Glycoform Quantification of Chondroitin/Dermatan Sulfate Using a Liquid Chromatography−Tandem Mass Spectrometry Platform†

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ABSTRACT: Chondroitin sulfate (CS) is a glycosaminoglycan consisting of repeating uronic acid, *N*-acetylgalactosamine disaccharide units {[HexAβ/α(1−3)GalNAcβ(1−4)]$_n$}. CS chains are polydisperse with respect to chain length, sulfate content, and glucuronic acid epimerization content, resulting in a distribution of glycoforms for a chain bound to any given serine residue. Usually, CS glycoforms exist, differing in sulfation position and uronic acid epimerization. This work introduces a novel LC−MS/MS platform for the quantification of mixtures of CS oligosaccharides. The CS polysaccharides were partially depolymerized and labeled with either the light (d_0) or heavy (d_4) form of 2-anthranilic acid (2-AA). Excess reagent was removed, and mixtures of the CS standard (d_0) and unknown (d_4) were made. The CS mixture was subjected to size exclusion chromatography (SEC) with on-line electrospray ionization mass spectrometric detection in the negative ion mode. Tandem mass spectra were acquired, and quantification of unknown samples within the mixture was made using relative ion abundances of specific diagnostic ions. The high accuracy and precision of the glycomics platform were demonstrated using glycoform mixtures made from standard CS preparations. The CS glycoform analysis method was then applied to cartilage extract, versican, and several dermatan sulfate preparations. This work presents the first application of a glycomics platform for the quantification of CS oligosaccharide mixtures for obtaining specific information about the positions of GalNAc sulfation and uronic acid epimerization.

Glycomics is applied biology and chemistry that focuses on the structure and function of carbohydrates. Carbohydrates have vital functions in the body; in particular, glycosylation serves to diversify protein functions. Despite their abundance, much less information is known about carbohydrates, as compared to genes and proteins, largely due to the absence of a simple code that determines their structures. Mass spectrometry (MS),[1] high-performance liquid chromatography, and other analytical tools have advanced rapidly to support the growth of biomolecular applications in the field of proteomics. However, technologies and methods for addressing the most challenging problems in glycomics, particularly with respect to glycoform quantitative measurements, remain undeveloped.

Glycosaminoglycans (GAGs) are linear polysaccharides that consist of repeating disaccharide units that are attached to proteoglycan core proteins on adherent animal cell surfaces and in extracellular matrices (*1*−*5*). GAGs play significant roles in the modulation of cellular signals through interactions with proteins, including growth factors and growth factor receptors (*6*−*8*). They mediate cell−cell and cell−matrix interactions and are crucial to cell development and function (*9*, *10*). GAG fine structure, consisting of patterns of sulfation and uronic acid epimerization, is variable and diverse. This is the result of maturation reactions involving sulfation, epimerization, and glycosylation, which are dependent on cell type and not the core protein. Depending on GAG type, disaccharide units may be epimerized at the C-5 position of uronic acid residues and may be N- or O-sulfated and/or N-acetylated. The mammalian GAGs are hyaluronan, dermatan sulfate (DS), chondroitin sulfate (CS), heparan sulfate (HS), heparin, and keratan sulfate (KS).

CS is a GAG linked to serine residues in core proteins by way of a xylosyl linker; it consists of repeating disaccharide units of HexAβ/α(1−3)GalNAcβ(1−4) that are polymerized in chains of size varying from 20 to 50 kDa depending on the core protein, tissue location, and disease contexts. The chains consist of mixtures of domains with high or low iduronic acid content with differing patterns of sulfation (*11*). Many types of CS exist; however, the three main classifications of CS found in higher animals include CS type A (CSA), CS type B (CSB, otherwise known as DS, or dermatan sulfate), and CS type C (CSC). CSA is most commonly sulfated (90%) at the 4-position of GalNAc, and CSC is most commonly sulfated (90%) at the 6-position of GalNAc. CSB contains a high percentage of repeats in which the uronic acid residue is epimerized to iduronic acid; a fraction of such repeats are sulfated at the 2-position of

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[1] Abbreviations: AMAC, 2-aminoacridone; CE, capillary electrophoresis; CID, collision-induced dissociation; CS, chondroitin sulfate; CSA, chondroitin sulfate type A; CSB, chondroitin sulfate type B; CSC, chondroitin sulfate type C; dp, degree of polymerization; DS, dermatan sulfate; GAG, glycosaminoglycan; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LIF, laser-induced fluorescence; SEC, size-exclusion chromatography; 2-AA, 2-anthranilic acid.

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iduronic acid. CSB is most often sulfated at the 4-position of GalNAc. Previous research has produced evidence that suggests that isomeric CS glycoforms differing in position and degree of sulfation play specific and distinct functional roles during development (*12*−*14*). The sulfation patterns of CS chains bound to the cartilage proteoglycan aggrecan have been shown to change with age. Mixtures of 4- and 6-sulfated GalNAc residues were shown to be present in juvenile cartilage, whereas that from adults contained almost entirely 6-sulfated GalNAc residues (*15*). For cartilage aggrecan, changes in sulfation pattern have been correlated not only with aging and development but also with the development of osteoarthritis (*16*). However, because of the lack of analytical techniques, it has not been possible to directly determine sulfation and uronic acid positional isoforms in CS oligosaccharides. Thus, detailed structural information about the variability of CS glycoform expression in extended sequences during biological processes is lacking.

MS is a valuable tool for structural and quantitative analysis of biomolecules, such as GAGs, as it offers high analytical versatility, sensitivity, and precision. Negative mode electrospray ionization (ESI) in the presence of ammonium salts has proven to be effective for the analysis of sulfated carbohydrates (*17*−*19*). Intact negative ions are formed without in-source fragmentation, thereby allowing tandem mass spectra to be acquired through the use of collision-induced dissociation (CID) (*20*). ESI is also advantageous because it allows for on-line coupling of mass spectrometry with chromatographic separation methods. Methods have already been developed for the use of size exclusion chromatography (SEC) and tandem mass spectrometry for the determination of sulfation position in CS oligosaccharides (*18*, *21*). It has previously been shown that CS disaccharides sulfated at the 4- or 6-position of the GalNAc residue, CSA or CSC, respectively, produce distinct CID product ion patterns (*18*, *22*, *23*). Oligosaccharides produced from the three glycoforms of CS (CSA, CSB, and CSC) may be differentiated on the basis of product ion abundance (*24*, *25*).

A specific challenge arises in the tandem mass spectrometric analysis of CS oligosaccharides. Lyase enzymes may be used to generate mixtures of oligosaccharides from intact CS or DS chains. The monoisotopic peaks for the most abundant charge state of CS oligosaccharides that differ in length are observed at the same *m*/*z* value (*m*/*z* 458), resulting in the observation of overlapping isotopic clusters. In this work, CS partial depolymerization products are derivatized by a reductive amination reaction. This step has two additional advantages. The first is that alkylated CS oligosaccharides have unique *m*/*z* values for their most abundant charge state. The second is that incorporation of the derivative offers a means for stable isotopic labeling. The use of a stable isotope allows direct comparison of two samples, one labeled with the light form and one with the heavy, present in a mixture. In this work, unknown samples are compared to standard preparations using stable isotope labeling.

This work demonstrates an analytical LC−MS/MS platform that incorporates a stable isotopic labeling technique for the quantification of CS glycoforms starting with a 1−10 μg quantity of sample. The platform introduced herein is advantageous in that the quantification of glycoforms within CS/DS samples is calculated directly from tandem mass spectral data. Extended sequence analysis and quantification of CS and DS chains have previously been investigated through the use of differential lyase digestion with chondroitinase ACI and chondroitinase B followed by ion-pair reversed-phase HPLC (IP-RP-HPLC). Following IP-RP-HPLC, 4,5-unsaturated uronic acid oligosaccharides (termed Δ-oligosaccharides) are further digested to Δ-disaccharides with chondroitinase ABC for structural and quantitative analysis by high-performance capillary electrophoresis (*26*−*28*). This platform follows a simpler analytical workflow, without the need for consecutive digestions and separations. The method is advantageous in that data for sulfation position and uronic acid epimers are produced simultaneously. Further, it is not necessary to purify the Δ-oligosaccharides. Presented here are results demonstrating the usefulness of a LC−MS/MS platform for the quantification of isomeric glycoforms of CS and DS from standard mixtures and applications to biologically relevant connective tissue samples.

EXPERIMENTAL PROCEDURES

Materials. CS type A (GlcA, GalNAc-4-sulfate), CSB (IdoA, GalNAc-4-sulfate), CSC (GlcA, GalNAc-6-sulfate), and chondroitinase ABC and AC1 were obtained from Seikagaku America/Associates of Cape Cod (Falmouth, MA). DS samples were generous gifts of A. Malmström (Lund University, Lund, Sweden). Versican was a generous gift from R. Perris (University of Parma, Parma, Italy). Purified Δ-disaccharide standards were purchased from V-labs (Covington, LA). 2-Aminoacridone (AMAC) and 2-anthranilic acid (d_0-2AA) were purchased from Fluka Chemika (Buchs, Switzerland); cartilage extract and sodium cyanoborohydride were from Aldrich Chemicals Co. (St. Louis, MO), and 2-anthranilic-3,4,5,6-d_4 acid (d_4-2AA) was from C/D/N Isotopes (Quebec City, PQ). Cellulose Packing Material Micro Spin Columns were purchased from Harvard Apparatus (Holliston, MA).

Release of GAG from the Core Protein. Sulfated GAGs were released from the core protein of both cartilage extract (from bovine articular cartilage) and versican (from bovine aorta) samples with a 1.0 M $NaBH_4$, 0.05 M NaOH solution at 45 °C for 16 h. The pH was adjusted to 5.0 using glacial acetic acid. The core protein was then separated from the GAG using a Pepclean C-18 spin column (Pierce, Rockford, IL). The sulfated GAGs (CS) were then separated from O-linked glycans by ethanol precipitation (−20 °C, overnight).

Preparation of Δ-Unsaturated Chondroitin Sulfate Oligosaccharides. CSA, CSB, or CSC (5 μL, 2 mg/mL) was mixed with water (87 μL), Tris-HCl buffer (5 μL, 1 M, pH 7.4), ammonium acetate (0.5 μL, 1 M), and chondroitinase ABC (2.5 μL, 4 milliunits/μL), digested at 37 °C to an absorbance value (232 nm) equal to 0.341, and boiled for 1 min. This degree of digestion was found to reproducibly depolymerize 10 μg of CSA, CSB, and CSC to 30% reaction completion, as determined by the absorbance at 232 nm. Partial depolymerization products were lyophilized for subsequent reductive amination.

Derivatization of Oligosaccharides with d_0- and d_4-2-AA. All oligosaccharides were derivatized with d_0- or d_4-2AA according to the method of Bigge et al. (*29*). Briefly, a dried

CSA sample was dissolved in 10 μL of a reaction reagent containing d_0-2AA in a DMSO/glacial acetic acid mixture (7:3) and 1.0 M sodium cyanoborohydride. All other partially depolymerized CS (unknown) samples were derivatized using d_4-2AA under the same conditions. The glycan solutions were then centrifuged for 3 min and incubated at 65 °C for 3 h. In an attempt to optimize the published reductive amination protocol, water and ammonium hydroxide were added to reaction mixtures 1 h into the incubation time to create a more neutral or basic environment. Also, two consecutive derivatizations were used to give the portion of the glycan that did not react the first time a more optimal opportunity to react during a second round. The relative derivatization yields were determined via mass spectrometry on a quadrupole ion trap mass spectrometer (QITMS). Samples were sprayed in a 30% MeOH, 0.2% NH_4OH solution under nanospray conditions (*24*). Mass spectra revealed peaks at *m*/*z* 459.1 and 578.8 representing the unlabeled and labeled CS disaccharides, respectively. The relative yield was found by calculating the percent total ion abundance (%TIA) of the labeled and unlabeled compounds. None of the procedure modifications improved on the published method.

Derivatization Sample Cleanup. Excess reagents were removed from derivatized glycans via cellulose microspin columns. The column was first hydrated with five 200 μL volumes of water and rinsed with five 200 μL volumes of a 30% acetic acid solution and then with three 200 μL volumes of acetonitrile. The 2-AA-derivatized reaction mixture was applied to the column, allowing 15 min for it to adsorb to the cellulose. Excess reagents were washed off with three 200 μL volumes of acetonitrile followed by two 200 μL volumes of 96% acetonitrile. The derivatized glycan was then eluted with two 100 μL volumes of water and dried.

LC−MS Analysis. The mixed, derivatized glycan samples were fractionated using high-performance SEC with on-line tandem mass spectrometric detection. Briefly, the column (Superdex Peptide 3.2/30, Amersham Biosciences, Piscataway, NJ) was equilibrated in a 10% acetonitrile, 0.05 M ammonium formate solution at a rate of 40 μL/min, and the oligosaccharide mixture (10 μL) was injected with UV detection at 310 nm.

The HPLC system was connected to a Bruker Daltonics (Billerica, MA) Esquire 3000 QITMS equipped with a standard electrospray ion source. The spray voltage was set at 3 kV; the capillary temperature was set to 250 °C. The nebulizer gas (He) was set to a pressure of 10 psi, and the drying gas (N_2) was set to a rate of 5 L/min. The capillary exit was set to −50.9 V and the skimmer potential to −10 V to prevent in-source fragmentation. The ion signal was optimized using a trap drive of 60. Introduction of the sample into the mass spectrometer was achieved by connecting the SEC column to the sample inlet of the QIT electrospray source with peek tubing. To minimize the dead volume, the column was clamped to a ring stand as close as possible to the MS sample inlet. The HPLC flow was split prior to the sample inlet, allowing 10 μL into the mass spectrometer per minute. All scans were acquired in the negative ion mode using the automated MS^n feature of the ion trap. To enable data-dependent scanning, specific parent ions (representing hexasaccharides, tetrasaccharides, and disaccharides) were listed into the auto MS^n method. The width of the isolation and fragmentation windows was set to 12.0 u so that CID spectra of both heavy and light forms of derivatized CS species within the mixture were acquired simultaneously. The ion trap was set at an accumulation time of 150 u/s. The instrument was set at a scan speed of 13 000 u per second, adequate to resolve heavy and light paired fragment ions and their isotopic distribution. The instrument was set to cycle between the MS and MS/MS modes until the entire mixture had eluted through the column. The MS/MS scans were summed for the most abundant charge state for the CS oligosaccharides; this corresponds to one negative charge per sulfate group.

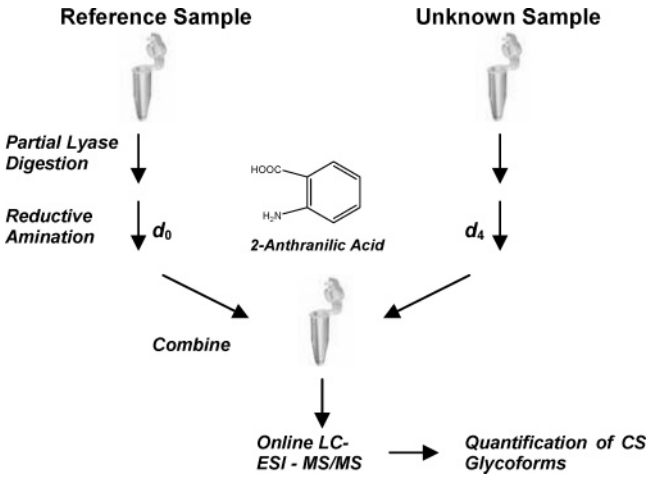

FIGURE 1: Protocol for the LC−MS/MS platform.

Calculation of Equation Coefficients. Tandem mass spectral data were collected on 2AA-labeled CSA, CSB, and CSC standards to establish diagnostic ions. An internal standard, d_0-2AA-CSA, was used for every sample. d_4-2AA-CSB and d_4-2AA-CSC were run with each series of samples, in triplicate. The percent total ion abundance of the diagnostic ions in each 2-AA-labeled standard was then calculated, and the results represent the equation coefficients, described further in the Results. The coefficients are therefore recalculated for every corresponding series of samples.

Exhaustive Digestion of CS and DS. CS or DS (10 mg) was mixed with 87 μL of water, 5 μL of 1 M Tris-HCl buffer (pH 7.4), 0.5 μL of 1 M NH_4OAc, 10 milliunits of chondroitinase ABC, 10 milliunits of AC1, and 2 milliunits of chondroitinase B, and digestion was allowed to proceed at 37 °C for 3 h. The reaction was stopped by incubating the mixture with boiling water for 2 min, and the mixture was then derivatized with AMAC.

AMAC Derivatization. Derivatization with AMAC was performed following the procedure described by Militsopoulou (*30*). Briefly, to the lyophilized CS/DS disaccharide sample were added 5 μL of 0.1 M AMAC solutions in acetic acid/DMSO mixtures (3:17, v/v) and 5 μL of a freshly prepared 1 M $NaBH_3CN$ solution in water. The mixture was vortexed for 3 min and was incubated at 45 °C for 4 h, after which 10 μL of DMSO was added. Excess reagent was removed using cellulose spin columns as described for 2-AA above.

Capillary Electrophoresis−Laser-Induced Fluorescence (CE−LIF) Analysis. The AMAC-derivatized CS/DS samples were analyzed using a Beckman Coulter (Fullerton, CA) P/ACE MDQ capillary electrophoresis instrument. The uncoated fused silica capillary tubing (75 μm inside diameter,

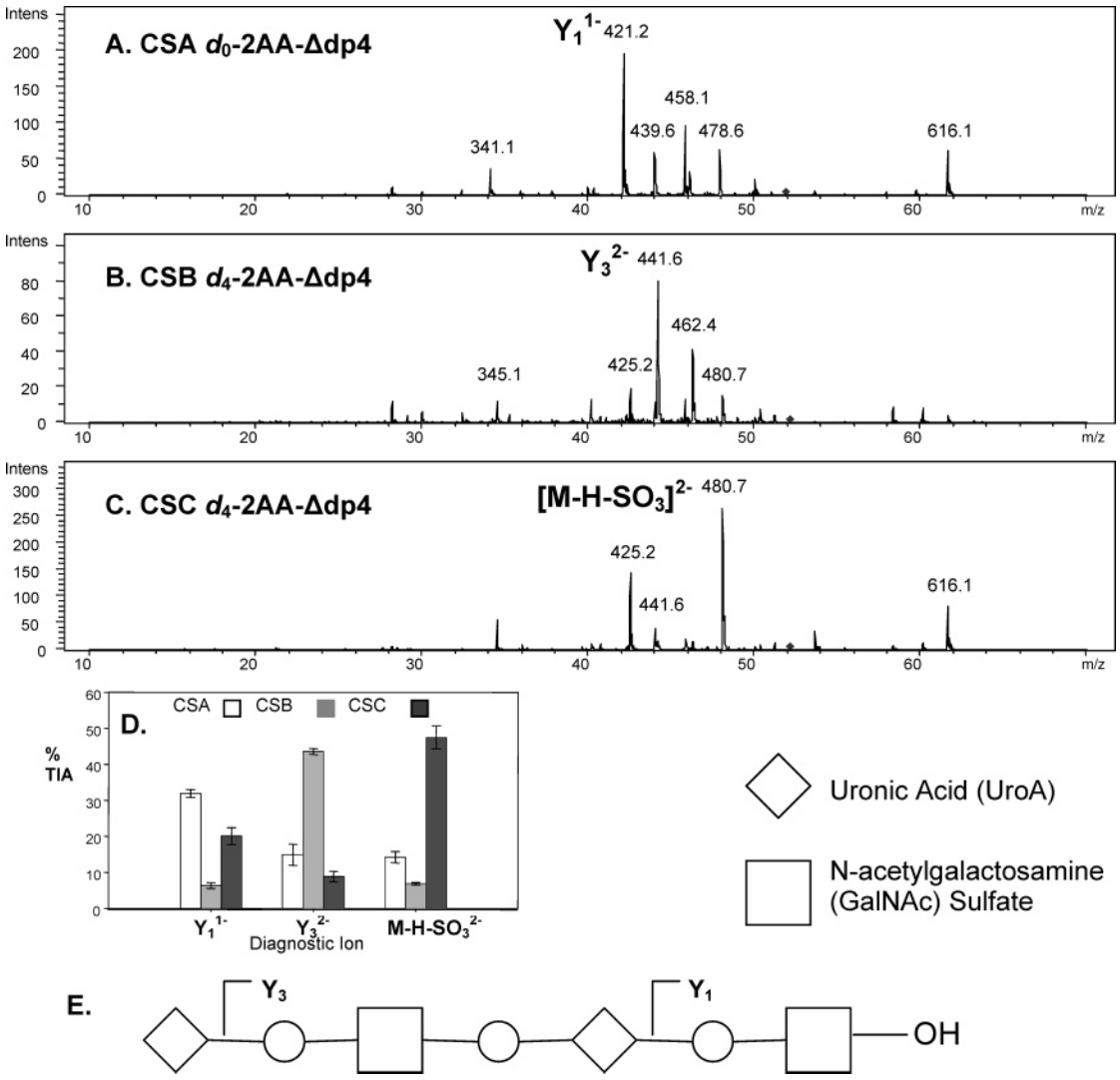

FIGURE 2: (A) Tandem mass spectrum of CSA d_0-2AA-Δdp4. (B) Tandem mass spectrum of CSB d_4-2AA-Δdp4. (C) Tandem mass spectrum of CSC d_4-2AA-Δdp4. (D) Bar graph representation of the contribution of each diagnostic ion in the pure CS standards: CSA (white), CSB (gray), and CSC (dark gray). (E) Structure of a CS tetrasaccharide and its diagnostic fragment ions.

60 cm total length) was regenerated with 1.0 M HCl, 0.1 M NaOH, and HPLC grade water before each run, and analyses were performed using 50 mM NaH_2PO_4 buffer (pH 3.5) at 30 kV using reverse polarity and detected using the AMAC fluorophore (λ_{ex} = 485 nm, λ_{em} = 510 nm). Baseline separation of the CS and DS isomers and good reproducibility were also obtained when fresh anode and cathode buffer solutions were used for each run. A trisulfated heparin disaccharide (ΔHSIS) was used as an internal standard for each run as it has a unique migration time compared to the CS/DS disaccharides.

RESULTS

LC−MS/MS Platform. Each unknown CS sample was divided into three equal aliquots, and each aliquot was subjected to the analytical workflow shown in Figure 1. Each aliquot was partially depolymerized using chondroitinase ABC, to produce a mixture consisting of abundant (4−5)-unsaturated (Δ-) hexasaccharides, tetrasaccharides, and disaccharides. The reference standard CS (CSA, Seikagaku) was then derivatized via a reductive amination reaction with d_0-2AA, while the unknown CS aliquots were derivatized via a reductive amination reaction with d_4-2AA. The excess derivatization reagent was removed using a cellulose microspin column. Mixtures of the standard (d_0) and unknown (d_4) CS samples were then made. The isotopically labeled CS mixtures were separated using SEC with on-line negative ion ESI-MS/MS detection.

Distinguishing Diagnostic Ions for Isomeric CS Glycoforms. It has previously been shown that native oligosaccharides yield tandem mass spectrometric fragment ions, the abundances of which are diagnostic of the CS type (*18*, *22*, *23*). Because this prior work utilized native CS oligosaccharides, it was necessary to show that reductively aminated CS yields different fragment ion abundances according to sulfation and epimerization patterns. Therefore, 10 μg samples of CSA $(GlcA\text{-}GalNAc4S)_n$, CSB $(IdoA\text{-}GalNAc4S)_n$, and CSC $(GlcA\text{-}GalNAc6S)_n$ standards were subjected to the analytical scheme shown in Figure 1. Tandem mass spectra for the 2-AA Δ-unsaturated depolymerized tetrasaccharide (termed Δdp4) peaks are shown in Figure 2 for (a) d_0-2AA-CSA, (b) d_4-2AA-CSB, and (c) d_4-2AA-CSC. From visual inspection, it can easily be determined that abundant fragment ions for 2-AA-Δdp4 oligosaccharides distinguish CS glycoforms, including Y_1^- for CSA, Y_3^{2-} for CSB, and $[M - H - SO_3]^{2-}$ for CSC. The Y_1^- ion is present in all three

Table 1: CID Diagnostic Product Ion Data for the 2-AA-Δdp4 CSA, CSB, and CSC Standards

ion	composition	calcd *m/z*	observed *m/z*
$[d_0\text{-2AA} - Y_1 - SO_3]^-$	$C_{15}O_7H_{22}N_2$	341.13	341.1
$[d_4\text{-2AA} - Y_1 - SO_3]^-$	$C_{15}O_7H_{22}N_2D_4$	345.16	345.1
$[d_0\text{-2AA} - Y_1]^-$	$C_{15}O_{10}H_{22}N_2S$	421.08	421.2
$[d_4\text{-2AA} - Y_1]^-$	$C_{15}O_{10}H_{22}N_2SD_4$	425.12	425.2
$[d_0\text{-2AA} - Y_3]^{2-}$	$C_{29}O_{24}H_{43}N_3S_2$	439.58	439.6
$[d_4\text{-2AA} - Y_3]^{2-}$	$C_{29}O_{24}H_{43}N_3S_2D_4$	441.59	441.6
$[d_0\text{-2AA} - {}^{0,2}X_1]^-$	$C_{17}O_{11}H_{19}N_2S_1$	458.06	458.1
$[d_4\text{-2AA} - {}^{0,2}X_1]^-$	$C_{17}O_{11}H_{19}N_2S_1D_4$	462.18	462.4
$[d_0\text{-2AA} - M - H - SO_3]^{2-}$	$C_{35}O_{26}H_{49}N_3S$	478.61	478.6
$[d_4\text{-2AA} - M - H - SO_3]^{2-}$	$C_{35}O_{26}H_{49}N_3SD_4$	480.62	480.7
B_3^-	$C_{20}H_{26}NO_{19}S$	616.08	616.1

species; however, it is significantly higher in abundance for the 2-AA-Δdp4 oligosaccharides derived from CSA relative to those from CSB and CSC and is therefore diagnostic of CSA. The same is true for the Y_3^{2-} and $[M - H - SO_3]^{2-}$ ions for CSB and CSC, respectively. To use such diagnostic ions as a means of quantification, it is necessary to calculate the percent total ion abundance of all three ions in the spectra of each CS species. The resulting percent total diagnostic ion abundances, for all three CS species, shown in Figure 2d, demonstrate that each form of CS Δdp4 oligosaccharide produces a unique signature; the pattern observed for an unknown is produced by a linear combination of product ion abundances reflecting the composition of the mixture with respect to Δdp4 oligosaccharides containing CSA-, CSB-, and CSC-like disaccharide repeats (*25*). A complete list of ion *m/z* values observed in the tandem mass spectra of 2-AA-Δdp4 CS standards is presented in Table 1.

Binary Mixtures of CS Tetrasaccharide Standards. To validate the LC−MS/MS platform, the same CSA, CSB, and CSC standards used above were subjected to the workflow shown in Figure 1. The isotopically labeled CS species were then mixed together in certain ratios, termed standard mixtures, to assess the validity of the method of quantification. All isotopically labeled mixtures were subjected to the entire analytical workflow in triplicate to determine the total experimental standard deviation. Each sample was mixed with d_0-2AA oligosaccharides derived from CSA. The first mixture that was analyzed was a 1:1 mixture of 5 μg of d_0-2AA-CSA and 5 μg of d_4-2AA-CSC. The CS mixture was separated by SEC with on-line ESI-MS/MS detection. The resulting MS total ion chromatogram can be seen in Figure 3a. The signal-to-noise ratio for 2-AA-Δdp6 was not high enough to yield confident results. The 2-AA-Δdp4 and 2-AA-Δdp2 spectra had sufficient signal-to-noise ratios and were therefore useful for glycoform mixture analysis. The Δdp4 oligosaccharides produce more information than the Δ-disaccharides due to the existence of an internal uronic acid residue; therefore, 2-AA-Δdp4 oligosaccharides will be discussed herein.

The tandem mass spectrum acquired for the 2-AA-Δdp4 mixture (1:1 d_0-2AA-CSA/d_4-2AA-CSC) is shown in Figure 3b. From the product ion isotopic ratios in the tandem mass spectrum, one can see that a mixture of glycoforms is present (see Figure 3c). The light (d_0) CSA and heavy (d_4) CSC precursor ions yield fragment ions that are paired by either 4 u if it is a singly charged ion or 2 u if it is doubly charged. Therefore, one can see in Figure 3c that two peaks exist at *m/z* 421.2 and 425.2 that represent the light and heavy Y_1^- ion, respectively. A pair of peaks differing by 2 u at *m/z* 478.7 and 480.7 are representative of the $[M - H - SO_3]^{2-}$ ion.

To quantify the mixture present in the sample, the percent total ion abundances of light and heavy diagnostic ions of interest were found by listing all ions and abundances within the spectrum and then removing all that do not contain the reducing end, therefore considering only ions paired by 4 or 2 units. These ions are then separated into heavy and light columns. The percent total ion abundances were calculated separately for heavy and light diagnostic ions. This resulted in two groups of ions, one significantly more abundant for CSA Δdp4 (light ions) and one more abundant for CSC Δdp4 (heavy ions). The 1:1 CSA/CSC mixture was subjected to the entire analytical workflow in triplicate to produce average percent ion abundances with standard deviations.

Quantification. To quantify glycoforms within the mixture, the percent total ion abundances of the diagnostic ions in both the pure standards and the standard mixture were used in a system of three equations and three unknowns. The diagnostic ion abundances for the three standard CS samples were used to determine the equation coefficients. The system of equations is a method originally used to quantify mixtures of oil distillates (*31*). The procedure was later adapted and specifically modified for the compositional analysis and quantification of glycosaminoglycans using electrospray ionization quadrupole ion trap mass spectrometry (*22*, *32*, *33*). It results in the determination of the relative quantity of each component in a given glycosaminoglycan mixture. A system of equations based on the same principles is given:

$$C_{Y_1} = aY_{1CSA} + bY_{1CSB} + cY_{1CSC}$$

$$C_{Y_3} = aY_{3CSA} + bY_{3CSB} + cY_{3CSC}$$

$$C_{M-H-SO_3} = a[M - H - SO_3]_{CSA} + b[M - H - SO_3]_{CSB} + c[M - H - SO_3]_{CSC}$$

where *a*, *b*, and *c* represent the percentage of CSA, CSB, and CSC, respectively, in a mixture. The constants Y_{1CSA}, Y_{1CSB}, and Y_{1CSC} represent the abundance of the Y_1^- diagnostic ion in the pure standards. Y_{3CSA}, Y_{3CSB}, and Y_{3CSC} represent the abundance of the Y_3^{2-} diagnostic ion in the pure standards, and $[M - H - SO_3]_{CSA}$, $[M - H - SO_3]_{CSB}$, and $[M - H - SO_3]_{CSC}$ represent the abundance of the $[M - H - SO_3]^{2-}$ diagnostic ion in the pure standards. C_{Y_1}, C_{Y_3}, and C_{M-H-SO_3} represent the abundance of the heavy and light diagnostic ions measured in the unknown CS sample.

Upon insertion of the values of the diagnostic ion abundances for both the pure standards and the 1:1 mixture of d_0-2-AA-CSA and d_4-2-AA-CSC, the system of equations yields

$$73.16 = a \times 31.93 + b \times 6.37 + c \times 20.09$$

$$38.84 = a \times 14.90 + b \times 43.56 + c \times 8.86$$

$$87.98 = a \times 14.20 + b \times 6.85 + c \times 47.48$$

where *a*, *b*, and *c* were then calculated to equal 1.368, 0.1338, and 1.424, respectively. The percentage of CSA in the mixture was then calculated by dividing the value of *a* by

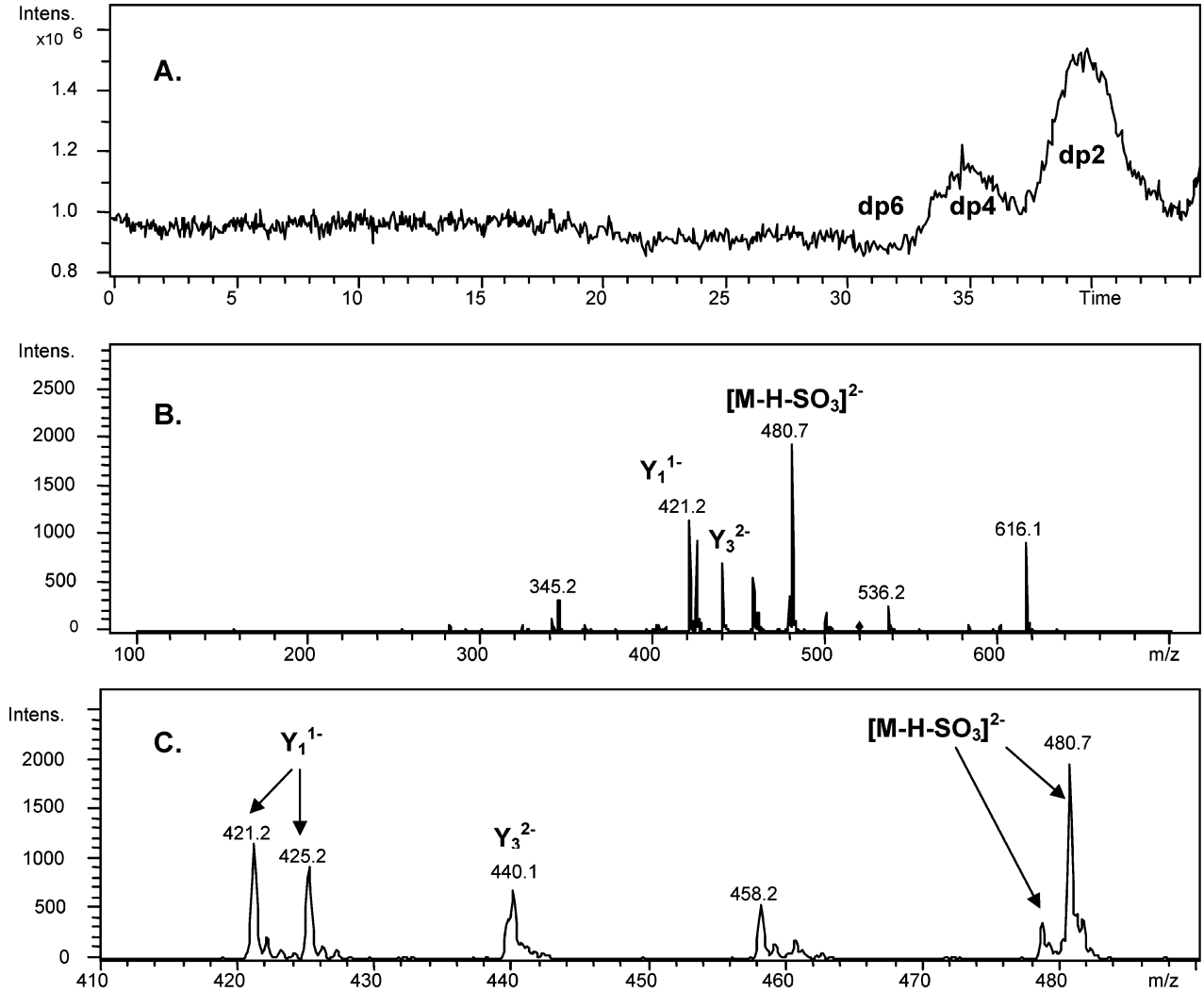

FIGURE 3: (A) Total ion chromatogram of a 10 μg binary 1:1 mixture of d_0-2AA-CSA and d_4-2AA-CSC. (B) Tandem mass spectrum of the 1:1 tetramer mixture of d_0-2AA-CSA and d_4-2AA-CSC. Diagnostic ions (Y_1^-, Y_3^{2-}, and $[M - H - SO_3]^{2-}$) are paired by 2 or 4 units depending on whether they are doubly or singly charged, respectively. (C) Expanded mass range of the diagnostic ions. CSA labeled with the light form (d_0) of 2-AA was incorporated as an internal standard. In the case of the CS standard mixture given above, the CSA standard sample labeled with d_0-2AA was mixed with a CSC sample (termed unknown) labeled with d_4-2AA.

the sum of the values of *a*, *b*, and *c*. The percentages of CSB and CSC were then calculated in the same manner. The percentages of CSA, CSB, and CSC in the first mixture were found to be 46.8, 4.5, and 48.7%, respectively. The original CS mixture was previously separated into three aliquots and subjected in parallel to the protocol shown in Figure 1. The final isomer percentages are represented as an average of the determination made for three aliquots of each mixture subjected to the entire analytical workflow to give a standard deviation that is representative of the total experimental error. Nine different binary mixtures created from CS standards were subjected to the LC−MS/MS platform in this manner, and the results are given in Table 2.

Quantification of Ternary Mixtures of CS Tetrasaccharide Standards. Following the quantification of nine binary mixtures, it was necessary to validate that the LC−MS/MS platform is also applicable to ternary mixtures. Therefore, a 1:1:1 isotopically labeled CS mixture of 3.3 μg of d_0-2AA-CSA, 3.3 μg of d_4-2AA-CSB, and 3.3 μg of d_4-2AA-CSC was generated and subjected in triplicate to the same protocol described previously. The resulting tandem mass spectrum of the tetrasaccharide mixture is shown in Figure 4a. Paired peaks representing the diagnostic ions are present at *m*/*z* 421.0 and 425.0 (Y_1^-), *m*/*z* 439.4 and 441.4 (Y_3^{2-}), and *m*/*z* 478.5 and 480.5 ($[M - H - SO_3]^{2-}$). The percent total ion abundance of each heavy and light diagnostic ion was inserted into the system of three equations and three unknowns given above, and the equations were solved for *a*, *b*, and *c*. The percentages of CSA, CSB, and CSC in the ternary mixture were found to be, as shown in Table 2, 34.9 ± 3.3, 34.7 ± 5.3, and 30.4 ± 2.3%, respectively.

Table 2: Percent Composition of CSA-like (GlcAGalNAc4S), CSB-like (IdoAGalNAc4S), and CSC-like (GlcAGalNAc6S) Isomeric Glycoforms in 10 Different Artificially Made CS Mixtures

mixture	CSA-like GlcAGalNAc4S	CSB-like IdoAGalNAc4S	CSC-like GlcAGalNAc6S
3:7 CSA/CSC	36.9 ± 1.5%	0.8 ± 0.8%	62.2 ± 0.5%
1:1 CSA/CSC	48.7 ± 1.7%	3.8 ± 1.2%	49.0 ± 3.3%
7:3 CSA/CSC	66.0 ± 3.1%	3.8 ± 1.3%	30.1 ± 1.9%
3:7 CSA/CSB	34.5 ± 2.7%	64.8 ± 2.4%	0.7 ± 1.0%
1:1 CSA/CSB	53.9 ± 1.7%	46.0 ± 2.3%	0.7 ± 0.5%
7:3 CSA/CSB	66.4 ± 1.8%	32.9 ± 1.9%	0.8 ± 0.4%
3:7 CSB/CSC	2.0 ± 1.2%	31.8 ± 3.1%	66.2 ± 4.3%
1:1 CSB/CSC	2.6 ± 2.5%	48.3 ± 1.9%	49.1 ± 2.9%
7:3 CSB/CSC	4.2 ± 2.1%	64.9 ± 1.1%	30.9 ± 2.4%
1:1:1 CSA/CSB/CSC	34.9 ± 3.3%	34.7 ± 5.3%	30.4 ± 2.3%

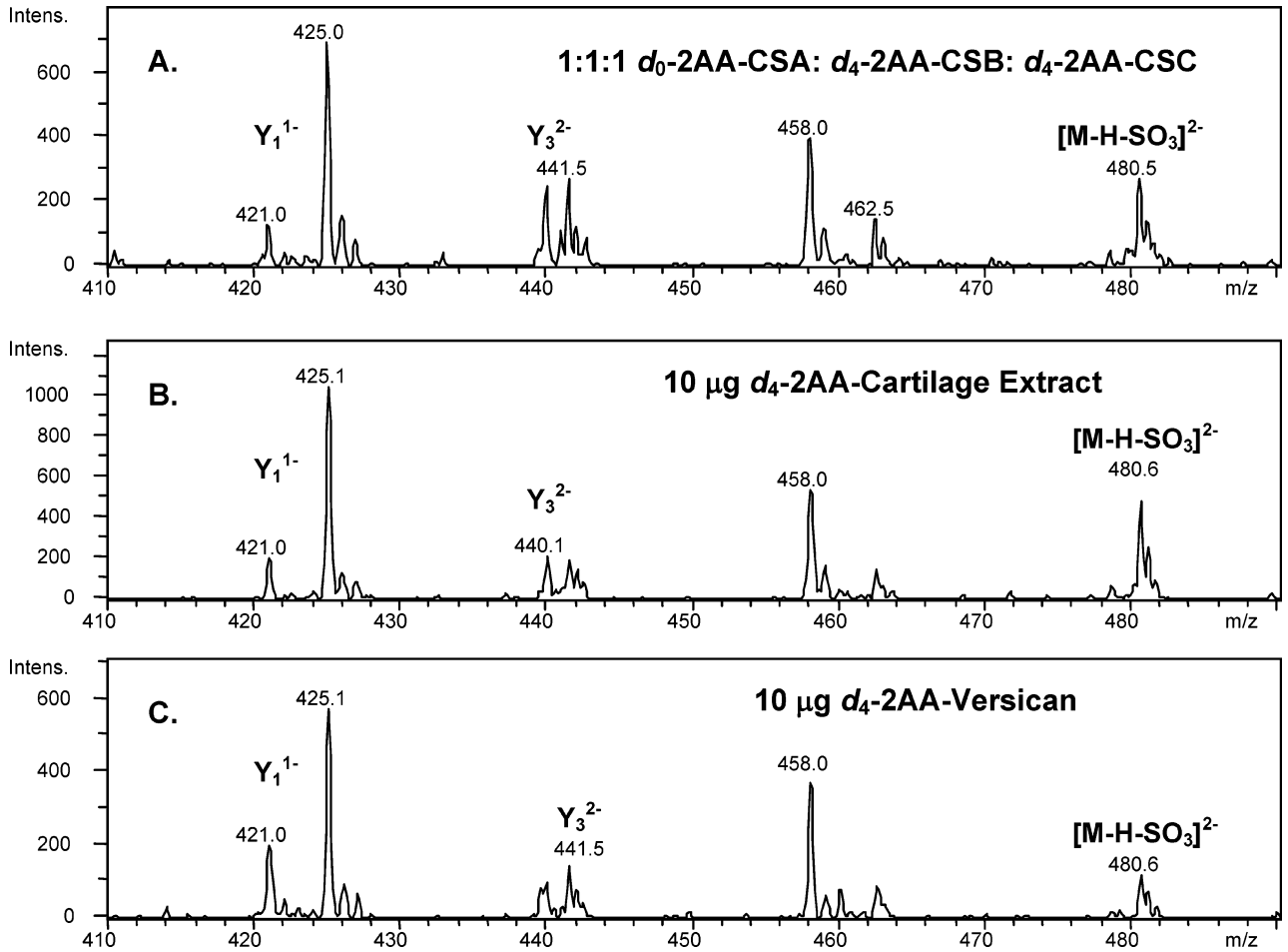

FIGURE 4: Expanded mass regions for diagnostic ions of different CS samples. (A) Tandem mass spectrum of a 10 μg tetramer 1:1:1 ternary mixture of d_0-2AA-CSA, d_4-2AA-CSB, and d_4-2AA-CSC. (B) Tandem mass spectrum of a 10 μg tetramer mixture of cartilage extract. Heavy (d_4) diagnostic ions used for quantification include Y_1^- at *m*/*z* 425.1, Y_3^{2-} at *m*/*z* 441.5, and $[M - H - SO_3]^{2-}$ at *m*/*z* 480.6. (C) Tandem mass spectrum of a 10 μg tetramer mixture of versican. CSA labeled with the light form (d_0) of 2-AA was incorporated as an internal standard. In the case of the CS standard mixture (A), the CSA standard samples labeled with d_0-2AA were mixed with CSB and/or CSC samples (termed unknown) labeled with d_4-2AA in varying ratios. In the case of proteoglycan samples (B and C), 3 μg of the CSA-d_0-2AA internal standard was added to each sample prior to LC−MS analysis. The CSA-d_0-2AA internal standard is represented by the peak at *m*/*z* 421.1.

Applying the LC−MS/MS Platform to the Quantification of Biologically Relevant Proteoglycan Samples. Cartilage Extract. CS and DS chains from triplicate 10 μg samples of bovine cartilage extract were released using alkaline borohydride and subjected to the analytical workflow described in Figure 1 using a d_4-2AA label. Prior to mass spectrometric analysis, each 10 μg d_4-2AA-derivatized sample was mixed with 3 μg of the d_0-2AA-derivatized CSA, termed the reference standard. The diagnostic ions representative of 2-AA-Δdp4 oligosaccharides derived from CSA, CSB, and CSC (Y_1^-, Y_3^{2-}, and $[M - H - SO_3]^{2-}$, respectively) are shown to be present and quantifiable in the tandem mass spectrum of the CS and DS chains released from cartilage extract, shown in Figure 4b. The percent total ion abundance of the heavy form of each diagnostic ion was calculated and inserted into the same system of three equations and three unknowns described for the CS standards. The contributions of the diagnostic ions from the d_0-2AA-Δdp4-derived CS standards were again inserted into the three equations and the equations solved for the percent of CSA, CSB, and CSC. The percentages of the isomeric glycoforms of CSA, CSB, and CSC were found to be 30.2 ± 2.8, 1.8 ± 0.7, and 68.0 ± 2.6%, respectively. The standard deviation is again representative of the entire experimental error within the method.

To minimize experimental variability, a reference standard, d_0-2AA-CSA, was spiked into each d_4-2AA-labeled proteoglycan or GAG sample that was quantified. The reference standard produced light (d_0) fragment ions in the tandem mass spectra (i.e., the Y_1^- diagnostic ion for CSA was produced at *m*/*z* 421.2, whereas the Y_1^- diagnostic ion for the proteoglycan sample was produced at *m*/*z* 425.2). The percent total ion abundance of the light diagnostic fragment ion was calculated in each proteoglycan CS/DS sample subjected to the LC−MS/MS platform. The abundance of the reference standard product ion was then compared with that of each sample to ascertain that, in fact, the samples produced similar ionization efficiencies, and therefore, these samples can be compared for quantification.

Versican. CS and DS chains from triplicate 10 μg samples of bovine aorta versican were next subjected to the LC−MS/MS platform with an internal standard, in the same manner as that of the cartilage extract samples. The diagnostic ions representative of CSA, CSB, and CSC (Y_1^-, Y_3^{2-}, and $[M - H - SO_3]^{2-}$, respectively) were found to be present and quantifiable in the tandem mass spectrum of

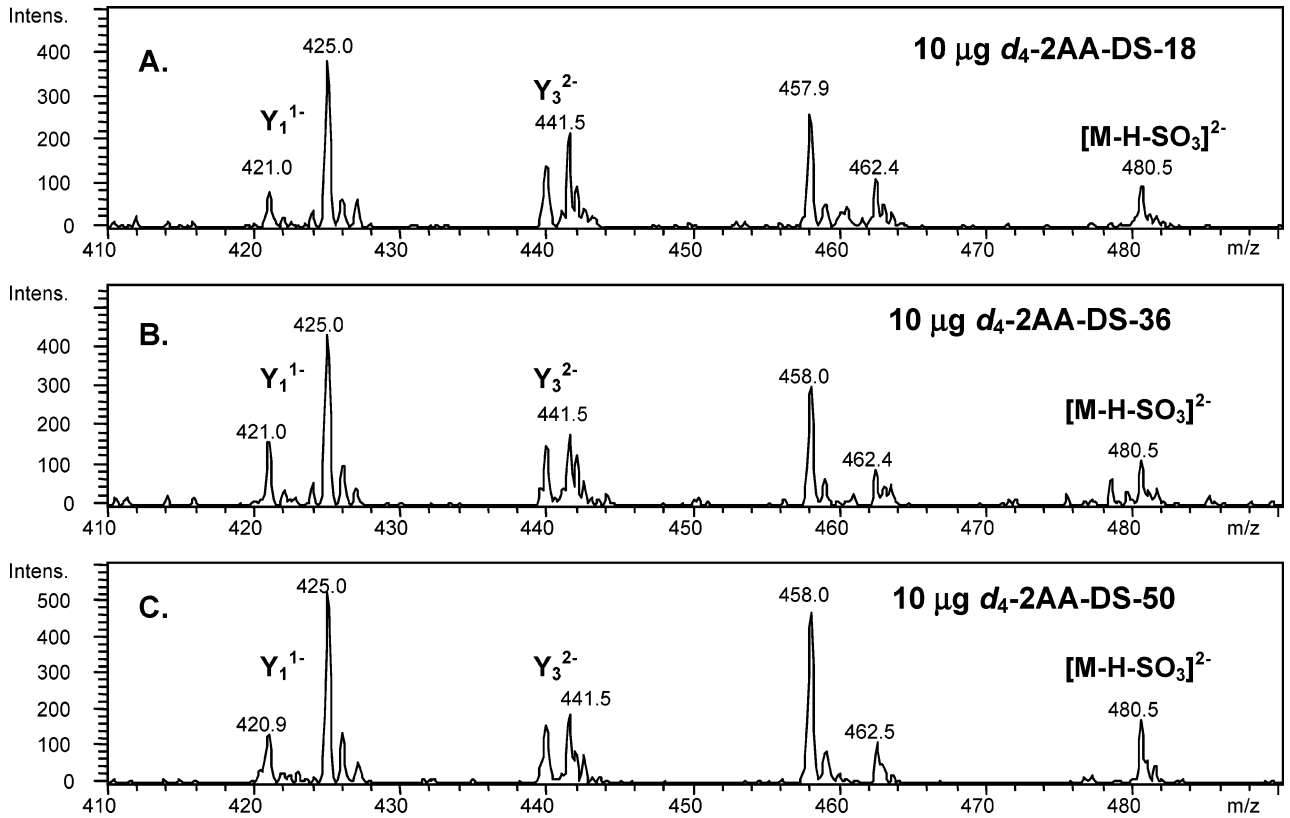

FIGURE 5: (A) Tandem mass spectrum of a 10 μg tetramer mixture of DS18. Heavy (d_4) diagnostic ions used for quantification include Y_1^- at *m*/*z* 425.0, Y_3^{2-} at *m*/*z* 441.5, and $[M - H - SO_3]^{2-}$ at *m*/*z* 480.5. (B) Tandem mass spectrum of a 10 μg tetramer mixture of DS36. (C) Tandem mass spectrum of a 10 μg tetramer mixture of DS50. CSA labeled with the light form (d_0) of 2-AA was incorporated as an internal standard. The CSA-d_0-2AA internal standard (3 μg) was added to each sample prior to LC−MS analysis. The CSA-d_0-2AA internal standard is represented by the peak at *m*/*z* 421.0.

the CS and DS chains released from versican, as shown in Figure 4c. The percentages of the isomeric glycoforms of CSA, CSB, and CSC were found to be 86.3 ± 2.1, 9.3 ± 2.5, and 4.7 ± 2.6%, respectively.

Dermatan Sulfate. Several DS preparations from bovine skin, namely, DS18, DS36, and DS50, were subjected in triplicate to the analytical workflow described previously. The DS chains were purified from skin, and the associated numeral refers to the percentage of ethanol at which the chains precipitated (*34*−*36*). The resulting 2-AA-Δdp4 tandem mass spectra for the DS preparations are shown in Figure 5a−c. From the signature ion patterns, DS18, DS36, and DS50 contain high percentages of the IdoA-GalNAc4S repeat. The complete results representing the quantification of the isomeric glycoforms of CSA, CSB, and CSC in each DS sample are presented in Table 3.

Disaccharide Analysis by CE-LIF. It is useful to compare the results of the analytical platform described here with traditional disaccharide analysis of the same samples. The MS ion signature methodology entails comparison of an unknown sample relative to standard CS/DS preparations. It was necessary to place these results in the context of those obtained using classical disaccharide analysis. Disaccharide analysis of standards and samples was achieved by exhaustive depolymerization of the whole polymer chain using combined chondroitinase ABC, chondroitinase AC1, and chondroitinase B digestion, followed by fluorescent derivatization using AMAC and CE-LIF analysis of the disaccharide compositions. Migration times of Δ-disaccharides generated from standard CS preparations CSA, CSB, and CSC and those from proteoglycan samples were verified against those of AMAC-derivatized commercially purified disaccharide standards. An AMAC-derivatized trisulfated disaccharide derived from heparin (ΔHSIS) was used as an internal standard for each run, as it has a unique migration time compared to the those of the CS/DS samples that were analyzed. Table 4 lists the Δ-disaccharide compositions of the whole polymer chain of every sample analyzed within this study. The Δ-disaccharide composition percentages were calculated using the CE fluorescence peak areas, normalized to the area of the internal standard.

Table 3: MS Ion Signature[a]

	CSA-like GlcAGalNAc4S	CSB-like IdoAGalNAc4S	CSC-like GlcAGalNAc6S
cartilage extract	30.2 ± 2.8	1.8 ± 0.7	68.0 ± 2.6
versican	86.3 ± 2.1	9.0 ± 2.5	4.7 ± 2.6
DS18	11.4 ± 5.2	85.4 ± 7.2	3.2 ± 2.5
DS36	26.1 ± 4.5	72.1 ± 4.5	1.9 ± 1.6
DS50	27.3 ± 2.9	68.5 ± 0.6	4.2 ± 2.4

[a] The percent composition of CSA-like (GlcAGalNAc4S), CSB-like (IdoAGalNAc4S), and CSC-like (GlcAGalNAc6S) isomers for biologically relevant tetramer proteoglycan samples known to contain chondroitin sulfate GAG chains. CSA, CSB, and CSC standards were used to compare the relative characteristic of each biological sample.

For the GlcA-containing CS standards, CSA is shown to contain approximately 96.6% ΔHexA-GalNAc4S and 3.5% ΔHexA-GalNAc6S while CSC is a mixture of approximately 20.6% ΔHexA-GalNAc4S, 74.1% ΔHexA-GalNAc6S, and 5.3% 2-sulfated disaccharide, ΔHexA2SGalNAc6S (Table 4). The CSB standard, which is an IdoA-containing com-

Table 4: CE-LIF Percentage Composition of ΔHexA-GalNAc4S, ΔHexA-GalNAc6S, ΔHexA2S-GalNAc4S, and ΔHexA2S-GalNAc6S after an Exhaustive Digestion of the Whole Polymer Chain of the Proteoglycan[a]

	ΔHexAGalNAc4S	ΔHexAGalNAc6S	ΔHexA2SGalNAc4S	ΔHexA2SGalNAc6S
CSA	96.56 ± 0.19	3.44 ± 0.20	0	0
CSB	93.67 ± 0.72	3.56 ± 0.14	2.75 ± 0.77	0
CSC	20.60 ± 0.51	74.06 ± 0.82	0	5.33 ± 1.10
cartilage extract	31.49 ± 1.02	68.26 ± 0.95	0.25 ± 0.08	0
versican	84.82 ± 3.37	3.82 ± 0.83	11.37 ± 4.20	0
DS18	95.16 ± 3.30	3.56 ± 3.01	1.28 ± 0.44	0
DS36	91.76 ± 0.27	1.51 ± 0.40	6.73 ± 0.14	0
DS50	86.60 ± 1.32	11.59 ± 1.48	1.79 ± 0.18	0

[a] Percentage Δ-disaccharide compositions were calculated using CE fluorescence peak areas normalized to the area of the internal standard.

pound, shows a high abundance of ΔHexA-GalNAc4S. Formation of Δ-disaccharides destroys information about the uronic acid epimerization; thus, the distinction between iduronic acid (IdoAGalNAc4S) and glucuronic acid (GlcAGalNAc4S) repeats is lost.

DISCUSSION

A novel feature of the LC−MS/MS platform introduced herein is the incorporation of a stable isotope for the quantification of CS glycoforms. It is necessary for CS partial depolymerization products to undergo derivatization for two primary reasons: to eliminate redundancy of *m*/*z* values for oligosaccharides of different lengths and to incorporate a means of stable isotopic labeling. The incorporation of a stable isotopic label allows the platform to be used with internal standard CS oligosaccharides in comparing glycoform variations, such as those associated with healthy and diseased states. All samples are run using the same internal standard CS oligosaccharides, thus compensating for variations in instrumental performance.

Tandem mass spectrometry is used to produce structural and quantitative information about sulfated GAGs, by virtue of the abundances of specific diagnostic product ions. Thus, it is possible to distinguish among the three isomeric forms of CS and DS based on 2-AA-Δdp4 product ion abundances. In previous work, diagnostic ions were generated from native CS/DS oligosaccharides (*25*). This work demonstrates that derivatization with 2-AA increases the abundances of diagnostic fragment ions (Y_1^-, Y_3^{2-}, and $[M - H - SO_3]^{2-}$) and thereby the certainty of glycoform quantification. In addition, the differences in diagnostic ion abundances between CS/DS Δdp4 glycoforms are greater in magnitude for the 2-AA-derivatized samples than for the native oligosaccharides.

The LC−MS methodology used herein provides the advantage that oligosaccharide sulfation and epimerization patterns are measured simultaneously using an on-line platform. The availability of CS standards lends specificity to the analysis given that the CSA, CSB, and CSC glycoforms are positional variants. The validity of our initial assumption that differences in ionization efficiency are negligible was demonstrated on a series of glycoform mixtures generated from CS standards.

The LC−MS/MS platform was first tested on CS standards to validate the use of the technique. The highest-purity commercially available CS standards are stated to be not less than 90% pure (*37*). Therefore, a potential 10% gap in purity allows for the detection of small percentages of other isomers not originally assumed to be in the mixture. Disaccharide analysis was performed as described in Experimental Procedures on each CSA, CSB, and CSC standard sample. CSA was found to be 96.56 ± 0.19% pure Δdi4S and CSC to be 74.06 ± 0.82% pure Δdi6S (complete set of results given in Table 4). Therefore, the LC−MS/MS data measure the extent to which a CS glycoform mixture resembles each of the three CS standards. This approach provides the advantage that uronic acid epimerization and GalNAc sulfation state are determined in a single direct measurement.

Disaccharide analysis by CE-LIF was accomplished not only to determine the purity of the CS standard samples used for quantification but also to validate the quantification of the proteoglycan samples proposed by our novel LC−MS/MS platform. The LC−MS/MS approach measures uronic acid epimers using CS-2AA-Δdp4 oligosaccharides. Epimerization information is destroyed when the CS chains are converted completely to disaccharides. Therefore, the ΔHexAGalNAc4S measured by CE-LIF is produced from both the GlcAGalNAc4S and IdoAGalNAc4S repeats. Disaccharide analysis of samples high in iduronic acid content shows a high percentage of the ΔDi4S disaccharide (Table 4). LC−MS/MS analysis of such samples shows much lower percentages of CSA-like and high percentages of CSB-like repeats (Table 3). Thus, the LC−MS/MS approach provides significantly more information than does disaccharide analysis. Both methods were used to analyze bovine aorta versican, bovine articular cartilage extract, and a series of bovine DS samples. These samples were chosen to correlate the 2-AA-Δdp4 LC−MS/MS results using CS and DS chains expressed in tissue with different glucuronic acid C5-epimerase activities.

The most abundant CS chains in cartilage are those on the large aggregating proteoglycan, aggrecan. In adult articular cartilage CS, 6-sulfation is known to dominate over nonsulfated and 4-sulfated GalNAc residues (*38*). The level of IdoA sulfation is also known to be low (*39*). Previous research has shown that the GAG composition of aggrecan in normal human adult articular cartilage was predominantly Δdi6S while Δdi4S was low in all normal samples (*40*). Disaccharides ΔdiS_B and ΔdiS_D (GlcA2SGalNAc4S and GlcA2SGalNAc6S, respectively) were detected in normal samples but only in minor quantities of approximately 1%. Following chondroitin lyase digestion, cartilage GAG products were shown to contain ∼70 ± 2% Δdi6S and ∼30% Δdi4S (*40*). These LC−MS/MS results show articular cartilage as 68.0 ± 2.6% CSC-like, 30.2 ± 2.8% CSA-like, and 1.8 ± 0.7% CSB-like. Such numbers establish clearly that in cartilage, Δdi4S is derived from CSA-like GlcAGalNAc4S repeats, given the low abundance of those

derived from CSB. It can be concluded that the LC−MS/MS methodology used herein provides another dimension of traditional analysis by determining epimerization positions directly. This capability will be applicable to studies of cartilage development and disease processes in which sulfation and epimerization levels change.

Versican is expressed in the extracellular matrix of a variety of tissues and organs (*41*) and has previously been reported to contain an L-iduronic acid content of ~15% in follicular fluid (*42*). These LC−MS/MS results show versican to contain a high percentage of CSA-like GlcAGalNAc4S (86.3 ± 2.1%) and a low percentage of CSC-like GlcAGalNAc6S (4.7 ± 2.6%) repeats. The CSB-like signature was found to be 9.0 ± 2.5%, slightly less than the value of 15% published previously. The ratio of the C5-epimerase activity to the 4-*O*-sulfotransferase activity has been shown to be 15-fold higher in fibroblasts, which produce versican, than in articular cartilage (*43*). The results are therefore consistent with the higher C5-epimerase activity resulting in versican containing a higher percentage of CSB-like signature ions than cartilage.

DS has previously been indicated to contain high levels of IdoA-GalNAc4S repeats, with lower levels of IdoA2S-GalNAc4S and GlcA(β1−3)GalNAc6S(β1−4) repeats (*35*, *36*, *44*−*46*). Previous tandem mass spectrometric analysis of the DS preparations investigated in this work showed that the abundances of CSB-like repeats was found to be 90.0, 75.0, and 50.0% for DS18, DS36, and DS50, respectively (*47*). These results were obtained using underivatized chondroitinase ABC-generated oligosaccharides. The results obtained in this work using the LC−MS/MS platform for the dermal DS samples show 85.4, 72.1, and 68.5%, respectively, have CSB-like IdoAGalNAc4S character (Table 3, column 2) and that CSC-like GlcAGalNAc6S repeats are detected in very low abundances. In this work, the reductive amination step was observed to increase the abundances of product ions that are diagnostic for different CS glycoforms. Further, the difference in the abundances of these product ions was observed to be greater than that for native oligosaccharides. On the basis of these observations, this method employing reductive amination and an on-line LC−MS/MS platform is thus shown to produce more accurate glycoform quantification. The corresponding disaccharide analysis of these DS samples indicates that ΔDi4S comprises 95.2, 91.8, and 86.6%, respectively, of the disaccharides formed by chondroitinase ABC digestion, and a small percentage of ΔDi6S is also detected (Table 4).

For the CE-LIF disaccharide analysis, the samples were subjected to exhaustive digestion using chondroitinases ABC, B, and ACI. For the MS-based analysis, the samples were subjected to limited digestion using chondroitinase ABC. The results for the CE-LIF measurement of ΔDi6S and the MS ion signature measurement of CSC-like isomers (Tables 3 and 4) are consistent for cartilage extract, versican, DS18, and DS36. For DS50, however, the measurement of ΔDi6S (11.6%) is significantly higher than that obtained for CSC-like isomer content (4.2%). Given the consistency of the MS ion signature method for standard mixtures (Table 2) and among the other GAG samples, it is likely that the discrepancy reflects structural differences between DS50 and the other samples. CS disaccharides other than those reported in Table 4 were not detected, and this is consistent with the tetrasaccharides analyzed in Table 3 composed entirely of HexAGalNAc4S and HexAGalNAc6S repeats. In theory, ΔDi6S may be produced from either a GlcAGalNAc6S or an IdoAGalNAc6S repeat. A DS repeat consisting of IdoA2S-GalNAc6S has been identified in invertebrates and antibodies against this repeat found to indicate its presence in mouse brain (*48*, *49*). A DS repeat of IdoA-GalNAc4S,-6S is found in hagfish (*50*). Although it is conceivable that an IdoA-GalNAc6S repeat is present in the porcine skin from which the DS samples were extracted, such a repeat has never been described in mammalian systems. If present, it is unknown whether such a repeat would produce a unique ion signature or one very similar to that of CSC-like GlcAGalNAc6S repeats. Efforts to obtain invertebrate DS from which a tetramer containing an IdoAGalNAc6S repeat may be prepared are underway to determine its tandem mass spectrometric diagnostic ion abundances.

The methodology introduced herein advances previous techniques for CS/DS analysis by producing information about both sulfation position and uronic acid epimerization in a single measurement. The stable isotopic labeling allows for internal standard CS oligosaccharides to be used in all quantification steps. The technique allows for a high-throughput analysis of CS GAGs on a 10 μg scale using high-performance SEC. The next step in this research is to further diminish the sample requirement by using a more sensitive chromatography method. Improved sensitivity will also allow analysis of oligosaccharides larger than Δdp4.

This work also demonstrates concepts that are likely to be useful as general analytical principles in glycomics. Specifically, it is valuable to measure changes in the expression of glycoforms through increases or decreases in the abundances of specific tandem mass spectrometric product ions. Thus, changes in glycoform expression are measured using reductive amination labels that include stable isotope labels to compare product ion abundances in a given sample against those of a reference glycan mixture. Standard preparations of CS were used as the references in this work to provide detailed information about the types of disaccharide repeats present in a given sample. For other glycan classes, the level of information that will result from the glycoform analysis will depend on how much structural detail is known about the reference glycan mixture. Even for glycan classes for which detailed information about the significance of the product ion patterns is lacking, an approach based on stable isotope labeling and tandem MS will be useful for producing ions that indicate changes in glycan expression that accompany biological perturbation. Thus, a LC−MS/MS approach similar to that described here will likely provide a general means of correlating the expression of glycan expression with biological change.

REFERENCES

1. Kraemer, P. M. (1971) Heparan Sulfates of Cultured Cells. I. Membrane-Associated and Cell-Sap Species in Chinese Hamster Cells, *Biochemistry 10*, 1437−1445.
2. Kraemer, P. M. (1971) Heparan Sulfates of Cultured Cells. II. Acid-Soluble and -Precipitable Species of Different Cell Lines, *Biochemistry 10*, 1445−1451.
3. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) Functions of Cell Surface Heparan Sulfate Proteoglycans, *Annu. Rev. Biochem. 68*, 729−777.

4. Perrimon, N., and Bernfield, M. (2000) Specificities of Heparan Sulphate Proteoglycans in Developmental Processes, *Nature 404*, 725−728.
5. Iozzo, R. V. (2000) *Proteoglycans: Structure, Biology and Molecular Interactions*, Marcel Dekker, New York.
6. Lander, A. D. (1998) Proteoglycans: Master Regulators of Molecular Encounter? *Matrix Biol. 17*, 465−472.
7. Kawashima, H., Hirose, M., Hirose, J., Nagakubo, D., Plaas, A. H., and Miyasaka, M. (2000) Binding of a Large Chondroitin Sulfate/Dermatan Sulfate Proteoglycan, Versican, to L-Selectin, P-Selectin, and Cd44, *J. Biol. Chem. 275*, 35448−35456.
8. Trowbridge, J. M., Rudisill, J. A., Ron, D., and Gallo, R. L. (2002) Dermatan Sulfate Binds and Potentiates Activity of Keratinocyte Growth Factor (Fgf-7), *J. Biol. Chem. 277*, 42815−42820.
9. Varki, A., Cummings, R., Esko, J., Freeze, H., Hart, G., and Marth, J. (1999) Proteoglycans and Glycosaminoglycans, in *Essentials of Glycobiology*, Cold Spring Harbor Laboratory Press, Plainview, NY.
10. Conrad, H. E. (1998) *Heparin Binding Proteins*, Academic Press, New York.
11. Malmström, A., and Fransson, L. A. (1975) Biosynthesis of Dermatan Sulfate. I. Formation of L-Iduronic Acid Residues, *J. Biol. Chem. 250*, 3419−3425.
12. Mark, M. P., Butler, W. T., and Ruch, J. V. (1989) Transient expression of a chondroitin sulfate-related epitope during cartilage histomorphogenesis in the axial skeleton of fetal rats, *Dev. Biol. 133*, 475−488.
13. Tufvesson, E., and Malmström, A. (2002) Biglycan Isoforms with Differences in Polysaccharide Substitution and Core Protein in Human Lung Fibroblasts, *Eur. J. Biochem. 269*, 3688−3696.
14. Kitagawa, H. K., Tsutsumi, K., Tone, Y., and Sugahara, K. (1997) Developmental Regulation of the Sulfation Profile of Chondroitin Sulfate Chains in the Chicken Embryo Brain, *J. Biol. Chem. 272*, 31377−31381.
15. Belcher, C., Yaqub, R., Fawthorp, F., Bayliss, M., and Doherty, M. (1997) Synovial Fluid Chondroitin and Keratan Sulphate Epitopes, Glycosaminoglycans, and Hyaluronan in Arthritic and Normal Knees, *Ann. Rheum. Dis. 56*, 299−307.
16. Shinmei, M., Miyauchi, S., Machida, A., and Miyazaki, K. (1992) Quantitation of chondroitin 4-sulfate and chondroitin 6-sulfate in pathologic joint fluid, *Arthritis Rheum. 35*, 1304−1308.
17. Chai, W., Luo, J., Lim, C. K., and Lawson, A. M. (1998) Characterization of Heparin Oligosaccharide Mixtures as Ammonium Salts using Electrospray Mass Spectrometry, *Anal. Chem. 70*, 2060−2066.
18. Zaia, J., and Costello, C. E. (2001) Compositional Analysis of Glycosaminoglycans by Electrospray Mass Spectrometry, *Anal. Chem. 73*, 233−239.
19. Kim, Y. S., Ahn, M. Y., Wu, S. J., Kim, D. H., Toida, T., Teesch, L. M., Park, Y., Yu, G., Lin, J., and Linhardt, R. J. (1998) Determination of the structure of oligosaccharides prepared from acharan sulfate, *Glycobiology 8*, 869−877.
20. Zamfir, A., Seidler, D. G., et al. (2003) Structural Investigation of Chondroitin/Dermatan Sulfate Oligosaccharides from Human Skin Fibroblast Decorin, *Glycobiology 13*, 733−742.
21. Zaia, J., McClellan, J. E., and Costello, C. E. (2001) Tandem Mass Spectrometric Determination of the 4S/6S Sulfation Sequence in Chondroitin Sulfate Oligosaccharides, *Anal. Chem. 73*, 6030−6039.
22. Desaire, H., and Leary, J. (2000) Detection and Quantification of the Sulfated Disaccharides in Chondroitin Sulfate by Electrospray Tandem Mass Spectrometry, *J. Am. Soc. Mass Spectrom. 11*, 916−920.
23. Lamb, D. J., Wang, H. M., Mallis, L. M., and Linhardt, R. J. (1992) Negative Ion Fast-Atom Bombardment Tandem Mass Spectrometry to Determine Sulfate and Linkage Position in Glycosaminoglycan derived Disaccharides, *J. Am. Soc. Mass Spectrom. 3*, 797−803.
24. Zaia, J., Li, X.-Q., Chan, S.-Y., and Costello, C. E. (2003) Tandem Mass Spectrometric Strategies for Determination of Sulfation Positions and Uronic Acid Epimerization in Chondroitin Sulfate Oligosaccharides, *J. Am. Soc. Mass Spectrom. 14*, 1270−1281.
25. Miller, M. J. C., Costello, C. E., Malmström, A., and Zaia, J. (2005) A Tandem Mass Spectrometric Approach to Sequence Determination of Chondroitin/Dermatan Sulfate oligosaccharide Glycoforms, submitted for publication.
26. Karamanos, N. K., Vanky, P., Syrokou, A., and Hjerpe, A. (1995) Identity of Dermatan and Chondroitin Sequences in Dermatan Sulfate Chains Determined by Using Fragmentation with Chondroitinases and Ion-Pair High-Performance Liquid Chromatography, *Anal. Biochem. 225*, 220−230.
27. Lamari, F. N., Militsopoulou, M., Mitropoulou, T. N., Hjerpe, A., and Karamanos, N. K. (2002) Analysis of glycosaminoglycan-derived disaccharides in biologic: Samples by capillary electrophoresis and protocol for sequencing glycosaminoglycans, *Biomed. Chromatogr. 16*, 95−102.
28. Karamanos, N. K., Axelsson, S., Vanky, P., Tzanakakis, G. N., and Hjerpe, A. (1995) Determination of Hyaluronan and Galactosaminoglycan Disaccharides by High-Performance Capillary Electrophoresis at the Attomole Level. Applications to Analyses of Tissue and Cell Culture Proteoglycans, *J. Chromatogr., A 696*, 295−305.
29. Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M., and Parekh, R. B. (1995) Nonselective and Efficient Fluorescent Labeling of Glycans Using 2-Amino Benzamide and Anthranilic Acid, *Anal. Biochem. 230*, 229−238.
30. Militsopoulou, M., Lamari, F. N., Hjerpe, A., and Karamanos, N. K. (2002) Determination of twelve Heparin- and Heparan Sulfate-Derived Disaccharides as 2-Aminoacridone Derivatives by Capillary Zone Electrophoresis using Ultraviolet and Laser-Induced Fluorescence Detection, *Electrophoresis 23*, 1104−1109.
31. Roboz, J. (1968) *Introduction to Mass Spectrometry: Instrumentation and Techniques*, Interscience, New York.
32. Desaire, H., and Leary, J. (2000) Utilization of MS^3 Spectra for the Multicomponent Quantification of Diastereomeric *N*-Acetylhexosamines, *J. Am. Soc. Mass Spectrom. 11*, 1086−1094.
33. Leary, J., and Saad, O. M. (2003) Compositional Analysis and Quantification of Heparin and Heparan Sulfate by Electrospray Ionization Ion Trap Mass Spectrometry, *Anal. Chem. 75*, 2985−2995.
34. Davidson, E., Hoffman, P., et al. (1956) The Acid Mucopolysaccharides of Connective Tissue, *Biochim. Biophys. Acta 21*, 506−518.
35. Fransson, L. A., and Roden, L. (1967) Structure of Dermatan Sulfate. I. Degradation by Testicular Hyaluronidase, *J. Biol. Chem. 242*, 4161−4169.
36. Fransson, L. A., and Roden, L. (1967) Structure of Dermatan Sulfate. II. Characterization of Products Obtained by Hyaluronidase Digestion of Dermatan Sulfate, *J. Biol. Chem. 242*, 4170−4175.
37. Schiller, S., Slover, G. A., and Dorfman, A. (1961) A Method for the Separation of Acid Mucopolysaccharides: Its Application to the Isolation of Heparin from the Skin of Rats, *J. Biol. Chem. 236*, 983−987.
38. Bayliss, M. T., Osborne, D., Woodhouse, S., and Davidson, C. (1999) Sulfation of Chondroitin Sulfate in Human Articular Cartilage. The Effect of Age, Topographical Position and Zone of Cartilage on Tissue Composition, *J. Biol. Chem. 274*, 15892−15900.
39. Lauder, R. M., Huckerby, T. N., and Neiduszynski, I. A. (2000) A Fingerprinting Method for Chondroitin/Dermatan Sulfate and Hyaluronan Oligosaccharides, *Glycobiology 10*, 393−401.
40. Plass, A. H. K., West, L. A., Wong-Palms, S., and Nelson, F. R. T. (1998) Glycosaminoglycan Sulfation in Human Osteoarthritis. Disease-Related Alterations at the Non-Reducing Termini of Chondroitin and Dermatan Sulfate, *J. Biol. Chem. 273*, 12642−12649.
41. Wu, Y. J., La Pierre, D. P., Wu, J. W., Yee, A. J., and Yang, B. B. (2005) The Interaction of Versican with its Binding Partners, *Cell Res. 15*, 483−494.
42. Eriksen, G., Carlstedt, I., Mörgelin, M., Uldbjerg, N., and Malmström, A. (1999) Isolation and Characterization of Proteoglycans from Human Follicular Fluid, *Biochem. J. 340*, 613−620.
43. Tiedemann, K., Larsson, T., Heinegard, D., and Malmström, A. (2001) The Glucuronyl C5-Epimerase Activity is the Limiting Factor in the Dermatan Sulfate Biosynthesis, *Arch. Biochem. Biophys. 391*, 65−71.
44. Hoffman, P., Linker, A., et al. (1957) The Acid Mucopolysaccharides of Connective Tissues. II. Further Experiments on Chondroitin Sulfate B, *Arch. Biochem. Biophys. 69*, 435−440.
45. Coster, L., Malmström, A., et al. (1975) The Co-Polymeric Structure of Pig Skin Dermatan Sulphate. Distribution of L-Iduronic Acid Sulphate Residues in Co-Polymeric Chains, *Biochem. J. 145*, 379−389.
46. Fransson, L. A. (1968) Structure of Dermatan Sulfate. 3. The Hybrid Structure of Dermatan Sulfate from Umbilical Cord, *J. Biol. Chem. 243*, 1504−1510.

47. Thorsson-Westergren, G., Onnervik, P. O., Fransson, L. A., and Malmström, A. (1991) Proliferation of Cultured Fibroblasts is Inhibited by L-Iduronate-Containing Glycosaminoglycan, *J. Cell. Phys. 147*, 523−530.
48. Mourão, P. A., Pavão, M. S., Mulloy, B., and Wait, R. (1997) Chondroitin ABC Lyase Digestion of an Ascidian Dermatan Sulfate. Occurrence of Unusual 6-O-sulfo-2-acetamido-2-deoxy-3-O-(2-O-sulfo-α-L-idopyranosyluronic acid)-β-D-galactose Units, *Carbohydr. Res. 300*, 315−321.
49. Bao, X., Pavão, M. S., Dos Santos, J. C., and Sugahara, K. (2005) A Functional Dermatan Sulfate Epitope Containing Iduronate(2-O-sulfate)α1−3GalNAc(6-O-sulfate) Disaccharide in the Mouse Brain: Demonstration Using a Novel Monoclonal Antibody Raised Against Dermatan Sulfate of Ascidian Ascidia Nigra, *J. Biol. Chem. 280*, 23184−23193.
50. Ueoka, C., Nadanaka, S., Seno, N., Khoo, K. H., and Sugahara, K. (1999) Structural Determination of Novel Tetra- and Hexasaccharide Sequences Isolated from Chondroitin Sulfate H (Oversulfated Dermatan Sulfate) of Hagfish Notochord, *Glycoconjugate J. 16*, 291−305.

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