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Characterization of the chemical structure of sulphated glycosaminoglycans after enzymatic digestion

Application of liquid chromatography–mass spectrometry with an atmospheric pressure interface

R. Da Col and L. Silvestro*

Res Pharma Pharmacological Research Srl, Via Belfiore 57, 10125 Turin (Italy)

A. Naggi and G. Torri

Istituto Scientifico di Chimica e Biochimica "G. Ronzoni", Via G. Colombo 81, Milan (Italy)

C. Baiocchi

Dipartimento di Chimica Analitica, Università di Torino, Via P. Giuria 5, 10125 Turin (Italy)

D. Moltrasio and A. Cedro

Crinos SpA, Villaguardia (Italy)

I. Viano

Istituto di Farmacologia e Terapia Sperimentale, Facoltà di Medicina, Università di Torino, Via P. Giuria 13, 10125 Turin (Italy)

ABSTRACT

Pneumatically assisted electrospray was demonstrated to be a powerful ionization source for the analysis of oligosaccharides. A mass spectrometer was interfaced to an HPLC system, using this interface, to determine oligosaccharides from the enzymatic digestion of heparin separated on a reversed-phase column. To set up the technique, and particularly to clarify the ionization process, purified disaccharides, from enzymatic digestion of chondroitin sulphates, were measured. The use of a suitable counter ion in the mobile phase, tetrapropylammonium (TPA), to optimize the HPLC separation, gave, with sulphated di- and oligosaccharides, adducts $[M + n\text{TPA} - (n + m)H]^{m-}$, which were unexpectedly stable to fragmentation; molecular ions $[M - (n + 1)H]^{n-}$, in the presence of the counter ion, were observed only with desulphated or monosulphated disaccharides. The stability of the adducts and the use of a deuterated ion-pair reagent permitted an exact evaluation of the molecular masses of disaccharides and oligosaccharides of unknown structure. Spectra obtained in the absence of the counter ion contained singly or multiply charged molecular ions and fragmentation ions mainly from loss of the sulphate groups; under these ionization conditions the exact mass determination and interpretation of the spectra were difficult. After removal of the counter ion, tandem mass spectra could be obtained with some interesting data for the characterization of these molecules. Complete spectral analyses were performed with amounts of samples of 50 μg but, using microbore columns, one twentieth of this amount may give good spectra.

* Corresponding author.

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INTRODUCTION

Sulphated glycosaminoglycans (GAGs), such as chondroitin sulphates (CS), dermatan sulphate (DeS), heparan sulphate (HS) and heparin (HEP), are heterogeneous polysaccharides that play important roles in all living organisms and sometimes have notable pharmacological activity [1].

Individual GAGs can be characterized by a variety of electrophoretic, chromatographic and spectroscopic methods, applied either to the intact polysaccharides or to their products of chemical or enzymatic depolymerization [2]. These degradative techniques are very important for obtaining sequence information and have been applied successfully to characterize the antithrombin binding site of heparin [3]. NMR spectrometry is one of the most effective techniques [4] in the analysis of the oligosaccharides from degradation, but it requires relatively large amounts of material and is not suitable for the analysis of very small samples, *e.g.*, most biological samples.

Attempts have been made to use mass spectrometry (MS) [5–7], generally using fast atom bombardment (FAB) [6,7] for ionization, to determine the structures of these oligosaccharides. The reported results were interesting, but some problems were evident: direct connection to high-performance liquid chromatography (HPLC) could not be used and the fractions, after HPLC, must be desalted before FAB analysis. Little information on the carbohydrate sequence could be obtained except with complex derivatization procedures that did not seem practical [6,7]. The extremely high polarity of GAGs is a major problem with other conventional MS techniques.

Recently we used a mass spectrometer equipped with a pneumatically assisted electrospray ionization interface, particularly effective for polar compounds [8], to determine HEP and DeS in human samples, with interesting results in terms of sensitivity and specificity [9]. Another group [10] achieved good results for the analysis of purified oligosaccharides, by enzymatic digestion of GAGs, with the same MS technique.

In this paper, we present the results, obtained with the same MS system, for the on-line characterization of sulphated disaccharides and oligosaccharides from enzymatic depolymerization of HEP, separated, as described previously [9], by ion-pair reversed-phase chromatography (IP-RP-HPLC) and analysed with on-line connection to the mass spectrometer. To set up the technique and elucidate the ionization process in the presence of an ion-pair reagent, purified disaccharides from enzymatic digestion of CS were analysed, dissolved in water or in the buffer used for the HPLC separation; a deuterated derivative of the ion-pair reagent was also used. The possible role of tandem MS (MS–MS) with collisionally induced dissociation (CID) to elucidate the structure and sequence of these molecules was also explored. Similar assays were carried out on real samples by enzymatic digestion.

MATERIALS AND METHODS

Chemicals

The following purified unsaturated disaccharides obtained by enzymatic digestion of CS were purchased from Seikagaku: 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-diS_d), 2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-diS_e) and 2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-O-sulpho-D-galactose (ΔDi-triS). The deuterium-labelled ion-pair reagent tetrapropylammonium bromide (d_{28}TPA), was purchased from MSD Isotopes. Heparinase I (Hepase) (EC 4.2.2.7), HEP from porcine intestinal mucosa sodium salt and all other chemicals of the purest grade available were obtained from Fluka. Research-grade water, produced with a Milli-Q system (Millipore), was used in all experiments.

Enzymatic degradation of HEP

HEP (1 mg), was digested for 24 h at 37°C with 1.5 mU of Hepase in 0.5 ml of 50 m*M* ammonium acetate buffer (pH 7.0) containing

1.0 m*M* calcium chloride [11]. At the end of the digestion, to remove the enzyme and the undigested HEP, the reaction mixture was loaded into Ultrafree MC tubes for ultrafiltration (molecular mass cut-off 10 000) and centrifuged at 2000 *g* for 45 min. Finally, the ultrafiltrates were lyophilized.

Sample introduction and HPLC separation

Purified unsaturated disaccharides were analysed by continuous infusion (Harvard Scientific syringe pump), at 5 μl/min, dissolved in water with 3.3 m*M* formic acid or in water with 3.3 m*M* tetrapropylammonium hydroxide (TPA), adjusted to pH 4.0 with formic acid, or with 3.3 m*M* d_{28}TPA, adjusted to pH 4.0 with formic acid to a concentration of 50 μg/ml.

HPLC separations of oligosaccharides from digestion of HEP were performed on a Spherisorb Hexyl reversed-phase column (250 × 4.6 mm I.D., 5 μm) from Phase Separations. A Gilson gradient HPLC system (Model 305 + Model 302 Pumps, both with a Model 5.SC head, analytical dynamic mixer and Model 231-401 autosampler with a sample loop of 20 μl) was used. The mobile phase was 3.3 m*M* TPA adjusted to pH 4.0 with formic acid in water (buffer A) and 3.3 m*M* TPA adjusted to pH 4.0 with formic acid in acetonitrile–water (90:10) (buffer B), with gradient elution as follows: 3 min isocratic at 100% buffer A then a linear gradient to 50% buffer B in 20 min and a final isocratic step of 7 min at that composition; the flow-rate was 1.00 ml/min. To decrease the mobile phase flow-rate to a level acceptable for the MS system (above 50 μl/min), splitting was performed, connecting to the splitting port of the MS interface a piece of silica capillary of appropriate length. In some experiments buffers A and B were prepared with the same characteristics using d_{28}TPA instead of TPA. All the HPLC–MS experiments were repeated also with a suppressor for cationic counter ions (Dionex MMPC), as described previously [12], with a regenerant composed of 0.1 *M* sulphuric acid at a flow-rate of 5.0 ml/min.

Mass spectrometric analysis

MS and MS–MS experiments were performed with a Perkin-Elmer–Sciex API III mass spectrometer equipped with a pneumatically assisted electrospray source (articulated ionspray source). Mass spectra were acquired in the negative-ion mode and the mass spectrometer was generally operated with scanning from *m/z* 200 to 2000. When MS–MS experiments were performed the mass spectrometer was operated in the daughter-ion scanning mode and argon was used to obtain CID. In these experiments the collisional energy was optimized at 70 eV and the collision gas had an effective target thickness of *ca.* 2.5×10^{14} atoms/cm^2.

RESULTS

Figs. 1 and 2 show the mass spectra obtained with the purified disaccharides by enzymatic degradation of CS dissolved in water with 3.3 m*M* formic acid (top), in water with 3.3 m*M* TPA adjusted to pH 4.0 with formic acid (middle) or in d_{28}TPA (bottom) in order to examine the ionization processes under different conditions.

The spectra from ΔDi-0S are presented in Fig. 1A–C; under the three different MS conditions only a relevant $(M\text{-}H)^-$ ion (*m/z* 377) can be observed and no adduct with TPA or fragments are present. Fig. 1D–F show the mass spectra obtained with ΔDi-4S.

The most relevant ion in all the spectra has *m/z* 457, corresponding exactly to the predicted *m/z* of the $(M - H)^-$ ion of this monosulphated disaccharide. In the spectrum obtained without TPA a second ion shifted of 22 u (*m/z* 479) can be observed and corresponds to an adduct with sodium, $(M + Na - 2H)^-$. Another ion with *m/z* 299, probably derived from fragmentation, is also present; this *m/z* value in fact corresponds to the mass of O-sulpho-N-acetylgalactosamine. In presence of TPA an ion of *m/z* 642 with an intensity similar to that of the deprotonated molecular ion is present; the difference in mass between these two ions is 185 u, corresponding exactly to the calculated mass increase for an adduct with the ion-pair reagent (TPA) used, therefore suggesting that this ion is $(M + TPA - 2H)^-$. This hypothesis is confirmed by the mass spectrum obtained with the sample dissolved in a solution of d_{28}TPA. In fact, with this buffer the $(M - H)^-$ ion (*m/z* 457) is still present, whereas

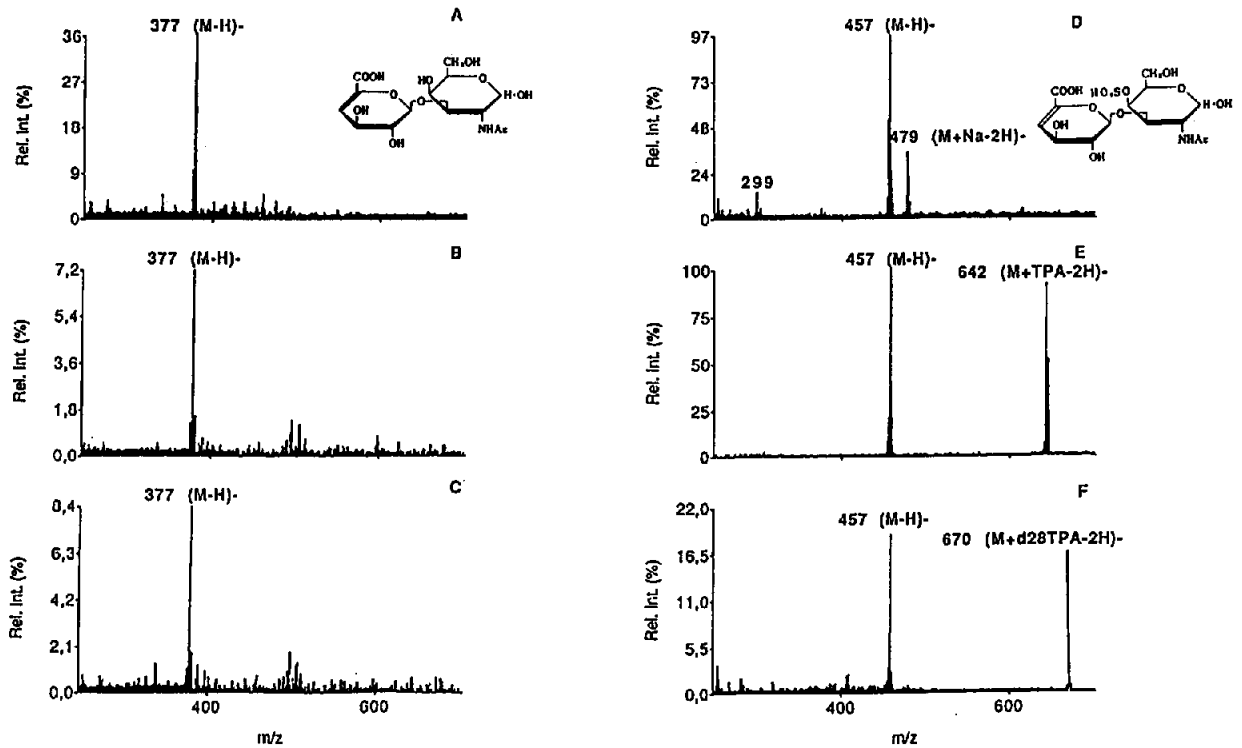

Fig. 1. Mass spectra obtained with the purified desulphated (ΔDi-0S, left) and monosulphated (ΔDi-4S, right) disaccharides by enzymatic digestion of CS. The mass spectra were recorded in the negative-ion mode by injections of the samples dissolved at 50 μg/ml in 3.3 m*M* formic acid in water (top), or TPA–formate buffer (pH 4.0, 3.3 m*M*) (middle) or d_{28}TPA–formate (pH 4.0, 3.3 m*M*) (bottom).

the ion at *m/z* 642 has disappeared and a strong ion at *m/z* 670 is present; the *m/z* difference between this ion and the adduct with TPA, $(M + TPA - 2H)^-$, is 28, corresponding to the molecular mass difference between TPA and d_{28}TPA. No ions from fragmentation can be observed. The mass spectra obtained from ΔDi-6S (data not reported) show the same ions as observed with ΔDi-4S and also the absolute intensity of ionization is similar.

The spectra from ΔDi-diS_d are summarized in Fig. 2A–C. In the spectrum without TPA, singly, $(M - H)^-$, and doubly charged, $(M - 2H)^{2-}$, molecular ions can be observed, with *m/z* 268–537. The adduct with a sodium ion, $(M + Na - 2H)^-$, is also relevant (*m/z* 559), followed (*m/z* 581) by a weak ion with two sodium ions, $(M + 2Na - 3H)^-$. Ions from fragmentation are also of relevant intensity; the ion with *m/z* 457 derives from the loss of a sulphate group, $(M - SO_3H - 2H)^-$, and others with $m/z < 300$ may be related to O-sulpho-N-acetylgalactosamine. In the spectrum obtained with TPA only ions formed by adducts with counter ions can be observed: at *m/z* 722 the adduct with one TPA, $(M + TPA - 2H)^-$, and at *m/z* 907 that with two TPA, $(M + 2TPA - 3H)^-$. Similar results were obtained with d_{28}TPA. The spectra obtained from the other disulphated disaccharide, ΔDi-diS_e, (data not shown) are very similar to those observed with ΔDi-diS_d.

The results from the trisulphated disaccharides, ΔDi-triS, are shown in Fig. 2D–F. In the spectrum without TPA the deprotonated $(M - H)^-$ ion, *m/z* 617, is present but the adducts with sodium, *m/z* 639, $(M + Na - 2H)^-$, and *m/z* 661, $(M + 2Na - 3H)^-$, are more important. Ions derived from fragmentation are relevant; the ion of *m/z* 559 corresponds to the loss of a sulphate group from a sodium adduct, $(M - SO_3H + Na - H)^-$, that with *m/z* 519 is due to the loss of sulphate from the molecular ion but

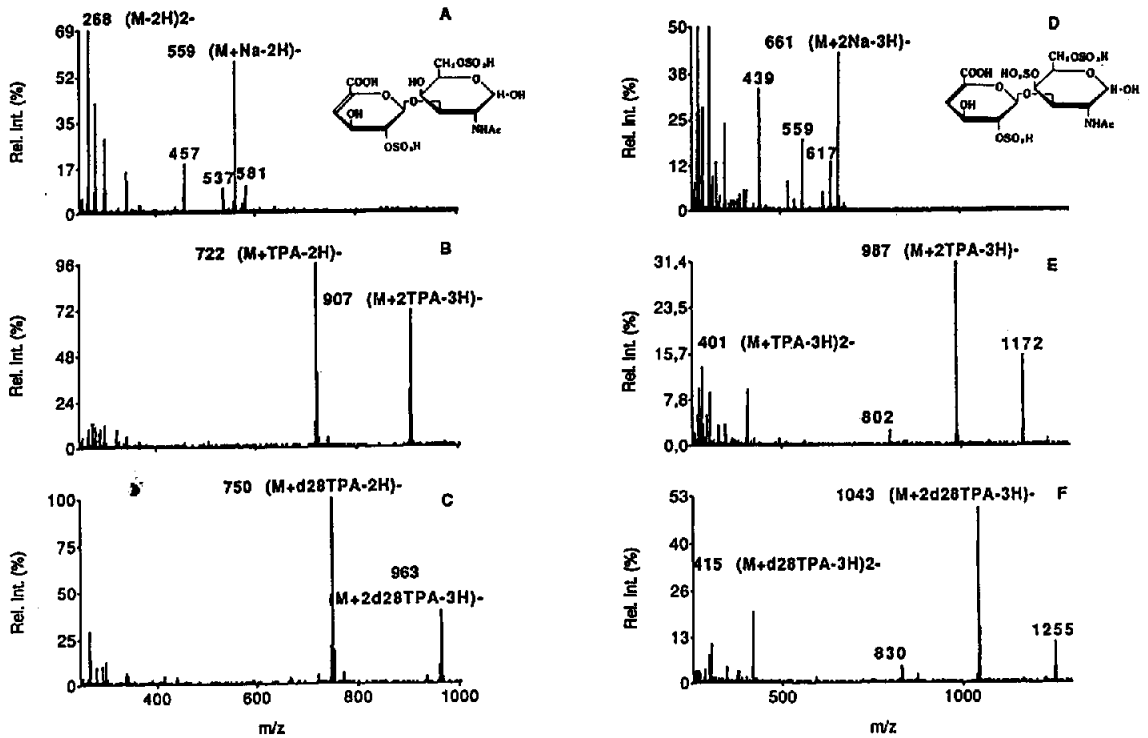

Fig. 2. Mass spectra obtained with the purified disulphated (ΔDi-DiS$_d$, left) and trisulphated (ΔDi-triS, right) disaccharides by enzymatic digestion of CS. The mass spectra were recorded using the same conditions as in Fig. 1 and are displayed in the same order.

with the oxygen involved in the ester bond, $(M - OSO_3H - 2H)^-$, and finally the loss of these two groups gives the ion of m/z 439, $(M - SO_3H - OSO_3H - 2H)^-$. In the spectrum with TPA the strongest ions correspond to the adduct with 2TPA, $(M + 2TPA - 3H)^-$, m/z 987, and other relevant adducts with TPA are present with m/z 1172 $(M + 3TPA - 4H)^-$, and m/z 802, $(M + TPA - 2H)^-$. The results obtained with d_{28}TPA confirm the interpretation given to this spectrum.

In Fig. 3 are summarized the tandem mass spectra obtained by CID of the deprotonated molecular ions from each of the previous disaccharides with the aim of understanding the possible role of this technique in characterizing the structure of these molecules. These spectra were acquired from samples dissolved in water with 3.3 mM formic acid because no results could be obtained with the same disaccharides dissolved with ion-pair reagents from both molecular and adduct ions. With ΔDi-0S (A) only a relevant daughter ion (m/z 174) can be observed; the m/z value corresponds to the hexuronic acid residue with the oxygen of the glycosidic bond. The tandem mass spectrum (B) obtained with ΔDi-6S shows the same fragment (m/z 299), related to the O-sulpho-N-acetylgalactosamine, as observed in the spectrum in Fig. 1D, and two other ions, m/z 281 and 341, derived from the same group; the first corresponds to O-sulpho-N-acetylgalactosamine with a further loss of water, in comparison with m/z 299, and the other (m/z 341) is the hexosamine group linked, by the glycosidic linkage, with a fragment (C_1–C_2) of the hexuronic acid. A daughter ion corresponding to the hexuronic acid, m/z 174, can also be observed in this spectrum as reported for ΔDi-0S. The CID mass spectrum (C) from ΔDi-4S, despite the results obtained with scanning, presents interesting differences compared with ΔDi-6S. All the ions

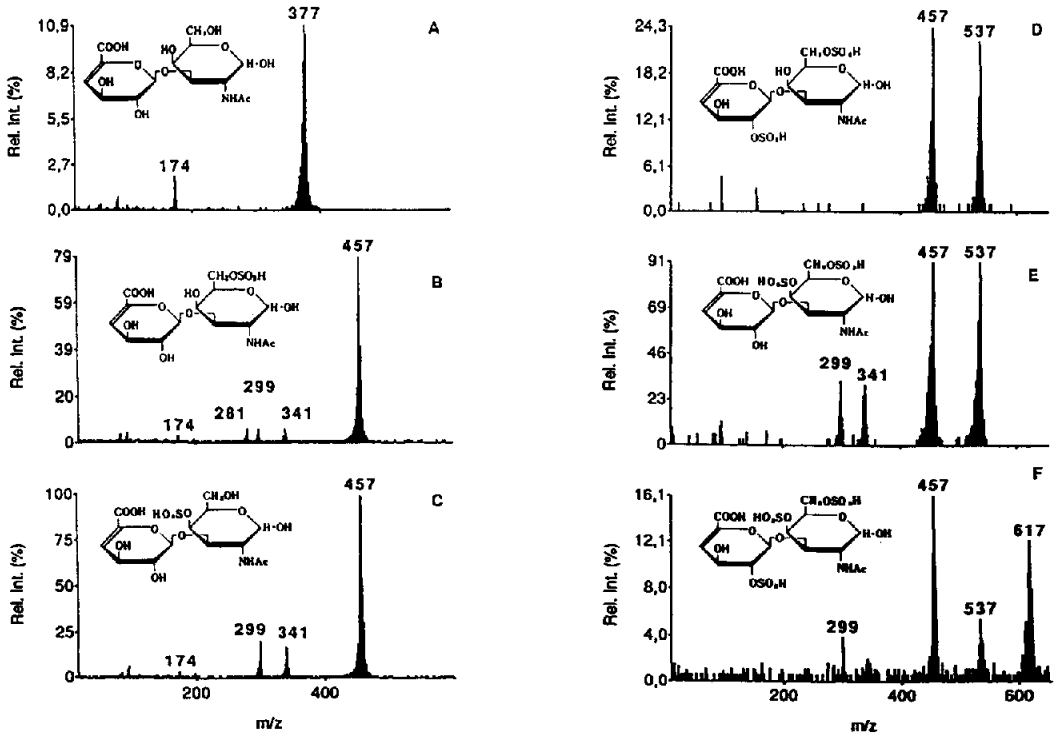

Fig. 3. Tandem mass spectra obtained from the following purified disaccharides by enzymatic digestion of CS : (A) ΔDi-0S; (B) ΔDi-6S; (C) ΔDi-4S; (D) ΔDi-diS_d; (E) ΔDi-diS_e; (F) ΔDi-triS. The spectra were acquired following injection of the samples dissolved in a mobile phase of 3.3 m*M* formic acid in water.

observed with this last disaccharide are present but the relative intensities of the daughter ions are different. First, the ion of *m/z* 281 is very weak, the intensity in comparison with the ion of *m/z* 299 is *ca.* 1/25, whereas with ΔDi-6S the intensities of these two ions are similar. The ions of *m/z* 299 and 341 have similar intensities and it is *ca.* 25% of that of the parent ion; in the spectrum of ΔDi-6S the relative intensity of these ions was only 5% of that of the parent ion. The fragment corresponding to hexuronic acid (*m/z* 174) is weak for both disaccharides, above 5% of the parent ion. The tandem mass spectrum (D) of ΔDi-diS_d shows only a relevant fragment of *m/z* 457 derived from the loss of a sulphate group. As observed with the monosulphated disaccharides, differences can be observed between the tandem mass spectrum (E) obtained with the two disulphated disaccharides; in fact, with ΔDi-diS_e (E), CID induces not only the formation of a fragment by desulphation but also cleavages such as those observed with ΔDi-4S giving O-sulpho-N-acetylgalactosamine (*m/z* 299) and the same group with a fragment of the hexuronic acid (*m/z* 341). Finally, the tandem mass spectrum (F) from ΔDi-triS is characterized by products of progressive desulphation, *m/z* 537 and 457, and by the fragment, already observed with the other disaccharides, corresponding to the sulpho-N-acetylgalactosamine.

In Fig. 4 is shown, on the left, the TIC chromatogram obtained for the separation of oligosaccharides from digestion of HEP with Hepase; 50 μg of depolymerized HEP were injected. Four major peaks can be observed and the mass spectra acquired with suppression of the ion-pair reagent are reported on the right. The spectra are very complex, perhaps owing to the presence of molecular and fragmentation ions with different numbers of charges. The spectra are presented with an *m/z* range of 200–1000 because no relevant ions was observed at higher *m/z*, including the expected *m/z* values of singly charged ions. The spectra are very differ-

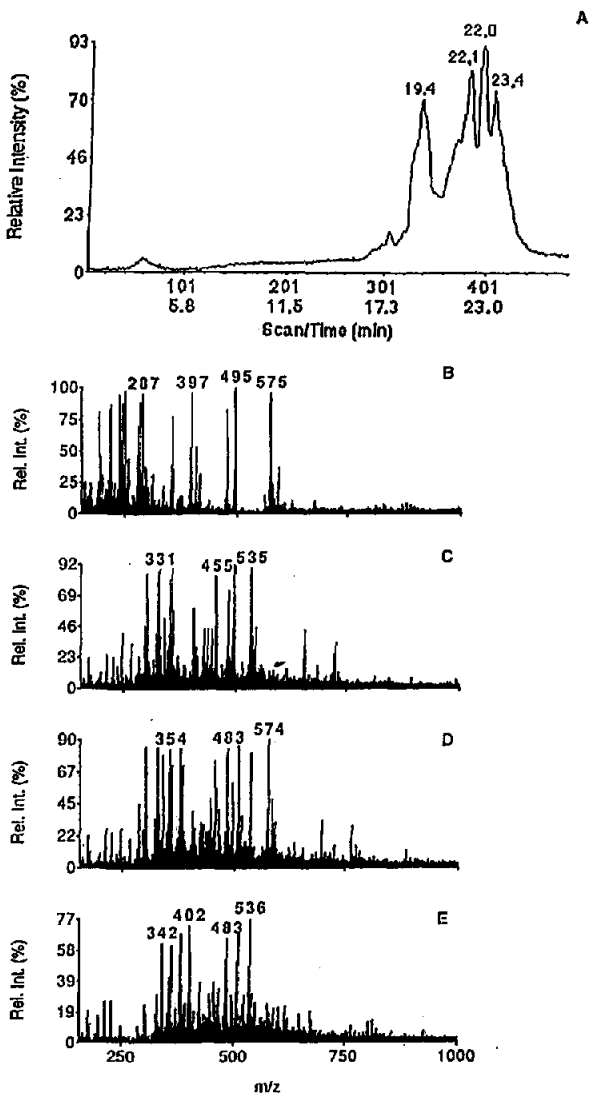

Fig. 4. (A) TIC chromatogram obtained during an RP-HPLC separation, with ion-pair reagent (TPA), of HEP (50 μg) degraded with Hepase. The mass spectra were recorded in the negative-ion mode scanning over the range m/z 100–1500. The mass spectra corresponding to the main peaks from the HPLC run in (A) are reported on the right; the ion-pair reagent was suppressed, from the mobile phase, with a membrane ion suppressor before the MS analysis. The spectra correspond to the peaks at (B) 19.4, (C) 22.1, (D) 22.8 and (E) 23.4 min.

ent in comparison with those obtained in the presence of ion-pair reagents, and the interpretation would be extremely complex and hazardous without the molecular mass assignments obtained from the spectra with TPA and d_{28}TPA.

In Fig. 5 are reported the mass spectra from these more relevant peaks in the presence of TPA (left) or d_{28}TPA (right). The spectral interpretations were obtained by comparing the spectra obtained with the different ion-pair reagents. A quick overview of the corresponding spectra, obtained with the different ion-pair reagents, shows that above all the ions are adducts; in fact, the m/z values of the relevant ions change completely when d_{28}TPA is used instead of TPA.

The spectra in the first row (A and E) were obtained from the peak eluting at 19.4 min. The ions, spectrum with TPA, of m/z 945 and 1130 are singly charged TPA adducts; in fact, the m/z difference between them is 185 (expected mass increase of an adduct with TPA); in the spectrum with d_{28}TPA a group of ions with similar characteristics can be easily identified (m/z 1001 and 1214) and the m/z difference is 213 (calculated mass increase of an adduct with d_{28}TPA). The difference in m/z between the ions of highest intensity in the spectra obtained with TPA or d_{28}TPA (945 − 1001) is 56, corresponding, in the case of singly charged ions, to the m/z difference between two molecules of d_{28}TPA and two molecules of TPA. Therefore, the ions of m/z 945 and 1001 are $(M + 2TPA - 3H)^-$ and $(M + 2d_{28}TPA - 3H)^-$, respectively. In these spectra two doubly charged ions of adducts with one or two molecules of ion-pair reagents are also relevant; m/z 380 and 472 with TPA and m/z 394 and 502 with d_{28}TPA. On the basis of the ion interpretations given to these spectra, the molecular mass of the molecule eluting in this peak is 576; this corresponds to the mass of a trisulphated disaccharide that is the main component of the oligosaccharides obtained by Hepase digestion of HEP [13] (see Fig. 6A).

Spectra B and F in Fig. 5 derive from the second relevant peak with a retention time of 22.1 min. In B a group of three intense ions with an m/z difference of 92–93, corresponding to doubly charged TPA adducts, can be observed (m/z 681, 773 and 866). As expected, in F three ions with an m/z difference of 106–107, deriving from doubly charged d_{28}TPA adducts, are present (m/z 710, 816 and 923); also ions with m/z differences corresponding to triply charged adducts with TPA and d_{28}TPA (very weak) can be

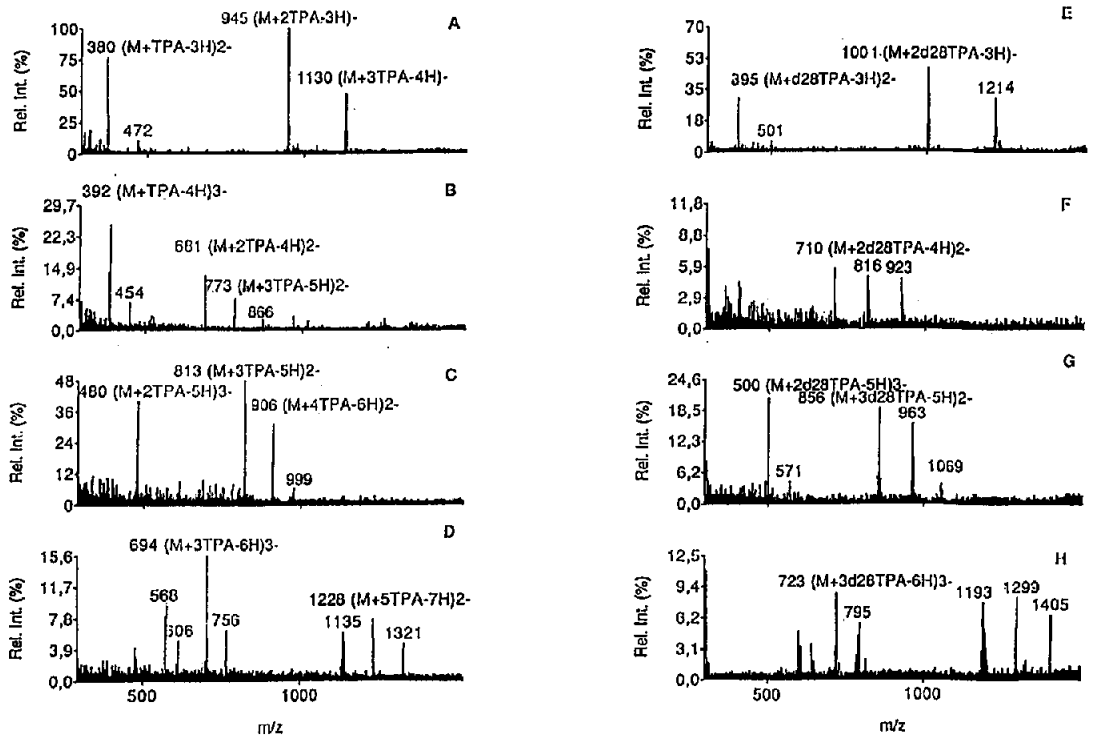

Fig. 5. Mass spectra on the left were recorded during an HPLC separation as in Fig. 4 but without using a membrane ion suppressor for TPA. The spectra on the right were acquired under similar conditions using d_{28}TPA instead of TPA. The spectra in the different rows correspond to those in Fig. 4, in the same order.

observed in both spectra. The *m/z* difference between the ions of *m/z* 710 and 681, multiplied according to the number of charges, corresponds to the mass difference between adducts with two TPA or two d_{28}TPA; in the same way it can be calculated that ions with *m/z* 816 and 773 are adducts with three TPA or three d_{28}TPA. The molecular mass of the oligosaccharide eluting in this peak, in accordance with the above-described spectral interpretation, is 994, corresponding to a tetrasulphated tetrasaccharide (see Fig. 6B) that may derive by enzymatic digestion of HEP [13].

Spectra C and G in Fig. 5 correspond to the peak eluting at 22.8 min. The most relevant ion with TPA, at *m/z* 813, is followed by an ion of *m/z* 906, with an *m/z* difference corresponding to a doubly charged TPA adduct. Two similar ions can be observed with d_{28}TPA (*m/z* 856 and 963) and the *m/z* difference corresponds to a doubly charged d_{28}TPA adduct. Considering the ions of *m/z* 813 (TPA) and 856 (d_{28}TPA), the *m/z* difference corresponds to three doubly charged ion-pair molecules, and therefore these ions are $(M + 3TPA - 5H)^{2-}$ and $(M + 3d_{28}TPA - 5H)^{2-}$, respectively. With this spectral interpretation the ions derive from an oligosaccharide with M_r 1074; a pentasulphated tetrasaccharide that may be derived from digestion of HEP with Hepase (see Fig. 6C) has exactly this mass [13]. In the same spectra ions corresponding to triply charged adducts are also present and the mass calculations performed from them give the same result.

Spectra D and H in Fig. 5 correspond to the peak at 23.4 min. Groups of ions derived from doubly or triply charged adducts are present in both spectra with the characteristic *m/z* differences. The *m/z* difference of the more intense doubly charged ions with TPA, *m/z* 1228, and with d_{28}TPA, *m/z* 1299, taking into account the number of charges, correspond to adducts with

five molecules of TPA or d_{28}TPA. The resulting molecular mass of this last-eluting oligosaccharide, calculated from the above interpretation, is 1534; this is characteristic of a hexasulphated hexasaccharide by enzymatic degradation with HEP (see Fig. 6D) [13]. The same result was reached on evaluating the other relevant ions in these spectra.

The proposed structures of the oligosaccharides with molecular masses corresponding to those calculated from the major peaks observed in these samples from enzymatic digestion of HEP are presented in Fig. 6. The positions of sulphation reported in these formulae are only indicative; in fact, from the present MS data, no definitive rule can be obtained.

The CID spectra obtained from some relevant ions during an HPLC separation of the same HEP enzymatic digest, with TPA suppression, are presented in Fig. 7. In spectrum A, acquired during the elution of the peak at 19.4 min corresponding to a trisulphated disaccharide, daughter ions were obtained by fragmentation of a parent of m/z 576 corresponding to the $(M-H)^-$ of this molecule. The spectrum is characterized by three relevant ions, including the parent ion, with m/z differences of 80, corresponding to the loss of sulphate groups.

Spectrum B was obtained under the same chromatographic conditions but from parent ions of m/z 287, therefore corresponding to $(M-2H)^{2-}$ ions of the disaccharide analysed in the first spectrum. Apart from the parent ion, the most relevant daughter ion has m/z 247, corresponding to a doubly charged ion of the molecule without a sulphate group.

Spectrum C was recorded at the HPLC retention time (22.8 min) corresponding to the pentasulphated tetrasaccharide from the parent ion of m/z 536, presumably corresponding to the $(M-2H)^{2-}$ ion of this oligosaccharide. The spectrum is characterized by four relevant ions, including the parent ion, with an m/z difference of 40 between them and therefore corresponding to the sequential loss of sulphate groups from the molecule, which always remains doubly charged. Another weak ion is present in this spectrum with m/z 257; presumably it derives from a

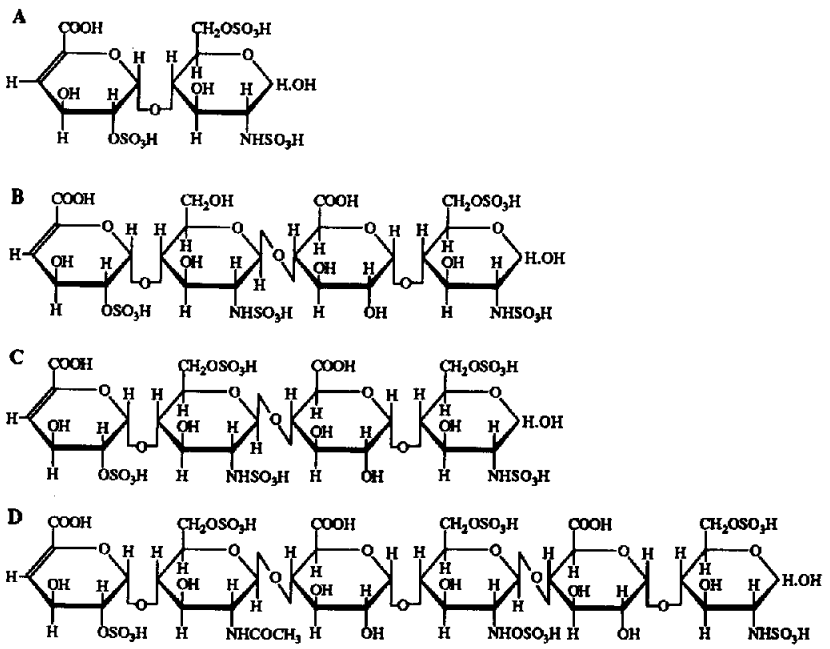

Fig. 6. Formulae of the di- and oligosaccharides that may be derived from HEP degradation with Hepase whose molecular masses correspond to those determined in the more relevant peaks of the separation in Fig. 4. Molecular masses: A = 577; B = 994; C = 1074; D = 1534.

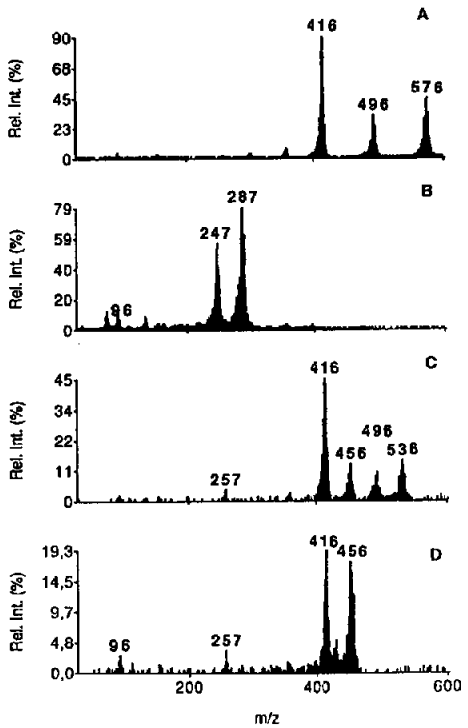

Fig. 7. Tandem mass spectra recorded during an HPLC separation as in Fig. 3 from some relevant ions. Two spectra were obtained from molecular ions of the trisulphated disaccharide (A) singly or (B) doubly charged. (C) Results for the $(M-2H)^{2-}$ ions of the pentasulphated tetrasaccharide and (D) for an ion of m/z 455 derived by fragmentation of this molecule.

singly charged fragment with the structure of a monosulphated glucosamine.

Spectrum D was acquired during the elution of the pentasulphated tetrasaccharide from a parent ion with m/z, 456, which seems to correspond to a fragment of the oligosaccharide with unknown charge. This spectrum is characterized by an intense parent ion followed by a second peak shifted by m/z 40 and therefore corresponding to the loss of a sulphate group from a doubly charged ion keeping the two charges. Two other weak ions are present with m/z 96, presumably corresponding to a sulphate group with the ester bond, and, already observed in the other spectra, m/z 257. From these findings the molecular mass of the fragment giving the doubly charged ion of m/z 455 is 910, corresponding to the calculated molecular mass of a tetrasaccharide from HEP digestion with three sulphate groups that derives from the fragmentation of the pentasulphated tetrasaccharide in the front-collision region.

The CID spectra obtained from these ions are in agreement with the proposed interpretations in Fig. 6.

DISCUSSION

The chromatographic separations were performed on a reversed-phase (C_6) column instead of a more conventional ion-exchange column to avoid the use of the non-volatile buffers normally used with such columns. The counter ion, TPA, as already adopted by Linhardt *et al.* [14], was buffered for use with a silica-based stationary phase. A pH below 4.0 was chosen in order to minimize the chemical reactivity of TPA on the column and wetted parts of the HPLC system. Attempts to use a more usual stationary phase such as C_{18} lead to unacceptably high retention times especially for the highly sulphated HEP fragments. In this study, to allow the use of a membrane ion suppressor that specifically removes the TPA counter ions from the mobile phase, we performed the separation on an analytical column (4.6 mm I.D.); in fact, at present, a membrane ion suppressor for quaternary ammonium ion-pair reagents optimized with the low flow-rate of microbore columns is not commercially available. Comparable separations (data not reported) have been obtained using analytical or microbore columns packed with the same stationary phase (Spherisorb Hexyl silica, 5 μm; Phase Separations).

The results obtained with purified disaccharides by enzymatic digestion of CS show that in the presence of ion-pair reagents their adducts with the whole molecules form the predominant ions; only with monosulphated disaccharides could molecular ions without TPA be observed. Interestingly, spontaneous fragmentations were not observed in the presence of the ion-pair reagent whereas without an ion-pair reagent fragmentations are evident mainly through the loss of the sulphate groups but also through the cleavage of the glycosidic bonds. Similar fragmentations have already been observed in studies performed on similar oligosaccharides using FAB [6,7], and therefore the use of ion-

pair reagents seems very important in facilitating the determination of the molecular masses of these oligosaccharides. The use of d_{28}TPA enabled us to confirm the spectral interpretations. Sodium adducts were frequently observed especially in the absence of an ion-pair reagent. Reported CID spectra were obtained from deprotonated molecular ions from samples analysed without an ion-pair reagent because no fragmentation could be obtained from adduct ions and also CID mass spectra from deprotonated molecular ions obtained in the presence of an ion-pair reagent were very weak. With molecules containing two or more sulphate moieties the most relevant daughter ions were derived from the loss of the sulphate groups; fragments corresponding to each hexose of the parent molecules can be observed with disaccharides containing one or no sulphate group. Generally the fragmentations observed under MS–MS conditions were similar to those observed in mass spectra acquired from samples in the absence of an ion-pair reagent. The daughter-ion spectra of mono- and disulphated disaccharides (ΔDi-4S, ΔDi-6S, ΔDi-diS_d and ΔDi-diS_e) showed interesting specific fragmentation patterns, depending on the position of the ester bonds, suggesting a possible role of MS–MS in discriminating positional isomers.

The results obtained with HEP were more complex to rationalize. Oligosaccharides from HEP have a very strong interaction with ion-pair reagents and therefore molecular ions cannot be observed when analysed under these conditions; moreover, in the absence of TPA or d_{28}TPA relevant fragmentations occur, giving spectra of complex interpretation. Only the combination of results obtained with TPA and d_{28}TPA permitted an unambiguous identification of the ions in these spectra and therefore the molecular masses of oligosaccharides can be determined accurately.

CID mass spectra of ions from oligosaccharides of HEP confirmed the preferential fragmentation at the ester bonds of the sulphate groups in comparison with the glycosidic bonds; in this study a specific evaluation of N–sulphate bond stability was not performed. Similar results were previously obtained on analysing sulphated oligosaccharides with FAB ionization. The use of MS–MS with these samples, even if did not give detailed information on sequence, was useful for improving the interpretation of some ions (*e.g.*, number of charges, presence of sulphate groups) obtained with the suppression of counter ions in the mobile phase, necessary to have effective fragmentation. The development of devices to remove quaternary ammonium counter ions from the mobile phase suitable for microbore HPLC will improve this technique; it is noteworthy that similar ion membrane suppressors are already available for applications to inorganic cations or anions (Dionex).

The sensitivity of the technique is interesting: 50 μg were injected to obtain the reported spectra and therefore, considering a splitting ratio of >1:20, 2.5 μg injected into a microbore column are sufficient to obtain clear scan spectra. In a previous study [9] we showed that, using single-ion monitoring, amounts of each oligosaccharide as low as 50 ng can be detected using a 1.0 mm I.D. column.

The use of the ionspray technique is one of the main reasons for the interesting results achieved in this study. Indeed, with this ionization method, no sample derivatization was required and on-line interfacing to an effective separative technique was easily achieved. The introduction of a counter-ion reagent, to obtain a good HPLC separation, was another unexpected and relevant improvement. In fact, highly sulphated oligosaccharides could be determined in complex mixtures, overcoming the previously observed problems of fragmentation and separation [7,10].

After this work using well known molecules, promising results have been already obtained by applying this HPLC–MS technique, in combination with enzymatic and chemical degradation, to characterize sulphated GAGs extracted from biological samples. These findings suggest a relevant role of this method in a "combined approach" [15] to elucidate the sequence of unknown GAGs.

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