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Liquid chromatography–electrospray mass spectrometry as a tool for the analysis of sulfated oligosaccharides from mucin glycoproteins

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Abstract

An approach for analyzing sulfated oligosaccharide alditol mixtures by liquid chromatography–electrospray mass spectrometry (LC–ESI-MS) is described. Two columns, an amino-bonded column and a porous graphitized carbon column (PGC) were used. Oligosaccharides were eluted with linear gradients of acetonitrile and water, with 5 m*M* ammonium hydrogencarbonate or formate buffers at a basic pH. The methods were evaluated on a mixture of sulfated oligosaccharide alditols prepared from mucin glycoproteins from pig stomach. Results from LC–ESI-MS of the mixture were compared with the structural information obtained by high energy collision fragmentation using fast atom bombardment tandem mass spectrometry (FAB-MS–MS). The separation ability of the two columns was also tested using a more complex mixture of sulfated oligosaccharides from pig colon, where several isomers were detected. The potential use of in-source collision-induced dissociation (CID) to gain sequence information of sulfated oligosaccharides was also evaluated. The major fragment ions obtained by in-source CID of the trisaccharide Hex-3HexNAcol6-HexNAc6-SO_3^- were sufficient for assigning the oligosaccharide sequence and the position of the sulfate group within the monosaccharide moiety. The LC–ESI-MS approach should be a valuable tool for characterization of mucin glycosylation and alterations during pathological conditions.

Keywords: Oligosaccharides; Glycoproteins; Proteins; Sulfates

1. Introduction

Characteristic of secreted mucin glycoproteins, the major protein component of the mucus protecting the surface epithelia of the body, is their large array of oligosaccharides *O*-linked via serine or threonine to the protein backbone, often making up 80% of the mass of the glycoprotein [1]. Mucins carry a large number of different oligosaccharide structures. The purpose of this diversity is unclear. Several diseases are related to the mucus, for example cystic fibrosis, where the patients suffer from an overproduction of mucus and bacterial infections in the respiratory tract [2]. Mucin glycosylation and sulfation have been shown to be altered in this disease [3–5], although the purpose and consequence of these alterations are at present subject to speculation. Oligosaccharide epitopes present on mucins are potential binding sites for microorganisms, and this initial interaction may be crucial in determining if the invading microbe will be trapped in the mucus and thus shedded from the lumenal tract.

In order to characterize the *O*-linked oligosac-

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charides on mucins, these are chemically released from the peptide or protein backbone and converted to alditols. The most common approach employed includes the purification of individual components by liquid chromatography followed by analysis with NMR, mass spectrometry, enzymatical techniques or chemical derivatization (see, for example, [6–8]). The use of these techniques require relatively large amounts of material, where quantitative information may be lost due to several purification steps.

Our group has focused on analytical techniques to allow profiling of mucin oligosaccharides. Using only a few mg of glycoproteins, extensive structural and quantitative information can be obtained, sufficient to identify differences between for example healthy and disease states [9]. Applying a strategy developed in our lab, the oligosaccharide alditols are fractionated into neutral, sialylated and sulfated species, facilitated by an on-column derivatization of the sialic acid groups [10]. The sulfated oligosaccharides have so far been characterized by fast atom bombardment (FAB) MS and FAB-MS–MS as acetylated derivatives [11]. Here we explore the possibility to analyze nonderivatized sulfated oligosaccharide alditols by LC–electrospray ionization (ESI) MS as part of the profiling strategy. The method has the advantage of including both an on-line desalting and clean-up of the sample, and also a separation and detection of isomers, thus characterizing the degree of glycan diversity. The performance of two liquid chromatography columns were studied, an amino-bonded column (aminopropyl silica, APS) and a porous graphitized carbon (PGC) column. Both types of columns have been used in previous studies for the separations of sulfated oligosaccharides with UV detection, the amino-bonded column mainly for preparative purposes (see, for example, [8] and the PGC column when analyzing sulfated mono- and disaccharide standards [12]. Trifluoracetic acid (TFA) has commonly been used as an additive for the analysis of oligosaccharides on the PGC column with UV detection. However, good separations and detection of sulfated oligosaccharides by LC–ESI-MS with a PGC column are shown here, using acetonitrile–water gradients with eluents buffered by 5 m*M* ammonium formate pH 9.3. This replacement did not only circumvent problems with lowered detection sensitivity due to TFA in the buffer, but also provided a basic pH when working with ESI-MS in the negative mode.

The possibilities of obtaining structural information on-line by fragmenting the oligosaccharides with in-source collision-induced dissociation (CID) was explored. Negative mode ESI-MS with in-source CID of nonderivatized neutral oligosaccharides has been shown before, where both linkages and anomeric configurations of disaccharides could be deduced [13,14]. It is now shown that it is possible to assign the sulfate position and terminal epitopes in sulfated oligosaccharide alditols by in-source CID.

2. Experimental

2.1. Materials

Acetonitrile (HPLC far UV grade) was obtained from Labscan (Dublin, Ireland). Ammonia and formic acid were from Merck (Darmstadt, Germany), and ammonium hydrogencarbonate from BDH (Poole, UK). Water was purified by a Milli-Q system from Millipore (Milford, MA, USA). The amino-bonded column (Hypersil APS-2, aminopropyl silica, 3 μm particle size, 250×2.1 mm I.D.) and the porous graphitized carbon column (Hypercarb, 5 μm particle size, 150×2.1 mm I.D.) were purchased from Hypersil, Runcorn, UK.

2.2. Samples and sample preparations

The highly glycosylated, trypsin resistant domains of mucins were purified from pig stomach [15] and from pig colon, essentially as described [16]. Oligosaccharides from 10 and 7.4 mg glycopeptide (from pig stomach and colon, respectively) were chemically released as alditols, separated into neutral, sialylated and sulfated oligosaccharide fractions [10], followed by desalting of the sulfated ones by gel filtration on Sephadex G-10 (Pharmacia). The obtained sulfated oligosaccharide fractions were dissolved in the HPLC mobile phase (400 μl, colon; 2 ml, stomach) before injection (2–5 μl) on the HPLC column.

2.3. HPLC–ESI-MS and FAB-MS–MS

The analyses were performed using a 2248 Pharmacia LKB pump and 2252 Pharmacia LKB controller. The amino-bonded column and the PGC column were used with gradients as specified in the figure captions. The eluents consisted of acetonitrile and water, containing 5 m*M* ammonium hydrogencarbonate, pH 8.0 (amino-bonded column) or 5 m*M* ammonium formate, pH 9.3 (PGC column). The liquid flows were 120 μl min^{-1}. The columns were connected to a single quadrupole LC–MS mass spectrometer, API 150EX (Perkin-Elmer Sciex, Thornhill, Canada) equipped with a Turbo Ionspray source. The operative parameters were set as follows: ionspray voltage: –4.8 kV, heater (turbo) gas

Table 1
Structures of sulfated mucin oligosaccharide alditols from pig stomach interpreted from FAB-MS–MS of the mixture

Name	Molecular ion $(M-H)^-$	Structures[a]
A	667	SO_3^--6HexNAc, Hex; 6, 3 HexNAcol
B	813	SO_3^--6HexNAc, Fuc-Hex; 6, 3 HexNAcol
C	829	Hex-4(SO_3^--6)HexNAc, Hex; 6, 3 HexNAcol
D	870	SO_3^--6HexNAc, Hex-HexNAc; 6, 3 HexNAcol
E[b]	975	Hex-4(SO_3^--6)HexNAc, Fuc-Hex; 6, 3 HexNAcol
F[c]	1032	Hex-4(SO_3^--6)HexNAc, HexNAc-Hex; 6, 3 HexNAcol
F[c]	1032	Hex-4(SO_3^--6)HexNAc, Hex-HexNAc; 6, 3 HexNAcol
G	1121	Fuc-Hex-4(SO_3^--6)HexNAc, Fuc-Hex; 6, 3 HexNAcol
H	1178	Fuc-Hex-4(SO_3^--6)HexNAc, Hex-HexNAc; 6, 3 HexNAcol

[a] Hex, hexose; Fuc, fucose; HexNAc, *N*-acetylhexosamine; HexNAcol, *N*-acetylhexosaminitol.
[b] Two peaks are observed by LC–MS.
[c] One peak is observed by LC–MS.

temperature 450°C; orifice voltage: −30 V (in-source CID: −140 V). Scan parameters: scan range ±5 u of the molecular ions of interest, step 0.4 u, dwell time 15 ms, scan speed 6.3 s. In-source CID: scan range m/z 100–1250, dwell time 2 ms, step 0.6 u, scan speed 3.8 s. Compressed air was used for nebulizer gas and for heater (turbo) gas and nitrogen for curtain gas. Mass spectrometric conditions were optimized with a reduced sulfated trisaccharide sulfo-Lewis a (Dextra Labs., Reading, UK). Detection limit of sulfo-Lewis a was approximately 0.5 n*M* (10 fmol) using a 20 μl loop. Detection limit was defined as three times the standard deviation of the blank.

The sulfated oligosaccharide alditol mixture from pig stomach was analyzed by FAB-MS–MS after perdeuteracetylation of the hydroxyl groups. The procedure, instrumentation and typical fragmentation patterns have been described [11].

3. Results and discussion

The aim of this study was to evaluate different chromatographic approaches for on-line separation and characterization of sulfated mucin oligosaccharide alditols by LC–ESI-MS. Approaches taken are illustrated by the analysis of two different mixtures of sulfated mucin oligosaccharide alditols prepared from pig stomach and colon.

3.1. LC–ESI-MS of sulfated oligosaccharide alditols

The mixture of sulfated mucin oligosaccharide alditols from pig stomach were characterized by FAB-MS–MS [11], the deduced structures are listed in Table 1. The same sample was chromatographed on an amino-bonded column (normal phase) coupled to ESI-MS. Extracted ion chromatograms of detected $(M–H)^-$ ions are displayed in Fig. 1. Results from the analysis of the sample on a PGC column are shown in Fig. 2.

Structures with $(M–H)^-=m/z$ 667 (marked A), 813 (B), 829 (C), 870 (D), 1121 (G) and 1178 (H) resulted in one peak each in the ESI-MS extracted ion chromatograms in Figs. 1 and 2, which is in agreement with the number of oligosaccharide iso-

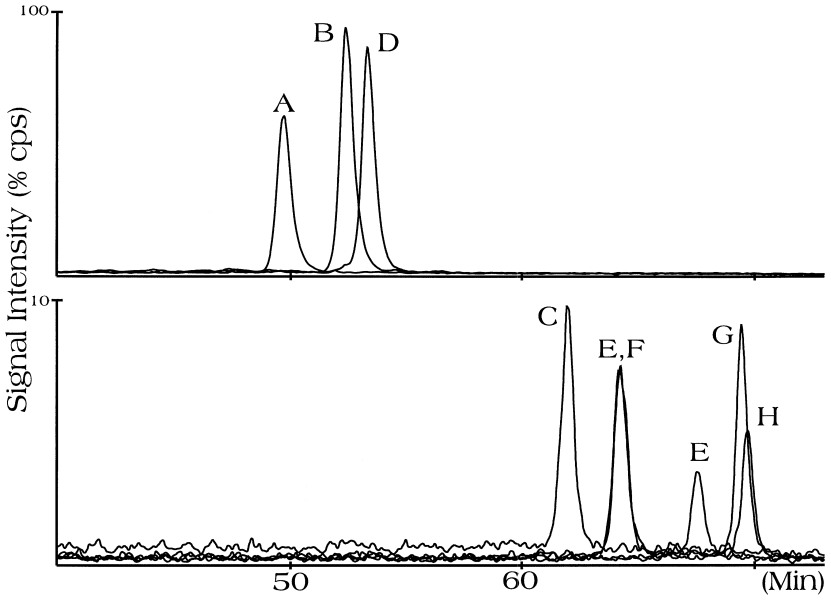

Fig. 1. Extracted ion chromatograms of sulfated oligosaccharide alditols from pig stomach, separated on an amino-bonded column and detected on-line as $(M–H)^-$ ions with ESI-MS. A linear gradient from 80 to 60% acetonitrile (60 min) was used, the eluents containing 5 m*M* ammonium hydrogencarbonate pH 8. The flow was 120 μl min^{-1}. The lower panel in the figure displays extracted ion chromatograms with signal intensity magnified ten times compared to the upper panel. Structures deduced by FAB-MS–MS of the peaks A–H in the mixture are listed in Table 1.

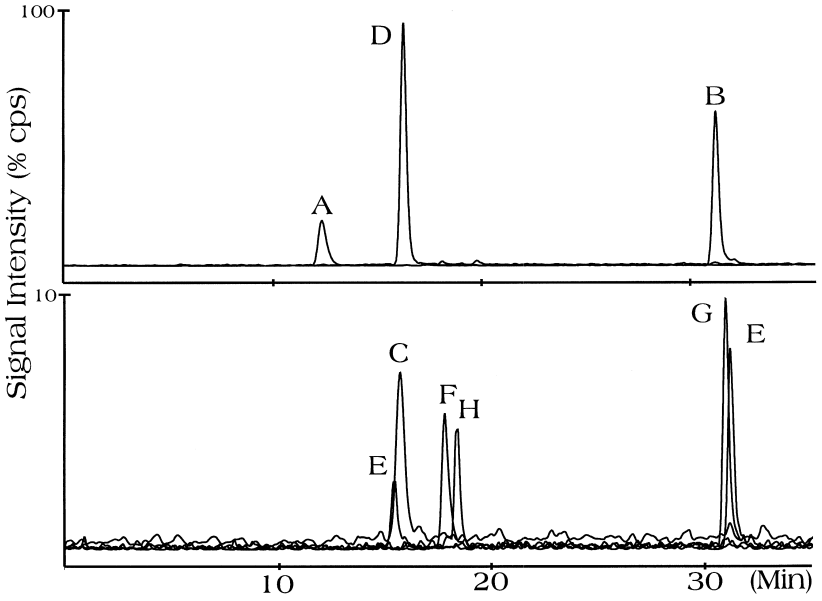

Fig. 2. Extracted ion chromatograms of sulfated oligosaccharide alditols from pig stomach, separated on a PGC column and detected as $(M–H)^-$ ions with ESI-MS. A linear gradient from 5 to 40% acetonitrile (50 min) was used, the eluents containing 5 m*M* ammonium formate pH 9.3. The flow was 120 μl min^{-1}. The lower panel in the figure displays extracted ion chromatograms with signal intensity magnified ten times compared to the upper panel. Structures deduced by FAB-MS–MS of the peaks A–H in the mixture are listed in Table 1.

mers detected by FAB-MS–MS (Table 1). However, there are some exceptions to this. Two peaks in the extracted ion chromatogram of *m/z* 975 (E) indicated the presence of at least two isomeric oligosaccharides which were separated on both liquid chromatography columns, while only one isomer with this molecular mass could be elucidated from the FAB-MS–MS spectrum (Table 1). In addition, FAB-MS–MS of the ion at *m/z* 1032 displays fragment ions originating from two possible structural isomers, but only one peak (F) could be detected when chromatographed in the two liquid chromatography systems.

Sulfated mucin oligosaccharide alditols from pig colon were analyzed on both the amino-bonded column (Fig. 3) and the PGC column (Fig. 4). The composition of the detected molecular ions is listed in Table 2, together with the number of peaks detected in the extracted ion chromatograms. Results from extracted ion chromatograms of ten molecular ions are presented here, where at least 33 structures were detected as separate peaks. The large number of peaks indicates a more complex mixture than in the sample from pig stomach, where nine peaks were detected, originating from eight molecular ions. Typical isomeric configurations reported for mucin oligosaccharides are not only different oligosaccharide sequences (branching), but also the presence of linkage isomers, for example type 1 and type 2 *N*-acetyllactosamine chains, and terminal GalNAc residues instead of GlcNAc [6–8]. In this sample, sulfate linked both to C-6 of HexNAc and to C-3 of Hex residues has been indicated from FAB-MS–MS of the oligosaccharide mixture. This explains the presence of at least two detected isomers of for example *m/z* 667 [17]. LC–ESI-MS of this sample demonstrates the diversity of isomeric sulfated glycans from pig colonic mucins. Here, six isomers of a tetrasaccharide [$(M–H)^-=m/z$ 870] and a pentasaccharide [$(M–H)^-=m/z$ 1016] could be detected. Such a complex mixture is not likely to be structurally elucidated by FAB-MS–MS of the whole mixture without a prior separation.

The presence of mucin oligosaccharides from pig colon containing more than one sulfate group is not addressed here, though these have been detected

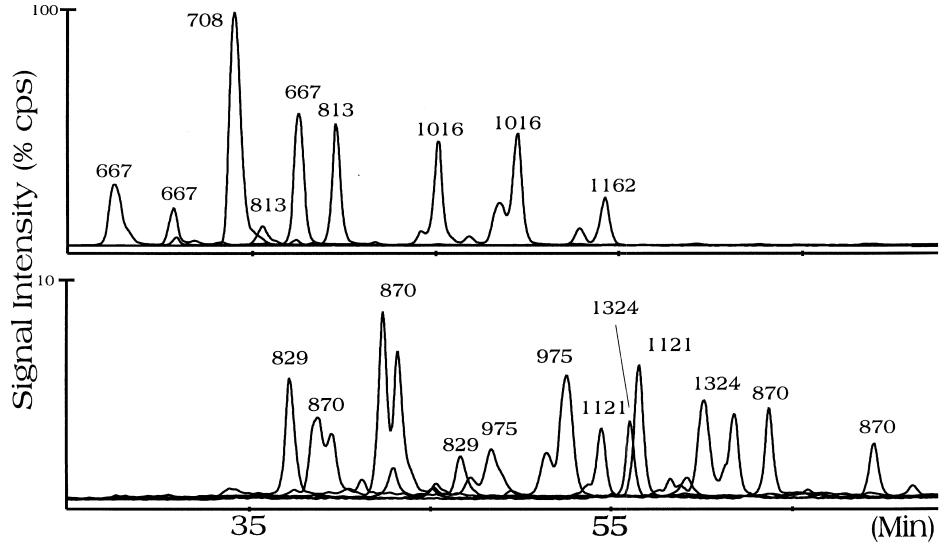

Fig. 3. Extracted ion chromatograms of sulfated oligosaccharide alditols from pig colon, separated on an amino-bonded column and detected as $(M-H)^-$ ions with ESI-MS. A linear gradient from 80 to 50% acetonitrile (90 min) was used, the eluents containing 5 m*M* ammonium hydrogencarbonate pH 8. The flow was 120 μl min^{-1}. The lower panel in the figure displays extracted ion chromatograms with signal intensity magnified ten times compared to the upper panel. The composition of the molecular ions is listed in Table 2.

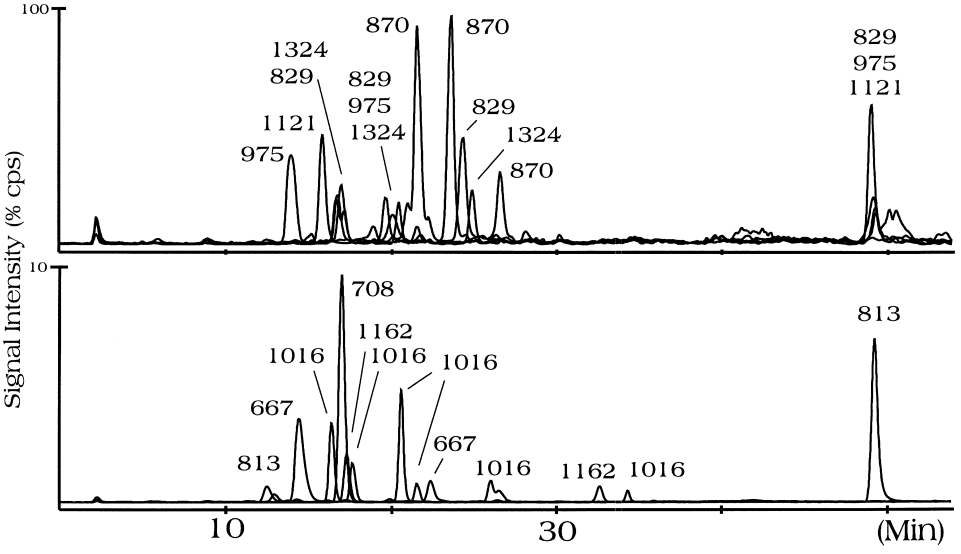

Fig. 4. Extracted ion chromatograms of sulfated oligosaccharide alditols from pig colon, separated on a PGC column and detected as $(M-H)^-$ ions with ESI-MS. A linear gradient from 5 to 25% acetonitrile (60 min) was used, the eluents containing 5 m*M* ammonium formate pH 9.3. The flow was 120 μl min^{-1}. The lower panel in the figure displays extracted ion chromatograms with signal intensity magnified ten times compared to the upper panel. The composition of the molecular ions is listed in Table 2.

Table 2
Composition of sulfated mucin oligosaccharide alditols prepared from pig colon

Molecular ion $(M-H)^-$	Composition[a]					No. of peaks detected with LC–ESI-MS	
	Hex	HexNAc	Fuc	HexNAcol	SO_3^-	PGC column	Amino-bonded column
667	1	1		1	1	2	3
708		2		1	1	1	1
813	1	1	1	1	1	2	2
829	2	1		1	1	4	2
870	1	2		1	1	3	6
975	2	1	1	1	1	4	4
1016	1	2	1	1	1	6	4
1121	2	1	2	1	1	2	2
1162	1	2	2	1	1	2	2
1324	2	2	2	1	1	3	3

[a] Hex, hexose; Fuc, fucose; HexNAc, *N*-acetylhexosamine; HexNAcol, *N*-acetylhexosaminitol.

[17]. The analytical problems involved in the analysis of multisulfated oligosaccharides have been discussed by other groups. Loss of sulfate when analyzing highly sulfated glycosaminoglycan derived fragments by mass spectrometry with various ionization modes has been observed [18]. As to ESI-MS, this loss has been shown to be minimized by controlling nozzle-skimmer voltage, when analyzing multisulfated oligosaccharides as ion-paired complexes with basic peptides and proteins [19]. In a study of heparin fragments analyzed by ESI-MS using sodium and ammonium additives, no or negligible sulfate loss from the oligosaccharides was detected [20]. Since the presence and location of sulfate groups on mucin oligosaccharides are of relevance for structure characterization, the use of ESI-MS for analysis of multisulfated mucin oligosaccharides must be further explored and the technique validated with regard to the process of sulfate loss during ionization.

The results presented in this study show the benefit of using both the amino-bonded and the PGC type chromatography in LC–ESI-MS to achieve separations of mixtures of sulfated oligosaccharide alditols. Compounds coeluting on one column could be separated on the other, indicated by the differing number of isomers separated in each system (Table 2).

Amino-bonded columns have been used extensively for the separation of oligosaccharides, compared to the more recent developed PGC column. This column has the advantage of a high physical and chemical stability and can be used throughout the entire pH range, in contrast to the silica-based amino-bonded ones with a more limited pH range and lifetime. Graphitized carbon columns also have the advantage that they can be used for desalting and clean-up of samples [21]. During LC–ESI-MS of sulfated oligosaccharides purified as in this study, traces of salt and byproducts from the previous on-column derivatization elute before the sulfated oligosaccharide alditols (authors, unpubl.). The elution order of sulfated oligosaccharides does not appear to be pH dependent on the PGC column. When sulfated oligosaccharide alditols from pig stomach were analyzed on the PGC column, the replacement of an acetonitrile–0.05% TFA gradient as previously used by others [12] with a gradient of acetonitrile–water with 5 m*M* ammonium formate pH 9.3, caused no change in the elution order of the oligosaccharides (not shown).

3.2. In-source CID of sulfated oligosaccharide alditols

With a single quadrupole mass spectrometer, structural information can be obtained of the saccharides by changing the orifice potential to create fragment ions. In Fig. 5, upper panel, the fragmentation spectrum of the saccharide Hex-3HexNAcol6-HexNAc6-SO_3^- is displayed. The spectrum was collected during a LC–ESI-MS run with the orifice potential kept at −140 V and

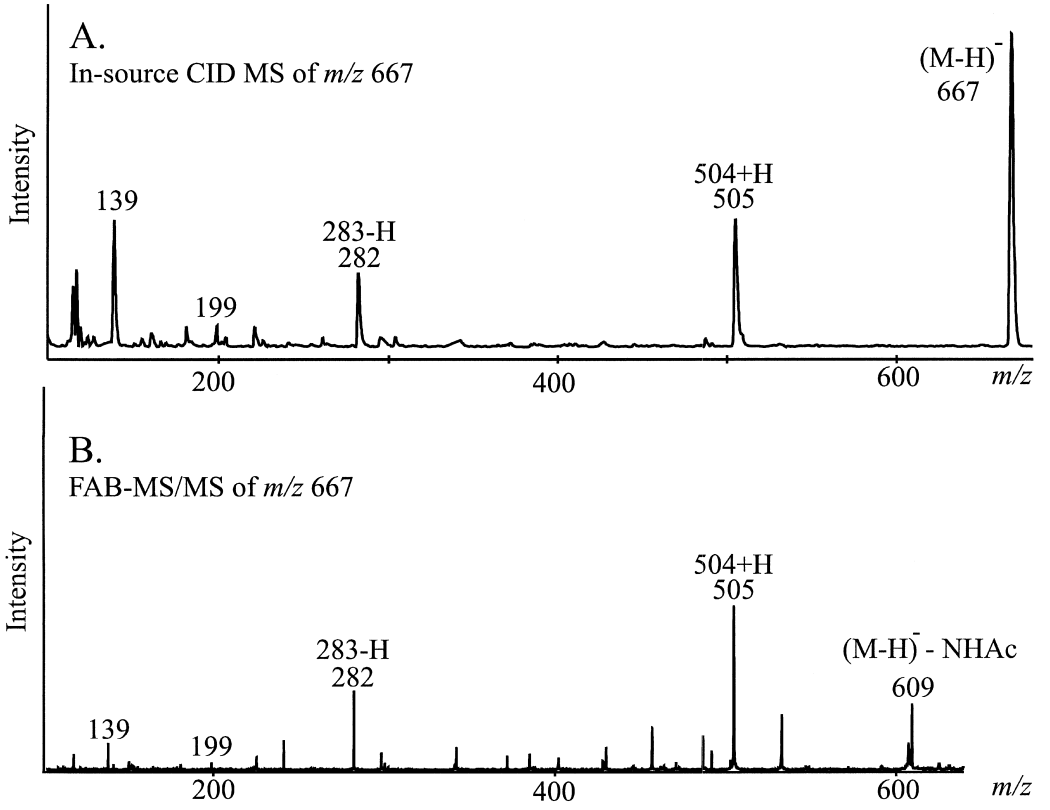

Fig. 5. In-source CID mass spectrum from LC–ESI-MS of the oligosaccharide Hex-3HexNAcol6-HexNAc6-SO_3^- (A) and FAB-MS–MS of the same compound (B). Background ions have been subtracted. Fragmentation is described in Fig. 6, the chromatographic conditions in Fig. 1.

detecting the fragments in the negative mode. The spectrum is compared with the corresponding high energy FAB-MS–MS spectrum of the same compound (Fig. 5, lower panel). Four fragment ions originating from the trisaccharide (Fig. 6) were detected, which also were present in the FAB-MS–MS spectrum of the same compound. The ions at m/z 139 and m/z 199 are due to internal cleavages of the sulfate-carrying monosaccharide unit, where the fragment at m/z 139 is indicative of the sulfate group positioned on C-6. Sulfate linked to C-4 of a HexNAc residue would also give rise to m/z 139, but this epitope has to our knowledge not been found on mucin glycans. The fragments at m/z 505 and m/z 282 are due to a loss of the terminal Hex unit and the Hex-HexNAcol, respectively. Daughter ion spectra from in-source CID of larger oligosaccharides indicated that the fragmentation was of similar type as in the example in Fig. 6, with cleavages in the glycosidic bonds and internal cleavages of the sulfate-carrying residue. Due to lower amounts and maybe also their size, larger oligosaccharides produced fewer fragment ions compared to the molecular ion.

Typical fragmentation patterns obtained from FAB-MS–MS of nonderivatized sulfated oligosaccharide alditols are more thoroughly described elsewhere [11]. The presence of these four fragments obtained by in-source CID, a fragmentation mechanism that appears similar to that obtained by FAB-MS–MS, is sufficient for assigning the sulfate position and the oligosaccharide sequence, and indicates the usefulness of this fragmentation mode to obtain structural information.

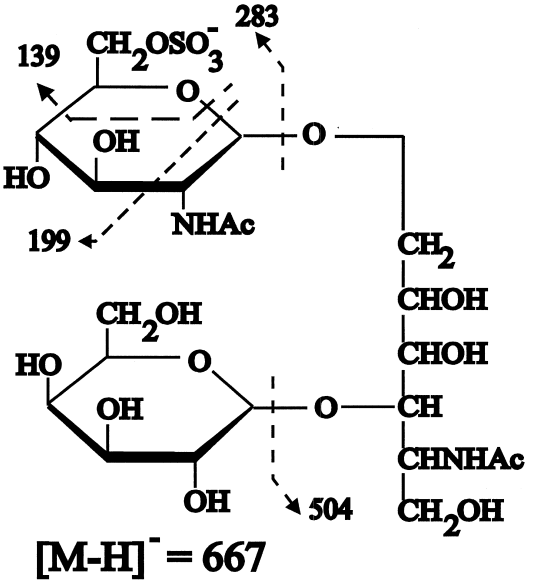

Fig. 6. Fragmentation pathways in negative mode collision-induced dissociation of the oligosaccharide Hex-3HexNAcol6-Hex-NAc6-SO_3^-, the spectra are displayed in Fig. 5.

4. General comments

Mass spectrometry could be regarded as an optimal on-line detector for liquid chromatography of sulfated oligosaccharide alditols, due to their lack of derivatizable groups and poor visibility in UV. The use of in-source CID or tandem mass spectrometry gives additional information regarding their structure. Mucin glycosylation is the result of the activity of several glycosyl- and sulfotransferases. In comparative studies, screening for differences between mucin populations or alterations due to pathological conditions affecting glycosyltransferase activity, the use of ESI-MS for quantitative detection should be investigated. This is a relevant task for further studies.

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