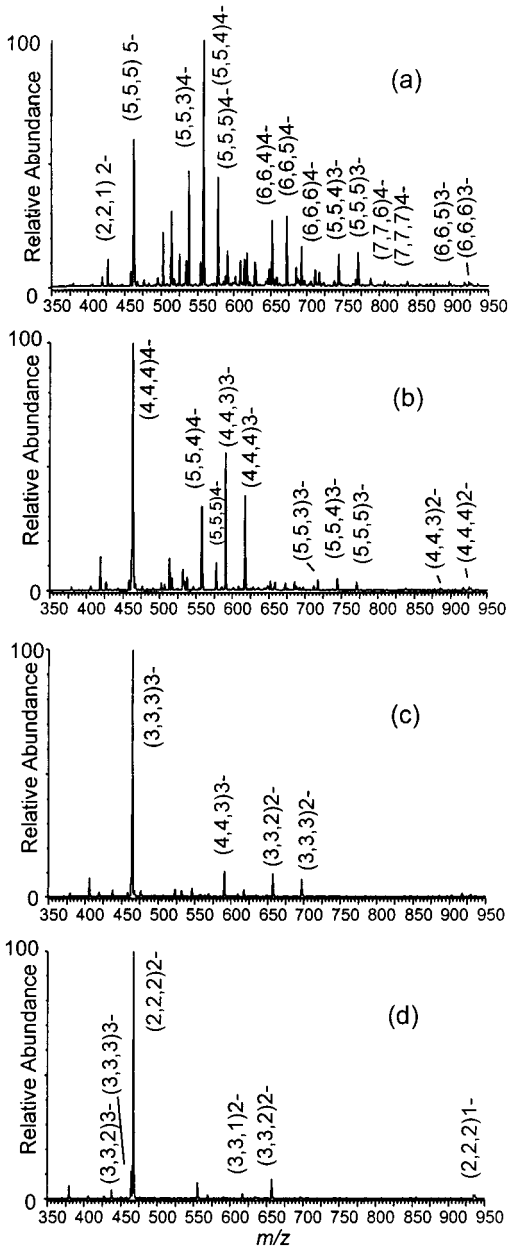

Figure 8. Summed spectra from the SEC fractionation of testicular hyaluronidase CS oligosaccharides: (a) region 1, (b) region 2, (c) region 3, and (d) region 4. The oligosaccharide compositions were calculated from the average m/z values, as shown in Table 2. The numbers in parentheses (X,Y,Z) refer to the oligosaccharide composition, where X = HexA, Y = GalNAc and Z = SO_4H.

The negative ion electrospray CID MS/MS spectra of ΔDi4S and ΔDi6S (Figure 5) are an interesting contrast to tandem mass spectra obtained using FAB ionization on a triple quadrupole mass spectrometer.[23] Linkage region fragmentation by electrospray results in a Y_1 ion for ΔDi4S and a Z_1 ion for ΔDi6S. FAB CID MS/MS spectra for ΔDi4S and ΔDi6S have been reported for the $[M - 2H + 3Na]^+$ ions in which both isomers produced Z_1 ions, with the greater relative abundance observed for ΔDi6S. No Y_1 ion was observed for either disaccharide. Results for negative FAB CID-mass-analyzed ion kinetic energy (MIKE) spectra have been

(23) Ii, T.; Okuda, S.; Hirano, T.; Ohashi, M. *Glycoconjugate J.* **1994**, *11*, 123−32.

Table 2. Observed and Calculated Average Masses for CS Oligosaccharides Detected by SEC LC/MS (Figure 8).

observed m/z	calculated m/z	composition
	Region 1 (Figure 8a)	
427.0	427.35	(2,2,1)2-
462.0	461.98	(5,5,5)5-
538.0	537.69	(5,5,3)4-
557.8	557.71	(5,5,4)4-
577.9	577.72	(5,5,5)4-
652.5	652.54	(6,6,4)4-
672.5	672.55	(6,6,5)4-
692.5	692.57	(6,6,6)4-
743.8	743.95	(5,5,4)3-
770.5	770.64	(5,5,5)3-
787.4	787.40	(7,7,6)4-
807.1	807.42	(7,7,7)4-
896.9	897.08	(6,6,5)3-
923.6	923.77	(6,6,6)3-
	Region 2 (Figure 8b)	
463.1	462.88	(4,4,4)4-
557.8	557.71	(5,5,4)4-
577.9	577.72	(5,5,5)4-
590.9	590.82	(4,4,3)3-
617.6	617.51	(4,4,4)3-
717.3	717.26	(5,5,3)3-
743.9	743.95	(5,5,4)3-
770.6	770.64	(5,5,5)3-
886.6	886.74	(4,4,3)2-
926.5	926.77	(4,4,4)2-
	Region 3 (Figure 8c)	
464.6	464.38	(3,3,3)3-
590.9	590.82	(4,4,3)3-
657.1	657.04	(3,3,2)2-
697.0	697.07	(3,3,3)2-
	Region 4 (Figure 8d)	
438.0	437.69	(3,3,2)3-
464.6	464.38	(3,3,3)3-
467.7	467.38	(2,2,2)2-
617.2	617.01	(3,3,1)2-
657.2	657.04	(3,3,2)2-
935.5	935.78	(2,2,2)1-

reported for m/z 458 for ΔDi4S and ΔDi6S.[24] The spectra are similar to those in Figure 5 in that an abundant Y_1 ion characterizes the spectrum generated from ΔDi4S and an abundant Z_1 ion characterizes that of ΔDi6S. Interestingly, the Z_1 ion is observed at ∼10% abundance relative to the Y_1 ion in the ΔDi4S spectrum and the Y_1 ion is similarly abundant relative to the Z_1 ion in the ΔDi6S spectrum. The absence of such pairs in the negative ESI spectra shown in Figure 5 may be due to the low energy of the collisions producing the fragmentation, relative to those used to generate CID-MIKES fragment ions. The negative ESI CID MS/MS mass spectra allow ΔDi4S and ΔDi6S in a mixture to be differentiated based on a selected reaction monitoring scheme, as shown in Figure 6. Analysis of 0.2−1.0 μg quantities of CS proteoglycan is demonstrated here, and it may be possible to improve these detection limits using a more sensitive analyzer (i.e., quadrupole time of flight).

Determination of the exact masses of sulfated GAG oligosaccharides is complicated by the large size and high degree of heterogeneity of these molecules. As a result, exact mass determination of intact GAG chains is presently not possible. Therefore, partial enzymatic or chemical digestion of GAGs is used to reduce the average molecular weight while structures with putative biological activity are retained. Even at reduced mass, GAG oligosaccharides still present formidable analytical challenges. For example, a pair of heparinoid oligosaccharides of composition $(HexA)_m(GlcN)_m(SO_3)_{2m}$ and $(HexA)_m(GlcN)_m(SO_3)_{2m-1}(Ac)_2$ have the smallest possible mass difference between two GAG chains of different composition, 4.065 Da. As m (the number of disaccharide units) increases, the complexity of the isotopic clusters also increases. Of particular importance is the isotope 4 nominal units above the monoisotopic mass (the A + 4 ion). The A + 4 ion of composition $(HexA)_m(GlcN)_m(SO_3)_{2m}$ differs from the monoisotopic ion of composition $(HexA)_m(GlcN)_m(SO_3)_{2m-1}$ by 0.051 Da. For 10-mers ($m = 5$), the A + 4 abundance is 38%, and for 24-mers, it is the most abundant isotopic peak. Separation of ions differing by 0.051 Da requires resolving power of 1:20 000 for tetramers and 1:116 000 for 24-mers. Clearly the quadrupole mass analyzer used in the present work will provide only average masses for GAG oligosaccharide mixtures, sufficient for assignment of CS oligosaccharide compositions up to ∼14-mers eluting from an SEC column (Figure 8a). This analyzer will be insufficient, however, for resolving spectra of longer CS oligosaccharides or more complex mixtures, particularly those resulting from limited digestion of heparinoids. A reflectron time-of-flight analyzer will be capable of resolving isotopes up to ∼10 kDa but will not resolve overlapping isotopic clusters. Thus, SEC LC/MS will be useful for limiting the complexity of GAG mixtures detected at a given time, allowing extraction of mass values. TOF analyzers are ideally suited for LC/MS and will be capable of resolving isotopes as long as the number of overlapping isotopic clusters is very few. FTICR analyzers will resolve isotopic fine structure but are not presently developed to the point where use for LC/MS is routine. SEC LC/MS has been used to profile heparin lyase I digests of heparin and HS, demonstrating that the approach will be useful for HLGAGS as well as CS.[25] Future efforts in this laboratory will be aimed at using high-resolution TOF and FTICR mass analyzers to define the capabilities and limitations of chromatography−analyzer combinations.

An important potential application of SEC LC/MS will be in the comparison of GAG chain compositions from different sources. It may be possible to compare the abundances of ions corresponding to specific compositions from the different GAG sources using selected ion chromatograms. Such comparisons would take advantage of the unique capability of mass spectrometry to collect data on all components in a mixture simultaneously. In this way, it may be possible to produce more detailed information on the macro- and microheterogeneity of GAG oligosaccharides than is presently available.

CONCLUSIONS

Effective new conditions are described for negative ion mass analysis of sulfated GAG oligosaccharides. By adding 10 mM HCl to the solvent it is possible to analyze disaccharides produced by chondroitinase ABC digestion directly and to determine ratios of sulfation position using multiple reaction monitoring. SEC LC/MS is a useful means for compositional mapping of oligosaccha-

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(25) Zaia, J.; O'Connor, P. B.; Costello, C. E. *Proc. 48th ASMS Conf. Mass Spectrom. Allied Top.*, Long Beach, CA, 2000; pp 939−940.

rides generated by hyaluronidase digestion of CS. The same technique will be applicable to chemical and enzymatic digestion of heparinoids and may provide a new means of comparing compositions derived from different sources. It is likely that some form of on-line chromatographic separation will be necessary even with high-resolution mass analyzers due to the difficulty of resolving overlapping isotopic clusters. Techniques for rapid comparison of GAG oligosaccharide compositions are greatly needed in the postgenome era as attention shifts to the functional implications of protein posttranslational modifications.

Abbreviations: CID, collisional-induced dissociation; CS, chondroitin sulfate; ΔDi4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; ESI, electrospray ionization; FAB, fast atom bombardment; HLGAG, heparin-like glycsoaminoglycan, GAG, glycosaminoglycan; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MIKE, mass-analyzed ion kinetic energy; MS, mass spectrometry.

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