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Discrimination of GalNAc (4S/6S) sulfation sites in chondroitin sulfate disaccharides by chip-based nanoelectrospray multistage mass spectrometry

Research Article

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Abstract: Sulfation pattern within chondroitin sulfate (CS) glycosaminoglycan (GAG) chains is an important post-translational modification that regulates their interaction with proteins. In this context, development of highly efficient and reproducible analytical methods for the investigation of CS sulfation patterns is of high necessity. In this study we report a novel method for straightforward determination of N-acetylgalactosamine (GalNAc) sulfation sites in chondroitin sulfate disaccharides. Our protocol involves combining fully automated chip-based nanoelectrospray (nanoESI) for analyte infusion and ionization in negative ion mode with multistage (MS^n) collision-induced dissociation (CID) high capacity ion trap (HCT) mass spectrometry for generation of sequence ions diagnostic for identification of sulfate ester group position within GalNAc residues. The feasibility of this approach is here demonstrated on chondroitin 6-O-sulfate and chondroitin 4-O-sulfate disaccharides. Fragmentation patterns obtained by MS^2 and MS^3 sequencing stages provided first mass spectrometric data from which sulfation site(s) within GalNAc monosaccharide ring could be unequivocally deciphered. Hence, the method allowed discriminating 4S/6S sulfation sites solely on the basis of MS and multistage MS evidence.

Keywords: *Chondroitin sulfate • Sulfation sites • Fully automated chip-nanoESI • High capacity ion trap mass spectrometry*

1. Introduction

Over the past several decades, genetic and biochemical studies have established the importance of glycosaminoglycans (GAGs) in regulating many physiological processes, including morphogenesis and development, viral invasion, cancer metastasis and spinal cord injury [1–4]. However, a key question is whether glycosaminoglycans use specific sulfation sequences to modulate biological processes [5-7].

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Chondroitin sulfate represents a particular class of GAGs consisting of a repeating disaccharide unit that contains two modified sugars *N*-acetylgalactosamine (D-GalNAc) and an uronic acid namely glucuronate (D-GlcA). A chondroitin chain can exhibit over 100 disaccharide units; each of these dissacharides can be unsulfated or sulfated in variable positions and quantities by chondroitin sulfotransferase enzymes [8]. CS heterodimers may be sulfated to hydroxyl groups on C4 of GalNAc, known as CS-A (Fig. 1a), C6 of GalNAc (Fig.1b) known as CS-C, C6 of GalNAc and C2 of GlcA (CS-D), or C4 and C6 of GalNAc (CS-E) [9].

Biological importance of CS gives rise currently to substantial efforts for development, improvement and implementation of efficient protocols and methods for their expression, structure and noncovalent interaction determination, among which, mass spectrometry (MS) is considered one of the most valuable [10].

Systematically optimized mass spectrometric methods based on electrospray (ESI) collision-induced dissociation (CID) in a single fragmentation stage (MS/MS) have demonstrated the MS technique capability to provide accurate information upon unsulfated and regularly sulfated chondroitin oligomers [11-16]. Recently, analysis of oversulfation in chondroitin sulfate oligosaccharides, with determination of sulfate position along the CS chain was accomplished by either nanoelectrospray ionization quadrupole time-of-flight, Fourier-transform ion cyclotron resonance mass spectrometry or combined capillary electrophoresis/electrospray ionization mass spectrometry (CE/ESI-MS) [17,18]. However, none of these studies reported on the identification of sulfate position inside the monomer. This state-of-art is attributable to the limitation of the employed single-stage dissociation methods. Tandem MS (MS/MS) with a single fragmentation event does not allow re-sequencing of small, sulfated fragments which is a requirement of absolute necessity for identification of sulfation site at GalNAc and/or GlcA ring.

In this work, we present the development of a novel method based on fully automated negative ion mode chip nanoESI high capacity ion trap (HCT) multistage mass spectrometry (MS^2-MS^3) for discrimination of GalNAc (4S/6S) sulfation sites in CS glycosaminoglycans. The method was applied to two commercially available standard CS (4S) and CS (6S) dissacharides. Obtained data revealed that by multistage MS with chip-based nanoESI infusion unambiguous identification of sulfation site(s) inside GalNAc was possible.

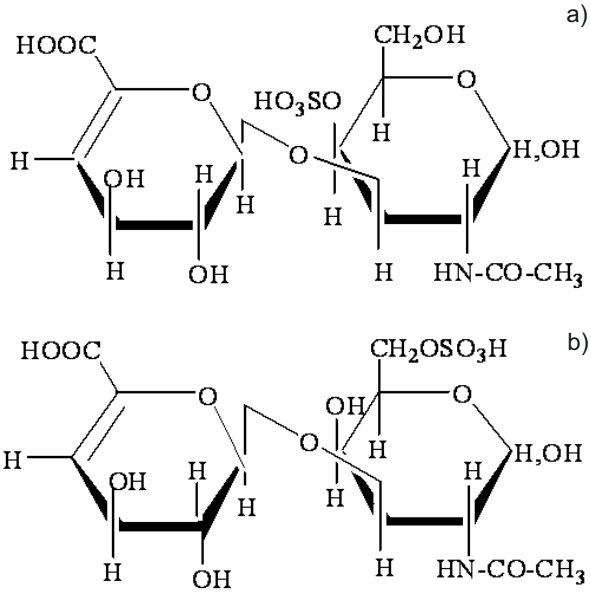

Figure 1. a) Chondroitin 4-O-sulfate; b) Chondroitin 6-O-sulfate.

2. Experimental Procedures

2.1. Materials

Analytical grade methanol was purchased from Merck (Darmstadt, Germany) and used without further purification. All sample solutions were dried in a SpeedVac Concentrator, SPD 111V-230 from Thermo Electron Corporation, (Asheville, NC, USA), coupled to a vacuum pump PC 2002 Vario with CVC 2000 Controller from Vaccubrand (Wertheim, Germany). Prior to chip-based MS analysis, the sample/methanol solutions were centrifuged for 2 h in a SIGMA 2-16 model centrifuge from Sartorius GmbH (Göttingen, Germany).

2.2. Samples

Standard disaccharides used in this study were Δ-4,5-GlcA-GalNAc 4-*O*-sulfate and Δ-4,5-GlcA-GalNAc 6-*O*-sulfate from bovine trachea both purchased from Sigma, Taufkirchen, Germany. Both standard CS samples were dissolved in pure methanol to yield the working solutions of 1 pmol μL^{-1} concentration.

2.3. Mass Spectrometry

Mass spectrometry was conducted on a High Capacity Ion Trap Ultra (HCT Ultra, PTM discovery) mass spectrometer from Bruker Daltonics, Bremen, Germany.

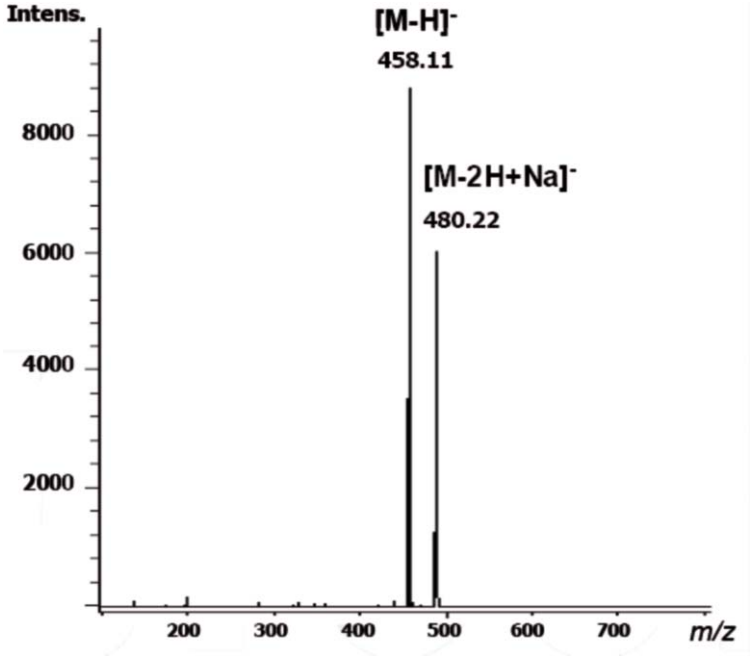

Figure 2. Fully automated chip (-) nanoESI HCT CID MS of monosulfated standard CS disaccharide having the composition [4,5-Δ-GlcA-GalNAc] (6S). Solvent: MeOH; chipESI: -1.4 kV; capillary exit: -50 V. Back nitrogen pressure 0.30 p.s.i. Nitrogen nebullizer on MS at 50 p.s.i.

HCT MS is interfaced to a PC running the Compass™ 1.2 integrated software package, which includes the Hystar™ 3.2.37 and Esquire 6.1.512 modules for instrument controlling and chromatogram/spectrum acquisition, and Data Analysis 3.4.179 portal for storing the ion chromatograms and processing the MS data.

Fully automated chip-based nanoelectrospray was performed on a NanoMate robot incorporating ESI 400 Chip technology (Advion BioSciences, Ithaca, USA) controlled and manipulated by ChipSoft 7.1.1 software operating under Windows system. The robot was coupled to the HCT Ultra mass spectrometer *via* an in-laboratory made interface as described by us before [19]. 10 µL aliquots of the working sample solutions were loaded into the NanoMate 96-well plate. The robot was programmed to aspirate the whole volume of sample, followed by 2 µL of air into the pipette tip and afterwards deliver the sample to the inlet side of the 400 microchip. To prevent possible *in-source* fragmentation which would lead to uncontrolled cleavage of labile sulfate group, HCT capillary exit was set to -50 V.

All mass spectra were acquired in the negative ion mode, within the mass range 100–2000 *m/z*, at a scan speed of 8000 *m/z* per second. Multistage mass spectrometry from MS^2 to MS^3 was carried out in manual mode of selection and fragmentation of the precursor ion by collision-induced dissociation (CID) at low energies using He as the collision gas. For multistage sequencing the precursor ions were selected within an isolation width of 2 u. To induce a fragmentation exhibiting high sequence coverage, MS^2-MS^3 fragmentation spectra were obtained by accumulating scans at variable RF signal amplitudes within 0.4-0.8 V.

The *m/z* scale of the mass spectrum was calibrated using an external calibration standard G2421A electrospray "tuning mix," from Agilent Technologies (Santa Rosa, CA, USA). The reference provided, in negative ion mode, a spectrum with a fair ionic coverage of the *m/z* range scanned in both MS and CID MS^2-MS^3 experiments. The obtained mass accuracy was situated within the normal range of an HCT MS instrument.

The assignment of the fragment ions followed the nomenclature introduced by Domon and Costello [20].

3. Results and Discussions

3.1. Chodroitin 6-O-sulfate dissacharide (CS-C)

MS screening of the standard 6-*O*-sulfate CS disaccharide is presented in Fig. 2. The spectrum exhibits two highly abundant singly charged ions detected at *m/z* 458.11 and 480.22 respectively. According to mass calculation the singly charged, monodeprotonated species at *m/z* 458.11 corresponds to a monosulfated 4,5-Δ-GlcA-GalNAc composition while the singly charged monodeprotonated ion detected at *m/z* 480.22 represents its sodiated counterpart. The ion assignment

to a structure containing unsaturated GlcA was based on the known mechanism of chondroitin AC lyase cleavage reaction, which leads to the formation of a 4,5 double bond in the GlcA moiety at the non-reducing end. As seen in Fig. 2, by optimized MS screening conditions, the *in-source* loss of the labile sulfate group was hampered and no desulfated dissacharide species were generated. Moreover, in spectrum in Fig. 2, no signals related to possible oversulfated ions were detected, which indicates that no oversulfated species are present in the analyzed CS disaccharide fraction.

To identify the sulfation site, the ion at *m/z* 458.11 was isolated within an isolation width of 2 u and submitted to stepwise fragmentation by multistage CID MS^2 and MS^3 experiments. ESI CID MS^2 spectrum obtained using as the precursor the singly charged ion at *m/z* 458.11 is shown in Fig. 3. Inspection of the spectrum indicates that cleavage of the glycosidic GlcA-GalNAc bond generated the highly abundant Y_1^- ion at *m/z* 300.09 corresponding to the cleaved monosulfated GalNAc$^-$ residue and Z_1^- ion at *m/z* 282.06 assigned, according to the *m/z* value, to the dehydrated form of GalNAc$^-$. (Y_1, Z_1) pair is diagnostic for GalNAc monosulfation. Further on, the C_1^- ion at *m/z* 175.00 and B_1^- at *m/z* 157.00 are both attributable to nonsulfated GlcA$^-$. The latter one is diagnostic for double bond formation and implicit unsaturation of the GlcA.

Besides the ions produced by glycosidic bond cleavage as discussed above, the spectrum shows evidence upon a number of nine ring cleavage ions detected at *m/z* 139.08, 180.99, 198.97, 242.00, 255.20, 335.40, 342.13, 396.17 and 412.40 (Fig. 3). However, none of these ions brings evidence upon SO_3^- position inside GalNAc moiety. Therefore, to identify the sulfation site, the fragment ion at *m/z* 300.09 corresponding to cleaved GalNAc monosaccharide bearing one sulfate ester group, was isolated within an isolation window of 2 u and subjected to further sequencing in CID MS^3 experiment. Obtained fragmentation spectrum is depicted in Fig. 4. Remarkably, in the MS^3 of the singly charged monodeprotonated Y_1^- ion at *m/z* 300.00, a ring cleavage ion at *m/z* 240.00 assigned according to Domon and Costello nomenclature to $^{0,2}X^-$ was detected. This ion, marked in the spectrum in Fig. 4 by (▼), is diagnostic for SO_3^- location at the C6 position of GalNAc (inset Fig. 4).

3.2. Chodroitin 4-O-sulfate dissacharide (CS-A)

Chondroitin 4-*O*-sulfate dissacharide was subjected to fully automated chip-based nanoESI MS screening and CID MS^2 sequencing under identical solution and instrumental conditions. The screening mass spectrum

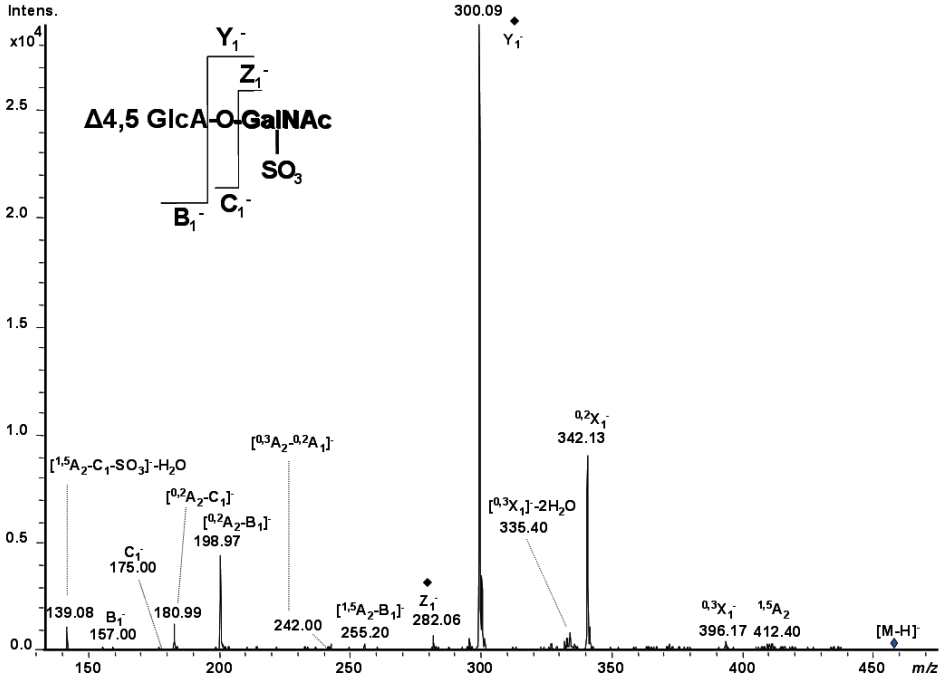

Figure 3. Fully automated chip (-) nanoESI HCT CID MS^2 of the singly deprotonated ion at m/z 458.11 assigned according to mass calculation to monosulfated standard CS disaccharide having the composition [4,5-Δ-GlcA-GalNAc] (6S). Fragmentation amplitude within 0.40-0.80 V. Diagnostic ions for GalNAc monosulfation are marked by (♦).

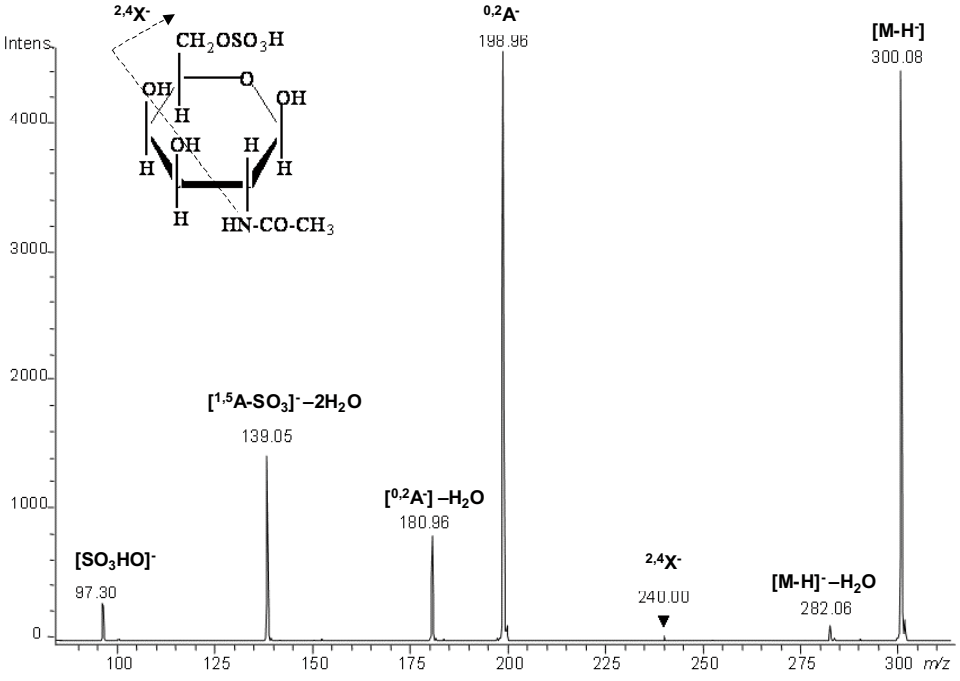

Figure 4. Fully automated chip (-) nanoESI HCT CID MS^3 of the singly deprotonated fragment ion detected in spectrum in Fig. 3 at m/z 300.09 and assigned according to mass calculation a composition of monosulfated GalNAc$^-$. Diagnostic ion for GalNAc (6S) is marked by (▼). Conditions are the same as for the experiment in Fig. 3.

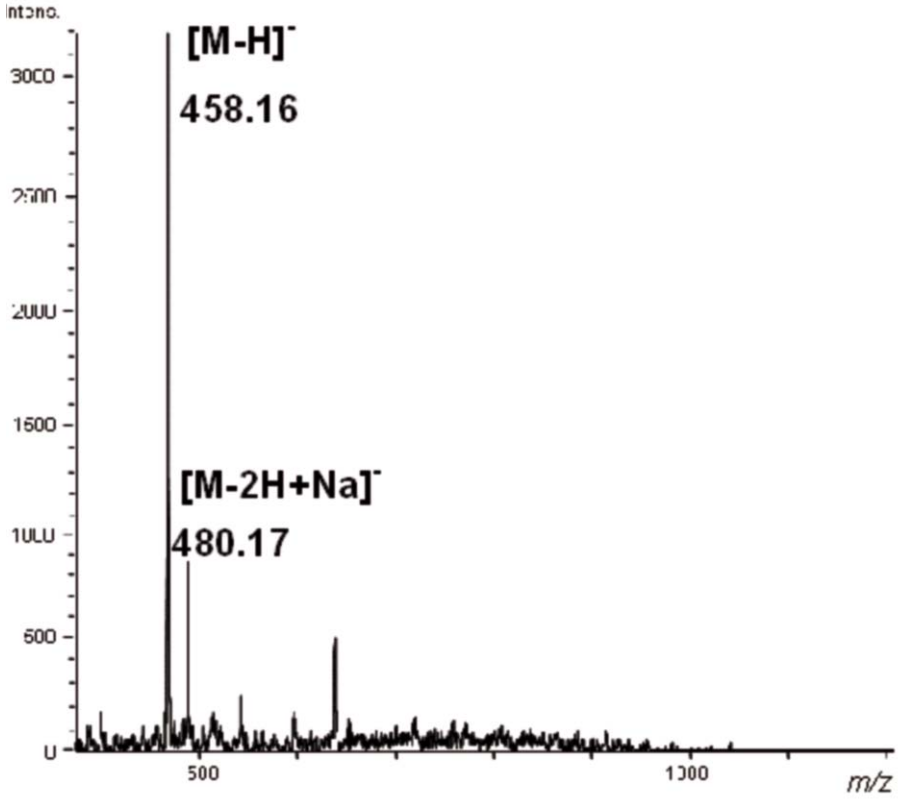

Figure 5. Fully automated chip (-) nanoESI HCT CID MS of monosulfated standard CS disaccharide having the composition [4,5Δ-GlcA-GalNAc] (4S). Conditions as for the experiment in Fig. 2.

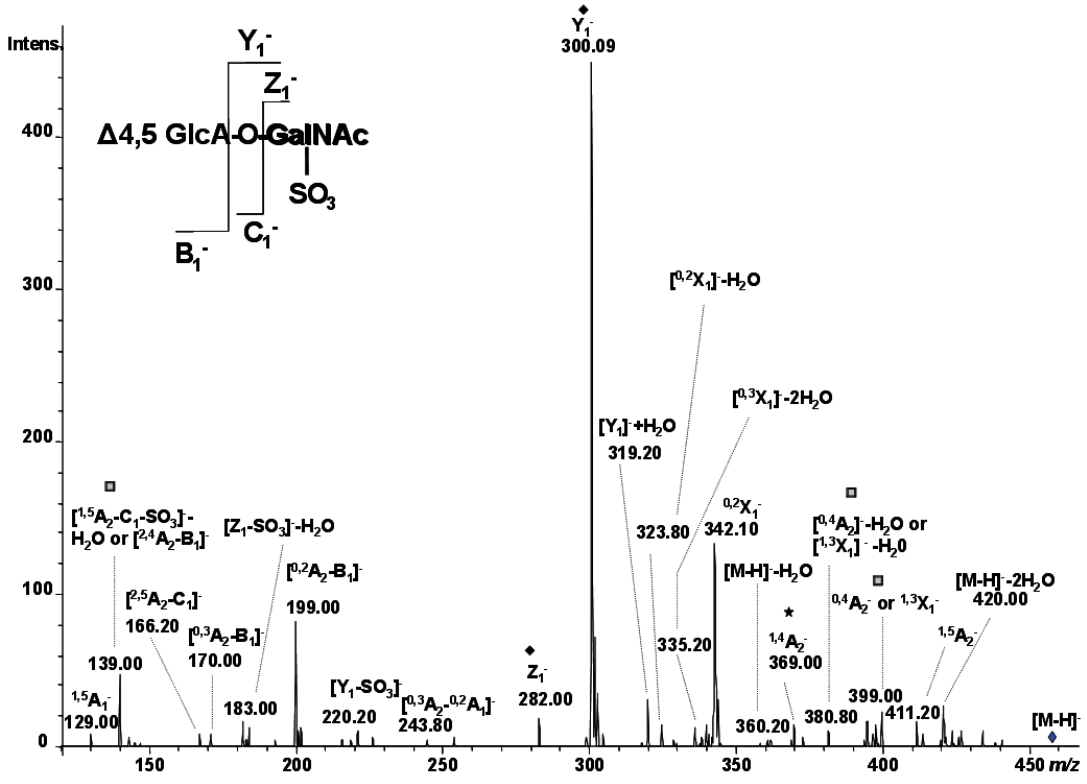

Figure 6. Fully automated chip (-) nanoESI HCT CID MS^2 of the singly deprotonated ion at m/z 458.16 assigned according to mass calculation to monosulfated standard CS disaccharide having the composition [4,5Δ-GlcA-GalNAc] (4S). Diagnostic ions for GalNAc monosulfation are marked by (♦); Diagnostic ions for GalNAc (4S) are marked by (*); Ions supporting GalNAc (4S) are marked by (■). Conditions as for the experiment in Fig. 3.

is presented in Fig. 5. Similar to 6-*O*-sulfate CS disaccharide, the spectrum is dominated by two highly intense signals at *m/z* 458.16 and 480.17. The signal at *m/z* 458.16 corresponds to $[M-H]^-$ singly charged monosulfated 4,5-Δ-GlcA-GalNAc species, while the signal at *m/z* 480.22 represents the same species ionized to yield the doubly deprotonated ion with sodium adduct $[M-2H+Na]^-$.

For tandem MS analysis the ion at *m/z* 458.16 was isolated within an isolation window of 2 u and submitted to fragmentation by CID MS^2. Generated product ion spectrum is depicted in Fig. 6 where to facilitate the distinction of sequence ions that are particularly relevant for the molecule sulfation status, diagnostic fragment ions for GalNAc monosulfation are marked by black diamond (♦), diagnostic ions for GalNAc (4S) by asterisk (*) and possible diagnostic ions for GalNAc (4S) by square (■).

As observed in spectrum in Fig. 6, chosen sequencing conditions induced single and double cleavages of several glycosidic bonds along with the occurrence of saccharide ring fragmentations, useful for sulfate group localization within GalNAc. Hence, highly abundant Y_1^- and Z_1^- ions detected at *m/z* 300.00 and 282.00, respectively, clearly confirm the monosulfation of GalNAc residue. Moreover, the signal at *m/z* 369.00 corresponds according to mass calculation to the $^{1,4}A_2^-$ ring cleavage ion bearing one sulfate ester group; therefore this ion is diagnostic for C4 sulfation site within GalNAc monomer. Sulfation site at C4 is further supported by the ions at *m/z* 139.00 assigned to $[^{2,4}A_2\text{-}B_1]^-$ type of fragment, at *m/z* 399.00 assigned to $^{1,3}X_1^-$ and at *m/z* 380.00 corresponding to the dehydrated form of $^{1,3}X_1^-$.

Following the procedure described for chondroitin 6-*O*-sulfate dissacharide, Y_1^- fragment ion detected at *m/z* 300.00 in spectrum in Fig. 6 was isolated within an isolation window of 2 u and submitted to CID MS^3 experiment under identical sequencing conditions. Obtained fragmentation mass spectrum is presented in Fig 7. The most abundant fragment ion was detected at *m/z* 198.80 and assigned to a $^{0,2}A^-$ ring cleavage type of ion, accompanied by its dehydrated form at *m/z* 181.20. Remarkably, besides these ions, three additional A-type of ring cleavages occurred during MS^3 fragmentation process. Thus, the fairly abundant ions at *m/z* 111.60,

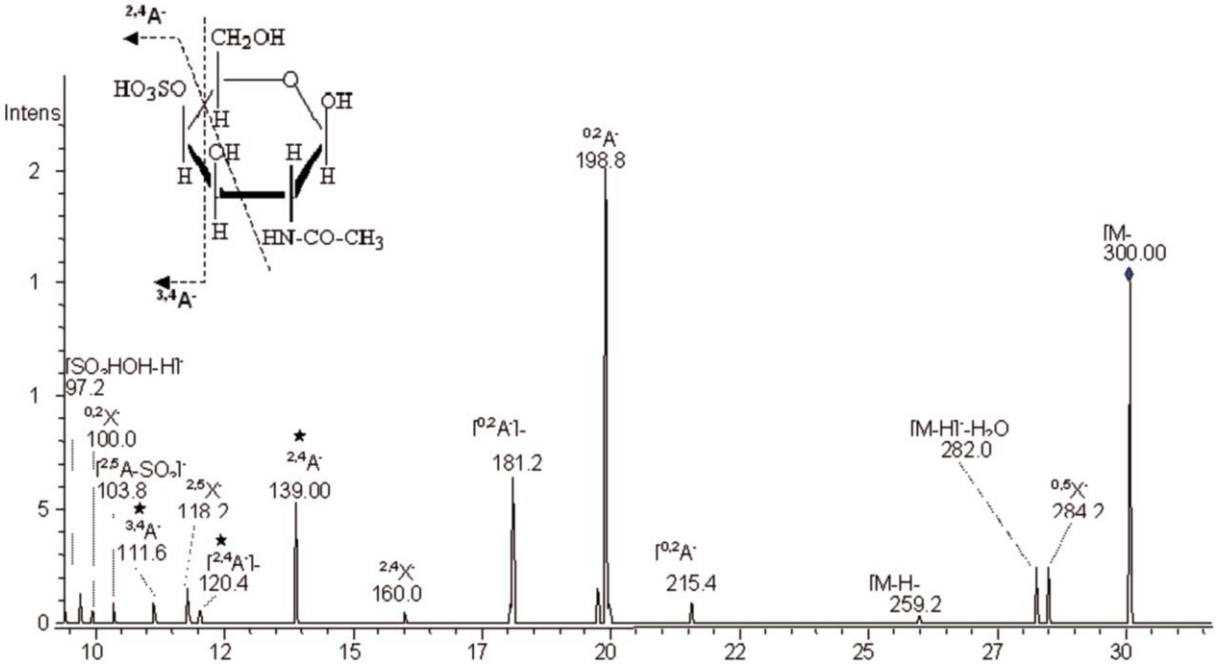

Figure 7. Fully automated chip (-) nanoESI HCT CID MS^3 of the singly deprotonated fragment ion detected in spectrum in Fig. 6 at m/z 300.00 and assigned according to mass calculation a composition of monosulfated GalNAc$^-$. Conditions and symbols are as in Fig. 6.

120.40 and 139.00 correspond to $^{3,4}A^-$, dehydrated $^{2,4}A^-$ and $^{2,4}A^-$ respectively. All three ions, marked in the spectrum by (*), are indubitably placing the sulfate ester group in the C4 position of the sequenced monosulfated GalNAc residue (inset Fig. 7).

4. Conclusions

In this study fully automated chip-based nanoESI CID HCT multistage mass spectrometry was for the first time introduced for discrimination of GalNAc 4/6 sulfation sites in chondroitin sulfate glycosaminoglycans. The feasibility of the approach was demonstrated on chondroitin 6-*O*-sulfate and chondroitin 4-*O*-sulfate disaccharides. Fragmentation patterns obtained by MS^2 and MS^3 sequencing stages provided a number of fragment ions diagnostic for GalNAc monosulfation and for localization of sulfate group at either C6 or C4 position within GalNAc ring. Based on this mass spectrometric evidence, location of sulfate group in chondroitin sulfate disaccharides, useful in discrimination of 4S/6S sulfation sites within GalNAc residue was postulated. The protocol developed here may constitute a practical alternative to the currently existing approaches in glycomics of glycosaminoglycans, which could be extended, optimized and applied to *de novo* identification of sulfation status and site(s) in isolated CS fractions containing longer chains or in complex mixtures extracted from various extracellular matrices.

Acknowledgements

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