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Determination of constituents of sulphated proteoglycans using a methanolysis procedure and gas chromatography/mass spectrometry of heptafluorobutyrate derivatives

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A major impediment in the analysis of glycosaminoglycans is the difficulty to cleave quantitatively the glycosidic bonds because of the stabilisation of glycosidic bonds and of the relative instability of the liberated constituents. This manuscript describes a modified procedure of methanolysis in the presence of barium acetate, reducing the destruction of uronic acids and increasing the cleavage yield. The reaction products could be identified and analysed quantitatively by GC and GC/MS of the heptafluorobutyrate derivatives of O-methyl glycosides of monosaccharides (for keratan sulphate and chondroitin sulphate B), or as a mixture of O-methyl glycosides of monosaccharides and of disaccharides (for the other sulphated glycosaminoglycans). Quantitative molar ratio between the different monosaccharide constituents (including the linkage region constituents) could be obtained, even when proteoglycans also contain classical N-glycans or O-glycans.

Keywords: **GLC, GC/MS, mass spectrometry, monosaccharide, GAGs**

Abbreviations: **CSA, CSB, CSC, chondroitin sulphate A, B, and C respectively; Xyl, xylose; Xyl-ol, xylitol; Gal, galactose; Man, mannose; GlcNAc, N-acetyl-glucosamine; GalNAc, N-acetyl-galactosamine; GlcN, glucosamine; GlcA, glucuronic acid; GalA, galacturonic acid; IdoA, iduronic acid; Lys, lysine. HFB, heptafluorobutyrate; HFBA, heptafluorobutyric acid; HFBAA, heptafluorobutyric anhydride; EI, electron impact; CI, chemical ionization; MS, mass spectrometry; TIC, total ion count; FID, flame ionization detector; RMR, relative molar response**

Introduction

There is an increasing interest to the carbohydrate moieties of proteoglycans since these compounds play essential roles in cell adhesion as constituents of the extracellular matrix, in cell signalling, and as ligands of growth factors [1–8]. As a first step of analysis of proteoglycans, a monosaccharide composition could provide an essential information on the nature of the oligosaccharide chains. Because of its easy coupling with mass spectrometry, gas chromatography of the monosaccharide constituents is a method of choice when working with minute samples of unknown composition [9]. However, the study of the glycan chains of the proteoglycans was difficult because the stability of the glycosidic bonds of glycosaminoglycans (GAGs), due essentially to the stabilising effect of the carboxyl group in position 6 of uronic acids, and frequently to the shielding effect of protonated free amino groups of hexosamines, on glycosidic bonds in acidic conditions. Circumventing this stability by the initial methyl-esterification followed by reduction of the methyl ester group of uronic acids [10] was possible but induces low recovery on macromolecules. Moreover, problems of desalting of minute samples impeded the use of this technique. As for glycoproteins or glycolipids [11], optimal conditions of analysis can be obtained when the initial sample remains in the same tube until the injection in a GC or GC/MS apparatus.

Different procedures were proposed in order to cleave these bonds: either 1 M sulphuric acid at 100°C during 2 h [12], or Dowex 50 H^+ form during 24 h at 100°C [13], or 90% aqueous formic acid for various periods of time at 100°C [14] or 1 M HCl for 4 h at 100°C [15]. The classical method of cleavage of glycosidic bonds, methanolysis [16–18] was

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poorly effective for cleaving some uronic acid-containing GAGs (heparin, heparan sulphate, chondroitin sulphate A and C). The use of higher HCl concentration, up to 4 M gaseous HCl dissolved in anhydrous methanol, was suggested to be more efficient in cleaving these bonds [15]. However, in all these acidic conditions (hydrolysis or methanolysis) significant degradations of the derivatives of uronic acids, of pentoses, of deoxy-hexoses, of galactose and of sialic acid were observed at various degrees, in such a way that a true molar ratio between uronic acids and hexosamines in most compounds (or between Gal and glucosamine for keratan sulfate) could not be quantitatively reached.

We previously observed by quantitative GC and using GC/MS that free glucuronic and galacturonic acids were stable for 20 h at 80°C in 0.5 M anhydrous methanolic HCl [11], forming the O-methyl glycosides of their methyl esters without apparent degradation. Since these compounds were not degraded in these conditions, we made the hypothesis that the degradation of uronic acids or of Gal observed for sulphated glycosaminoglycans was, at least in part, due to the release of sulphuric acid from these compounds. In order to prevent these artefacts, we decided to perform the methanolysis step in the presence of barium salts, known to provoke a quantitative neutralisation of sulphate as a stable precipitate of barium sulphate [19]. In fact, the presence of barium salt not only prevented the degradation of uronic acids and of other monosaccharides, but also significantly increased the yield of cleavage of glycosidic bonds of GAGs. When the reaction products are analysed by GC as heptafluorobutyrate derivatives [11], all constituents of GAGs (except iduronic acid, which is partially degraded) can be quantitatively recovered as their O-methyl glycosides or as O-methyl glycosides of disaccharides. This method associated with mass spectrometry in the electron impact mode provides a qualitative and quantitative information on the composition of sulphated GAGs and can be applied to proteoglycans having other types of glycans.

Material and methods

Chemicals

Standard glycosaminoglycans chondroitin sulphate A (sodium salt from whale cartilage; 70% purity; contaminated with CSC), chondroitin sulphate B (sodium salt from porcine skin; 85% purity; contaminated with CSA and CSC) and chondroitin sulphate C (sodium salt from shark cartilage; 90% purity; contaminated with CSA), heparin (sodium salt from porcine intestinal mucosa; grade II) and keratan sulphate (sodium salt from bovine cornea) and monosaccharides were from Sigma Chem. Co (St Louis, Mo, USA). HPTLC Silicagel 60 glass plates (20 × 10 cm) were from Merck (Darmstadt, Germany). Barium acetate was from Merck (Darmstadt, Germany) and heptafluorobutyric anhydride (HFBAA) was from Fluka (Buchs, Switzerland). It should be stressed that some batches of HFBAA, provided into 1 ml Teflon-lined screw cap vessels were not tightly closed and the cap was in part dissolved in HFBAA. This induced enormous peaks of HFB derivatives of phtalates.

Methanolysis and acylation with heptafluorobutyric anhydride

Samples of standard glycosaminoglycans (0.1–10 μg) in heavy walled Pyrex tubes with a Teflon lined screw cap were supplemented or not with the desired quantity of a water solution of the internal standard lysine, then lyophilised. The samples were suspended in 100–250 μl of methanolic 0.5 M HCl [11] supplemented with solid barium acetate (13.5 mM final concentration). Samples were heated for 20 h at 80°C and cooled. The samples were evaporated under a stream of nitrogen in a ventilated hood. The dry sample was taken up into 200 μl of acetonitrile (HPLC grade), followed by the addition of 25 μl of heptafluorobutyric anhydride (HFBAA). After heating for 15 min at 150°C in a sand bath, the samples were cooled, evaporated to dryness in a ventilated hood under a stream of nitrogen, then solubilised in the desired volume of acetonitrile kept dry on calcinated calcium chloride [11] and injected in the Ross injector of the GC or GC/MS apparatus.

Gas chromatography and mass spectrometry

For analytical purposes, analyses were performed on a Shimadzu GC-14A gas chromatograph equipped with a Ross injector and a 25 m long capillary column (25QC3/BP1; 0.5 μm film phase; SGE France SARL; Villeneuve St Georges (France)). Injector and flame ionisation detector temperatures were 260°C and the temperature program was 1.2°C/min between 100 and 140°C, followed by 4°C/min from 140°C to 240°C then maintaining this temperature for 10 min [11]. The carrier gas (helium) pressure was 0.8 bar. The second part of the temperature program is necessary for the separation of disaccharides released from incompletely cleaved GAGs.

For GC/MS analysis, the GLC separation was performed on a Carlo Erba GC 8000 gas chromatograph equipped with a 60 m × 0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 μm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 260°C and the samples were analysed using the following temperature program: 90°C for 3 min then 5°C/min until 260°C, followed by 10 min at 260°C. The column was coupled to a Finnigan Automass II mass spectrometer or, for mass larger than 1000 amu, to a Riber 10-10H mass spectrometer (mass detection limit 2000 amu). The analyses were performed routinely in the electron impact mode (ionisation energy 70 eV; source temperature 150°C) or, for measurement of the mass of the derivatives, in the chemical ionisation mode in the presence of ammonia (ionisation energy 150 eV; source temperature of 100°C). In the chemical ionisation mode, the detection was performed for positive ions or for negative ions, in separated experiments, the latter allowing the quite specific

detection of heptafluorobutyrate derivatives with a higher sensitivity.

HPTLC analysis of the effect of methanolysis on the different GAGs

After methanolysis, starting from mg amount of the different GAGs, the different compounds were analysed by thin-layer chromatography. Samples were grossly neutralised with KOH, concentrated under vacuum, and analysed using TLC on HPTLC plates in the solvent system: methyl acetate-chloroform-methanol-*n*-propanol-0.25% aqueous KCl solution: 25/20/20/20/17 by volume [20]. After chromatography, the plates were sprayed with the orcinol/sulphuric acid reagent and revealed at high temperature on electric plates.

Results and discussion

In a previous study [11], we reported a new method for the analysis of constituent monosaccharides from glycoproteins and glycolipids as the heptafluorobutyrate derivatives of the O-methyl glycosides formed after methanolysis. The acylation with heptafluorobutyric anhydride blocks in a single step all amino and hydroxyl groups (except the semi-acetalic OH group), forming derivatives poorly interacting with the classical GC liquid phases and, consequently, separated at relatively low temperature on capillary GC columns [11]. Since this technique allowed the quantitative determination of all monosaccharide constituents of glycoproteins and of all constituents of glycolipids in a single experiment [11], we tried to extend this method to the constituents of proteoglycans.

In fact, using the classical methanolysis conditions (0.5 anhydrous methanolic HCl during 20 h at 80°C; [11]), it was observed that standard glucuronic and galacturonic acids formed stable O-methyl glycosides of their methyl esters which can be quantitatively recovered (4 different peaks for each uronic acid) and analysed by GLC after derivatisation with heptafluorobutyric anhydride. Studies by GC/MS in the chemical ionisation (Cl; NH3 gas) and either positive or negative ion detection indicated that the four peaks of glucuronic acid and the four peaks of galacturonic acid gave the same pseudo-molecular ion at $[M + NH4]^+ = 828$ in the Cl positive mode and $[M]^{\cdot -} = 810$ and $[M - HF]^{\cdot -} = 790$ in the Cl negative mode (Table 1). This demonstrated that all of them corresponded to O-methyl glycosides of their methyl esters and not to lactones. In fact, EI/MS studies of the derivatives of GlcA and GalA indicated the presence of two furanic forms and two pyranic forms for glucuronic acid (see later).

Our initial trials for cleaving the glycosidic bonds of GAGs using the standard methanolysis conditions showed that the cleavage was largely incomplete (1–50% depending on the compounds), except for keratan sulphate (complete cleavage) and for chondroitin sulphate B (80% cleavage). For compounds showing an incomplete cleavage, specific peaks with relatively high retention times (Rt) and eluted at temperatures compatible for the stability of the capillary column were detected. Preliminary studies suggested that these compounds could be O-methyl glycosides of disaccharides.

Although the monosaccharide constituents of the GAGs could be identified using GC/MS, it was observed that the

Table 1. Retention times, percentage of isomers and reporter ions obtained by mass spectrometry

Compound	*Rt*	*% of total*	*EI/MS*	*CI^+/MS*	*CI^-/MS*
Xyl-ol	12.06	100.00	919-679	1150	1132
Xyl	15.20	66.48	479-265	770	752
Xyl	15.54	33.52	479-265	770	752
Gal	19.21	19.75	519-525*	996	978-958
Gal	21.90	6.18	519-525*	996	978-958
Gal	24.30	49.38	519	996	978-958
Gal	26.56	24.69	519	996	978-958
Man	25.20	93.05	519-509	996	978-958
Man	28.35	6.95	519	996	978-958
GlcNAc	34.28	6.67	276	995	977
GlcNAc	35.28	83.83	276	995	977
GlcNAc	36.33	9.50	276	995	977
GlcN**	20.17	48.90	476	981	963
GlcN**	22.29	51.10	476	981	963
GalNAc	28.45	18.13	276-524*	995	977
GalNAc	31.20	24.30	276-524*	995	977
GalNAc	34.80	54.59	276	995	977
GalNAc	36.53	4.98	276	995	977
GlcA	24.44	29.63	537-525*	828	810-790
GlcA	26.52	8.32	537-525*	828	810-790
GlcA	31.01	9.00	537-597	828	810-790
GlcA	31.42	53.05	537-597	828	810-790
IdoA	19.80	9.83	478	600	582
IdoA	21.30	43.04	478	600	582
IdoA	23.96	13.20	537-525*	828	810-790
IdoA	27.17	5.26	537-597	828	810-790
IdoA	27.92	17.55	537-597-525*	828	810-790
IdoA	31.67	16.42	537	828	810-790
IS (Lys)	38.65	100.0	520-280	570	552
Di-Hep	49.95		947-732		
Di-Hep	51.74		947-732		
Di-CSA-C	51.53		779		
Di-CSA-C	51.81		779		
Di-CSA-C	53.42		779		
Di-CSA-C	53.90		779		

Rt are indicated for the temperature program used for GC. *corresponds to the E1 ions specific for the furanic forms of the different compounds. **These peaks correspond to the HFB derivatives of glucosamine (not the O-methyl glycoside of glucosamine). As previously described (14), it is the major product of the cleavage of the N-glycosidic bond. The proportion of the two anomers is varying with time in acetonitrile. IS = internal standard lysine. Di-Hep, Di-CSA-C are the disaccharides characteristic of heparin and of CSA and CSC, respectively. No disaccharides are found for KS and for CSB. Xyl-OH = xylitol (obtained when GAGs are obtained by reductive β-elimination).

ratio between GlcA to GalN in chondroitin sulphate A (CSA) and the ratio between Gal and GlcN in keratan sulphate (KS) was much lower than expected (about 0.5/1 instead of 1/1). This suggested that GlcA in CSA and Gal in KS were partially destroyed. Since this destruction was not observed when standard GlcA and Gal were submitted to methanolysis, we made the hypothesis that the destruction was due to the presence of significant amounts of sulphuric acid, resulting from the cleavage of sulphated groups.

Methanolysis in the presence of barium acetate

In order to test this hypothesis, equimolar mixtures of Gal, GlcA and GlcNAc supplemented or not with Na_2SO_4 were prepared. After methanolysis of the samples and formation of HFB derivatives, GC analyses indicated that the yield of Gal and GlcA relative to GlcNAc was reduced by about 50% in the sample supplemented with Na_2SO_4 relative to the other, whereas the quantity of hexosamine was unchanged. This indicated a strong destructive effect of sulphuric acid despite its low concentration in the methanolysis reagent (10^{-5}–10^{-3} M in the range of concentration tested). Gal was destroyed to 53%, its pyranic forms of Gal being preferentially destroyed. A small proportion of Gal (3%) was recovered as free Gal, suggesting that sulphuric acid impeded the formation of O-methyl glycosides. GlcA was destroyed at the same level as Gal and was recovered as the four anomers of the O-methyl glycosides of its methyl esters and as two peaks of lactone (M = 582). The quantity of lactone was equivalent to that of GlcA, in contrast with results obtained when methanolysis performed in the absence of sulphate.

Consequently, these experiments demonstrated that the small amount of sulphuric acid liberated from sulphated GAGs during methanolysis (between 10^{-5} to 10^{-3} M in our experiments) was responsible for the intense degradation of the most labile monosaccharides, i.e. Gal and uronic acids and that the level of degradation was not dependent upon the sulphuric acid concentration within the range mentioned above.

We then made the hypothesis that one way to circumvent this destructive effect was to precipitate the liberated sulphuric acid as soon as it is formed using barium salts, a method for the quantitative determination of sulphate ions. Because of its easy solubility in the methanolysis reagent (0.5 M HCl in anhydrous methanol), barium acetate was preferred to barium chloride. Its final concentration in the methanolysis reagent (13.5 mM) was chosen in order to have a minimal 3 fold molar excess of barium ions relative to sulphate present in the more sulphated GAG (i.e. heparin) when present at the concentration of 1 mg/ml in the methanolysis reagent When the mixtures mentioned above were submitted to methanolysis in the presence of barium ions, the correct yield of Gal and GlcA relative to GlcNAc was restored, as did the normal proportions of the isomers of Gal. The formation of lactones for GlcA was avoided. For the mixtures depleted from Na_2SO_4, the results were identical when methanolysis was performed both with and without barium acetate. Consequently, this procedure of cleavage was systematically applied to glycosaminoglycans as described below.

Composition of keratan sulphate

The methanolysis products of keratan sulphate (Fig. 1a) showed predominant peaks corresponding to the isomers of the O-methyl glycosides of Gal and GlcN. Using electron impact (EI) mass spectrometry, the A3 ion at 519 (and absence of the ion at 509) identified all O-methyl glycosides of Gal, the two furanic forms being identified by the additional E1 ion at 525 (Fig. 2a). The O-methyl-glycosides of GlcN (the N-acetyl group of N-acetylated compounds is removed during methanolysis, and transformed into an N-HFB derivative) were revealed by the presence of the intense specific ions at 490 and 276 (Fig. 2b). None of the GlcN peaks corresponded to furanic forms (revealed by a specific E1 ion at 524 and characteristic of two furanic forms of GalNAc (Table 1)). It should be emphasised that the specific ion corresponding to the HFB derivative of free GlcN (not the O-methyl glycoside) at M = 476 [11] was less than 4% that of the O-methyl glycoside of GlcN, indicating that the glycosidic bond was essentially cleaved before the de-acetylation of GlcNAc. Indeed, due to the shielding effect of the $NH3^+$ group, the O-methyl glycoside of GlcN can not be formed [11,21–22]. Mannose was also detected in this sample and separated from the other peaks. The anomers of the HFB derivatives of the O-methyl glycosides of Man were characterised by the higher abundance of the ion at 509 relative to the ion at 519; Figure 2c.

Calculations of the quantity of the liberated compounds relative to the internal standard lysine indicated a complete cleavage of the glycosidic bonds. This point was also verified by the absence of high molecular weight compounds (dimers) on the GC or TIC chromatograms, in agreement with HPTLC analysis of the reaction products (not shown). Calculations of the degree of polymerisation of the keratan sulphate based on the assumption that is was associated to a bi-antennary N-glycan (three mannose residues; as expected from this standard keratan sulphate) gave between 13 and 14 dimers, i.e. a mass of 3100–3300 for each sulphated chain. The validity of this determination was reinforced by the identification of minor peaks of GlcN (not the O-methyl glycoside) as the product of the cleavage of the glycosylamine of GlcNAc involved in the N-glycosidic bond [22]. Indeed this compound represented between 3–4% of the O-linked GlcNAc derivatives, i.e. one residue for 28–30 O-linked GlcNAc residues.

The complete cleavage of the keratan sulphate chain was expected since these compounds did not contain uronic acids (know to stabilise the O-glycosidic bonds at its reducing end) and since the glycosidic bonds of these compounds behave as in poly-N-acetyl-lactosaminic chains. However, in contrast with methanolysis in the absence of barium acetate, Gal was not destroyed to a significant level as evidenced by a molar

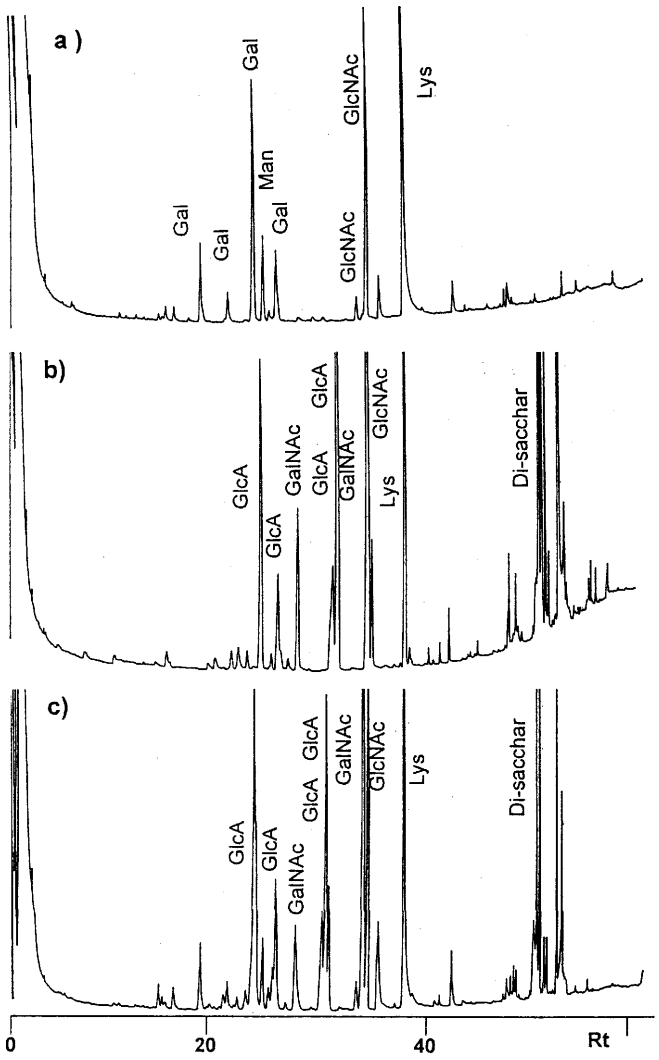

Figure 1. FID chromatograms of the heptafluorobutyrate derivatives of the methanolysis products of sulphated glycosaminoglycans. a) Profile obtained from keratan sulphate. Note that the predominant peaks corresponded to Gal and GlcNAc. The absence of Xyl and the presence of Man indicated that the KS chains were part of a N-glycan. Note the absence of peaks with high Rt. b) and c) Profiles obtained for chondroitin sulphate A and C respectively. Note that major peaks corresponded to GlcA and GalNAc, the presence of GlcNAc being representative of a contamination of the samples by heparan sulphate-like substances. Note also the presence of four peaks with high Rt corresponding to disaccharides. Lys = lysine used as an internal standard. Rt are expressed in min.

ratio of the O-methyl glycoside of Gal to that of GlcN of 1/1 (1.0008/1.000 in repetitive GC analyses, using the relative molar response (RMR) factors determined previously [11]).

Composition of chondroitin sulphate A and C

As shown in Figure 1b, chondroitin sulphate A gave major peaks corresponding to the O-methyl glycosides of the methyl ester of glucuronic acid and to the O-methyl glycosides of galactosamine, in the area corresponding to monosaccharides. Based on Cl data (see above), the four peaks derived from GlcA corresponded to O-methyl glycosides (two pyranic and two furanic forms (Fig. 2d and 2e)) and not to lactones of GlcA. The specific search by GC/MS for ions derived from lactones as well as the identification of all peaks in the TIC chromatogram indicated the absence of lactones in a ratio higher than 1% relative to the sum of the other compounds. The O-methyl glycosides of galactosamine were identified by their Rt and the presence of the ions of hexosamines at 490 and 276, the furanic forms of GalN being identified by the specific EI ion at 524 (Fig. 2f). However, other peaks were observed. One with a Rt slightly higher than that of GalN was identified as the O-methyl glycoside of GlcN (see above the data for keratan sulphate). It represented 7% of the GalN suggesting a contamination by heparan sulphate like substances. In fact, careful analysis by GC/MS indicated the presence of the ions characteristic of iduronic acid $[M]^+ = 537$ and iduronic acid lactones $[M]^+ = 478$ (see below).

Two important peaks with higher Rt were detected (Fig. 1b), presenting the characteristic ions of both hexosamines and GlcA by mass spectrometry in the EI mode. Based on their fragmentation patterns (Fig. 3a), the major peaks corresponded to the two anomers of the O-methyl glycosides of dimers. The presence of the A1 ion at 779 indicated that the GlcA residue was in the non-reducing position and, consequently, GlcN in the reducing position.

Quantitative analysis by comparison with the internal standard lysine as well as HPTLC analysis (not shown) indicated that the methanolysis of CSA resulted in a mixture of O-methyl glycosides of the monosaccharide constituents and of dimers. Based on the proportions of the different peaks on the GC chromatograms, it could be calculated that the CSA was cleaved into monosaccharides with a yield of 53.32%, the remaining material being quantitatively recovered on the GLC chromatograms as dimers. The calculated ratio of GlcA relative to GalN was 0.946, indicating again that GlcA was not significantly destroyed during the methanolysis step.

As shown in Figure 1c, chondroitin sulphate C presented a complex GLC profile. The characteristic peaks of the derivatives of the O-methyl glycosides of GlcA and of GalN were the major peaks. The molecular ratio between these compounds (1.0006 GlcA for 1.000 GalN) indicated again that GlcA was not destroyed during methanolysis. As for chondroitin sulphate A, these compounds were identified by their specific ions in the EI/MS mode. However, CSC showed an unexpected high amount of O-methyl glycosides of GlcN (15.30% of the areas of the GalN peaks). As already described for CSA, the characteristic ions of the products of IdoA allowed identifying the extraneous peaks observed on the chromatograph (see below). High Rt compounds were also detected, having exactly the same Rt and the same characteristic ions as those derived from CSA (Fig. 3a). They

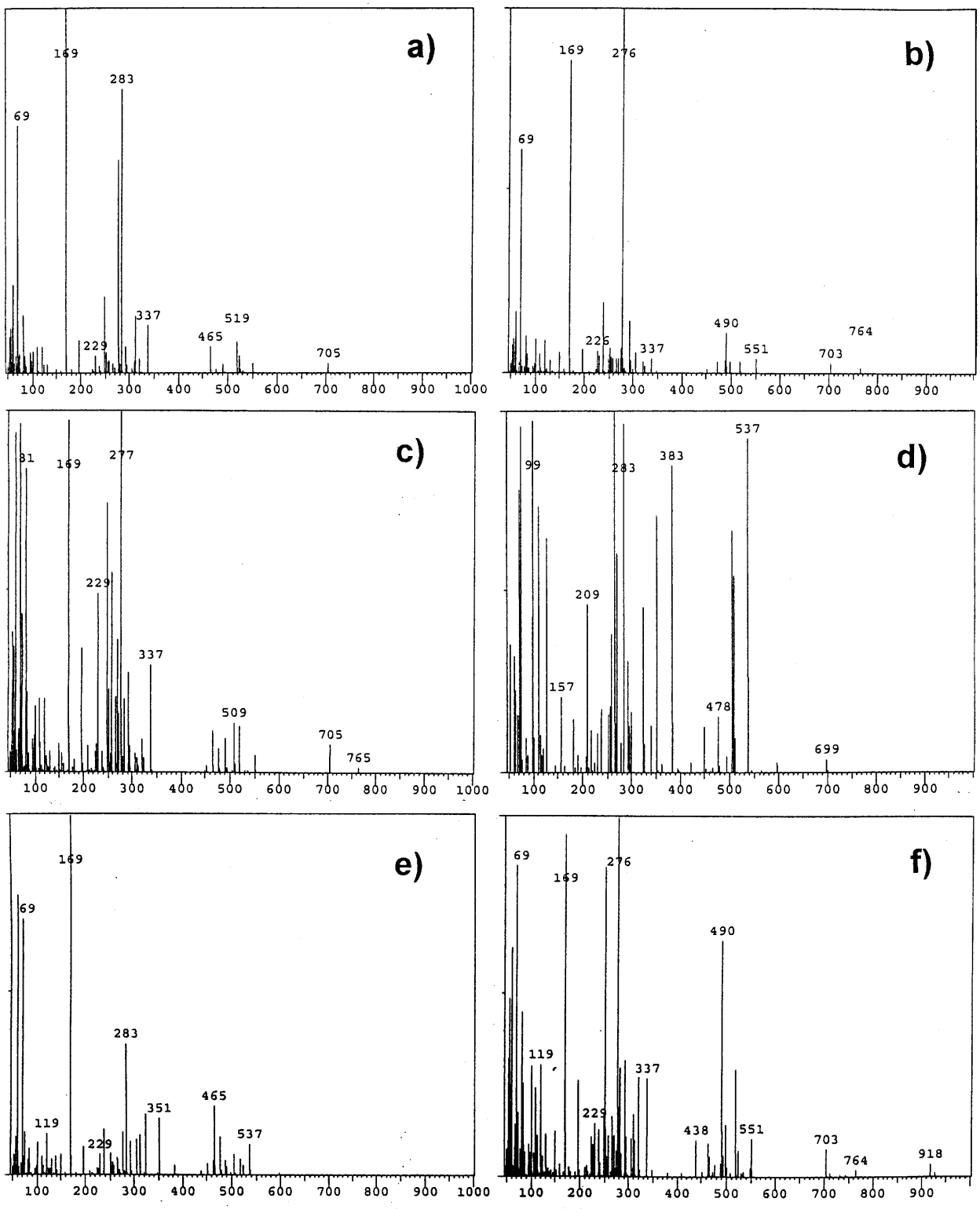

Figure 2. Mass spectra in the electron impact (EI) mode of the heptafluorobutyrate derivatives O-methyl glycosides of: a) = furanose forms of Gal; b) = pyranose forms of GlcN; c) = pyranose forms of Man; d) = pyranose forms of the GlcA methyl ester; e) = furanose forms of GlcA; f) = furanose forms of GalN. The spectra of pyranose or furanose forms of the same O-methyl glycosides are not significantly different (weak differences in intensities of the same fragments). Note that Man and Gal could be easily distinguished since the ion at 509 of Man anomers shown in c) was almost not detectable in Gal anomers (a). Glc, a frequent contaminant, gave an intermediate pattern. Furanic forms of GlcA and Gal were characterised by an ion at 525, absent from pyranic forms; furanic forms of hexosamines were characterised by the ion at 524 absent in pyranic forms. Note in d) and e) the intense ion at 537 characteristic of GlcA (and of all uronic acids).

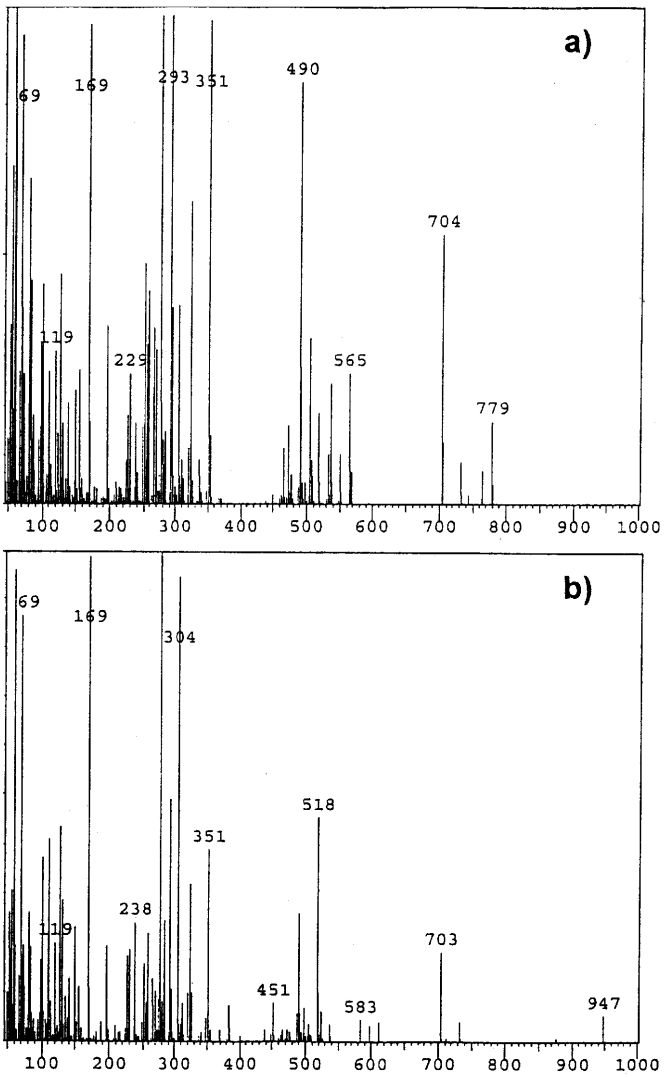

Figure 3. Mass spectra in the EI mode of the dimers present a) in chondroitin sulphate A and C and b) in heparin or heparan sulphates. Note the intense high mass ions at 779 and 947 characteristic of these different dimers remaining after methanolysis of the different compounds. Note that both types of dimers have in common most of the characteristic ions of hexuronic acid and hexosamine derivatives.

corresponded to the same dimers as those derived from CSA, an expected result since these compounds should give the same dimers after O-de-sulphatation. However, the proportion of dimers relative to the monosaccharides was much lower than for CSA, indicating that the cleavage was more pronounced for CSC than for CSA (80.92% instead of 53.32%, respectively).

Chondroitin sulphate B

As shown in Figure 4a and 4c, the methanolysis products of chondroitin sulphate B showed an almost complete absence of characteristic peaks of disaccharides, concomitantly with the presence of numerous peaks in the area of monosaccharides. The analysis of this GAG presents difficulties because of the instability of iduronic acid (IdoA) derivatives, resulting in a multiplicity of peaks. This complexity was also increased by the presence of the peaks characteristic of GlcA. In fact, although IdoA was partially destroyed during methanolysis, the use of heptafluorobutyrate derivatives allowed identifying the products using GC/MS analysis. Two peaks of compounds formed during the methanolysis of CSB were lactones of iduronic acid identified by their mass in the CI mode and positive and negative detection (M = 582). Specific ions were detected in the EI mode: a weak molecular ion at 582 and an intense characteristic ion at 478, allowing the identification of these lactones formed from IdoA in an analysis resulting from the methanolysis of a proteoglycan mixture (Fig. 5c). Based on these ions and on the presence of four peaks with the uronic specific ions at M = 537 (Fig. 5a and 5b), corresponding to different Rt than those of GlcA and GalA peaks, a total of six peaks specific for IdoA were detected, all different from those of GlcA. Based on the presence of GlcA peaks, it could be calculated that this CSB contained only 16.92% of GlcA. In contrast with CSA and CSC, the GlcA present in this sample was entirely liberated from the dimers. Taking into account the sum of the areas of the uronic acid peaks, a molar ratio of relative to GalNAc of 1/1 could only be obtained affecting the sum of the IdoA peaks with a relative molar response coefficient (RMR) of 0.711. Because the RMRs of GalA and GlcA submitted to methanolysis were higher (0.930 and 0.950, respectively [11]), it was suggested that a part of IdoA (24–25%) was lost during methanolysis. Nevertheless, affecting the sum of the areas of the different peaks resulting from IdoA with the RMR of 0.711 allowed to obtain a correct molar ratio between the sum of GlcA and IdoA relative to GalN, reproducibly.

Heparin

The profile of the GLC separation of the methanolysis products of heparin (Fig. 4b and 4d) showed a large complexity. Both the peaks of the anomers of GlcN (represented by the specific ion in the EI/MS mode at 476) and of the O-methyl glycosides of GlcN (ions at 276–490) were detected. This indicated that a significant portion of the substituent of the NH2 group of glucosamine residues was lost before the cleavage of the glycosidic bonds. Indeed, it is almost impossible to produce the O-methyl glycoside of GlcN from GlcN with a protonated amino group [21,22]. The characteristic peaks of to GlcA and IdoA were also detected. Gal and Xyl were present in a molar ratio of 2.013/1.000, providing a correct representation of the linkage region. GC/MS analysis showed the presence of specific peaks of dimers composed of IdoA (Fig. 3b) and of GlcA. In contrast with the dimers derived from CSA and CSC, the dimers presented a characteristic A1 ion at 947 (Fig. 3b), indicating that the hexosamine was in the non reducing position of the dimers. As contrasted from the previous

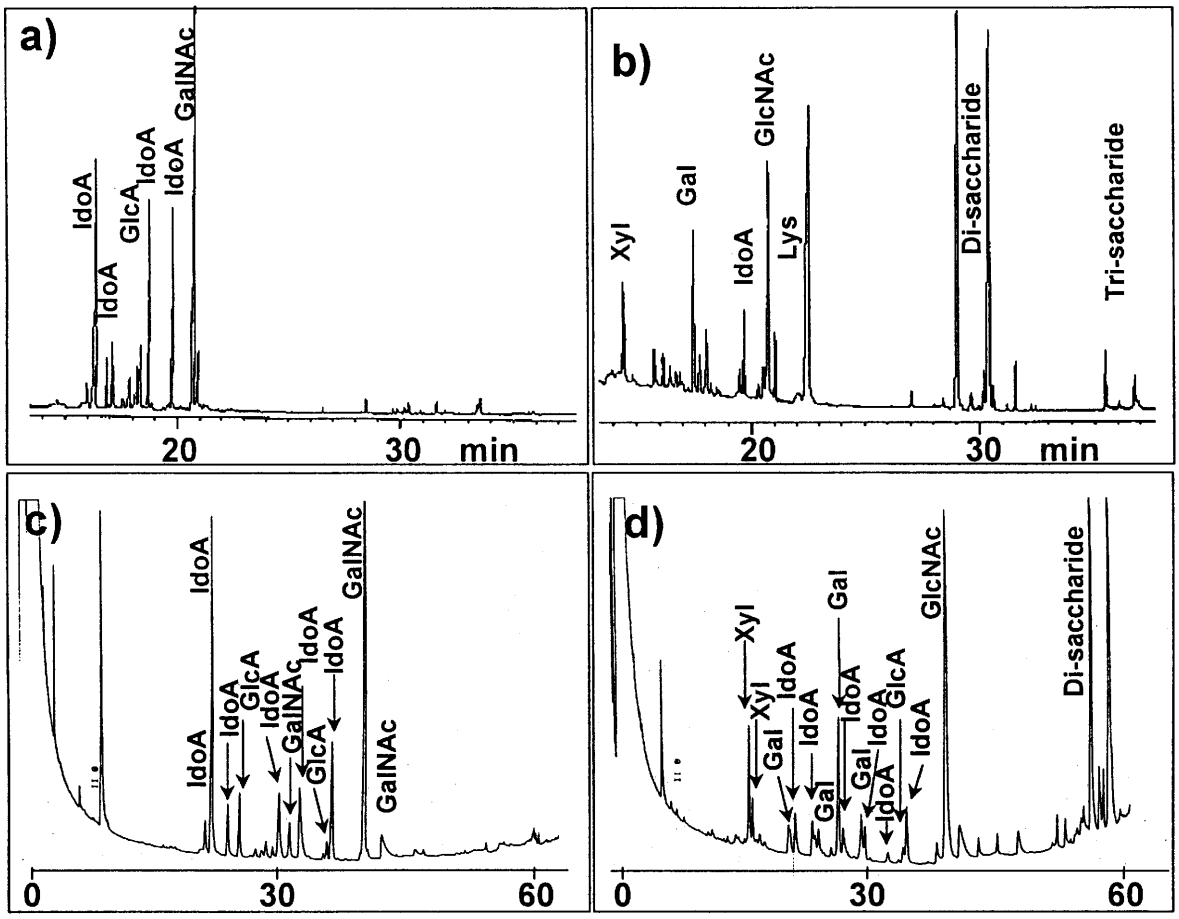

Figure 4. Comparison of TIC (a) and b)) and FID (c) and d)) chromatograms of the HFB derivatives of the methanolysis products of chondroitin sulphate B (a) and c)) and of heparin (b) and d)). In the TIC profiles, only the major peaks shown on the FID profiles are indicated. Note the complexity of the monosaccharide profiles in large part due to the products from IdoA. Note that the cleavage of CSB into monosaccharide is quantitative in contrast with that of heparin, for which peaks of di- and tri-saccharides are detected. Quantitation is performed on the FID chromatogram when samples contain disaccharides. Indeed, with a MS apparatus with a mass limit of 1000 amu, higher mass ions are not integrated and the TIC response is about one half of the correct value.

compounds, small peaks representing about 3% of the disaccharides were observed at higher Rt than the disaccharides. From their Rt, they likely corresponded to tri-saccharides (Fig. 4b). The major of these compounds presented a relatively intense ion at 779, suggesting that a hexuronic acid was at the non reducing end. Based on the proportion of the disaccharides relative to the level of the peaks of the O-methyl glycosides of GlcN obtained by GC analysis, it was calculated that heparin was cleaved into monomers to a level of 48%. Affecting peaks of IdoA of the RMR 0.750, calculated from CSB, resulted in a ratio between the sum of GlcA and IdoA relative to GlcN close of 1/1. Assuming that three sulphate groups were present for each disaccharide unit, the molecular mass of this heparin was 12800 Da.

The study of this compound suggest that heparin-like substances present in CSA, and CSC and revealed by the presence of GlcN and IdoA may be compounds in which the glucosamine is N-acetylated and not N-sulphated. Indeed, sulphate groups should be rapidly removed by methanolysis from the N-sulphated GlcN and, due to the shielding effect of the protonated $NH3^{+}$ group on the cleavage of the O-glycosidic bond [21], this bond would not be cleaved. This assumption was supported by the finding that disaccharides had the hexosamine at the non-reducing hand. In contrast, in the case of GlcNAc instead of N-sulphated GlcN in the chain, the O-glycosidic bond of the reducing GlcNAc would be cleaved before de-acetylation [22], giving the O-methyl glycoside of GlcN and only monomers. This suggested that an approximation of the level of N-sulphated GlcN could be deduced from the proportion of disaccharides having GlcN in a non-reducing position and of O-methyl glycosides of GlcN in heparin and heparan sulfate.

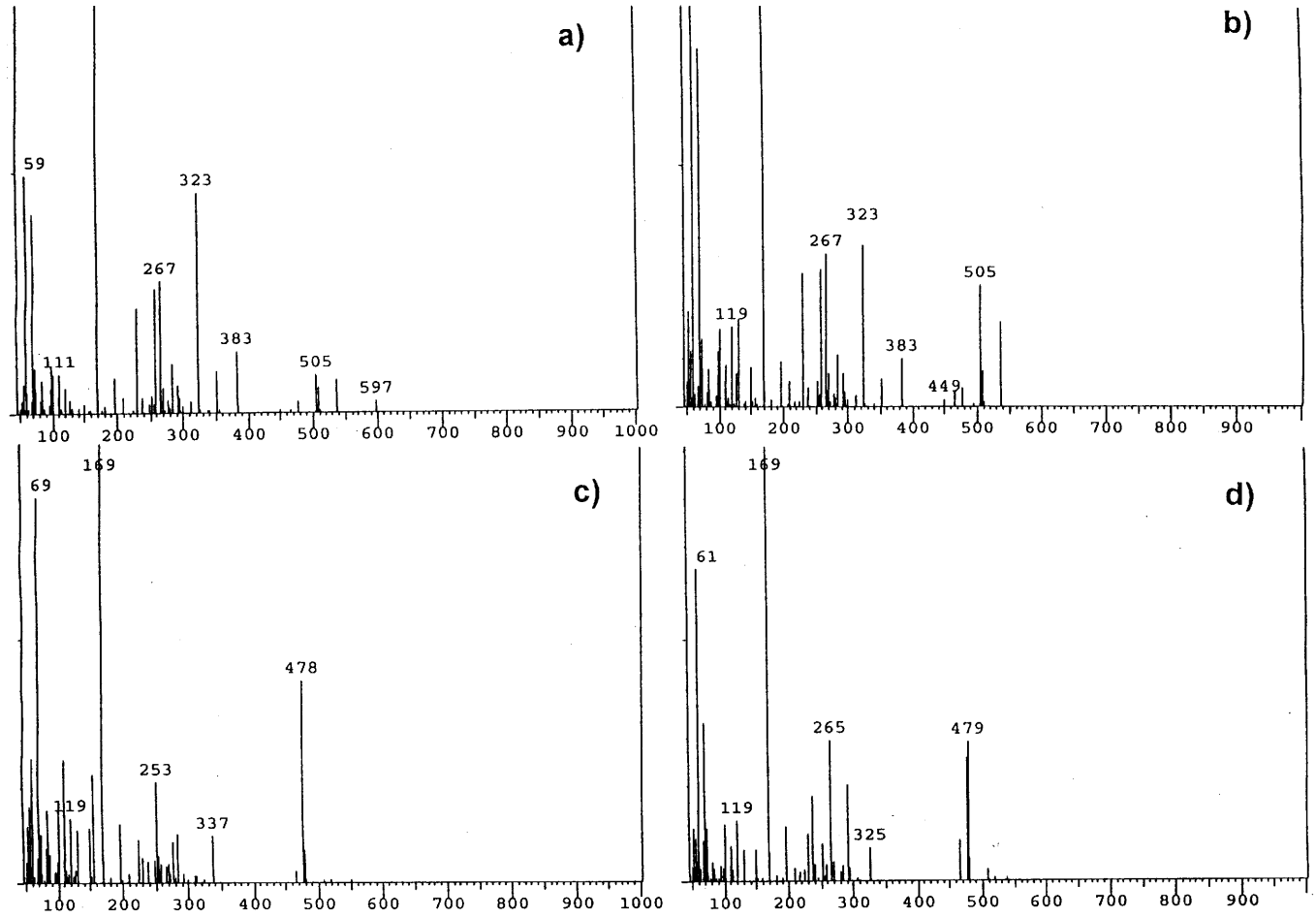

Figure 5. Mass spectra in the EI mode of the HFB derivatives of; a) and b) O-methyl glycosides of the methyl esters of IdoA; c) O-methyl glycosides of lactones derived from IdoA; d) O-methyl glycoside of xylose.

Additional data on the cleavage conditions of sulphated GAGs or sulphated oligosaccharides

It was previously reported that high HCl concentrations in the methanolysis reagent increased significantly the yield of cleavage of the glycosidic bond of GAGs [15]. We therefore tested this point using HFB derivatives. Methanolysis of heparin in the presence of 3 M HCI did not increase the yield of cleavage. In fact, lower level of disaccharides (70%) and extremely reduced yield of monosaccharides were observed (down to 10% for GlcN and lower percentage for the others). These yields were not significantly changed upon addition of barium acetate, probably because the degradation was essentially due to the action of HCl (it should be kept in mind that HCl is considered as a catalyst of methanolysis).

As discussed above, the de-acetylation of the amino group of GlcNAc impedes the cleavage of the glycosidic bond [21, 22]. Consequently, one way to increase the facility of cleavage was to re-acylate the amino group [9], especially when it was initially free or N-sulphated. Because acylation of such NH2 group could be achieved using HFBAA, we tried to perform a second methanolysis after the first step of methanolysis/HFB derivatisation. This procedure appeared efficient for the cleavage of the GlcN-inositol bond of glycosyl-phosphatidyl-inositols (Pons et al., in preparation). For heparin, this procedure did not increase significantly the percentage of monosaccharides relative to disaccharides, the reason being an increased degradation of monosaccharides.

Because the introduction of barium ions did not modify the yield of methanolysis when sulphate was absent, it could be suggested to systematically perform methanolyses in these conditions. However, repetitive injections of such samples implicate frequent cleaning of the injector part of the GC apparatus. Therefore, this procedure should be applied only when the GAG nature of the compounds is certain or when uronic acids have been identified after normal methanolysis.

Conclusions

Performing acid methanolysis in the presence of a small excess of barium acetate relative to quantity of sulphate present in

sulphated glycosaminoglycans resulted into two major improvements: i) the precipitation of the liberated sulphuric acid as an insoluble barium sulphate salt eliminates the degradation of the less stable compounds (uronic acids, but also hexoses, pentoses and deoxy-hexoses, when present). GlcA was found to be stable in these conditions, whereas the extremely unstable IdoA was recovered with a yield about 75%; ii) for a still undefined reason, barium seemed to modify the electronic environment of the carboxyl groups of uronic acid, facilitating the cleavage of the glycosidic bonds (more than 50% cleavage into monomers was observed for all compounds, the yield being of 100% for keratan sulphate and chondroitin sulphate B).

Although the liberation of the monosaccharide constituents of CSA, CSC and heparin was not complete, the presence of disaccharides (tri- and tetra-saccharides are eluted within the chromatographic run (unpublished results)) could propose an essential information on the structure of the glycosaminoglycans using GC/MS analysis, especially in the electron impact mode. Indeed, some disaccharides derived from heparin presented the A1 ion at 947 absent from CSA and CSC, whereas the latter were characterised by the A1 ion at 779. The sensitivity of the detection of these specific ions allowed defining the nature of both the major and the minor compounds, independent from the analysis of the monomers, the exact monosaccharide composition being deduced from the molar ratio between the uronic acids and hexosamines (and from monosaccharides of the linkage region, when present). Using this technique, the constituents of the linkage region of O-linked GAGs (Xyl-Gal-Gal) can be quantitatively determined and the chain length of the GAG chain deduced from the sum of the monosaccharides and disaccharide relative to one Xyl and two Gal residues. Xyl could be easily identified through characteristic peaks (Fig. 4b) with intense ions at 479 (Fig. 5d). Recent experiments indicated that analyses could be performed on proteoglycans with mixed glycan compositions (i.e. GAGs and N-glycans and O-glycans). The presence of barium ions fully protected sialic acid from destruction in such a way that its recovery was quantitative. In two different compounds having CSB and CSA chains besides N-glycans,

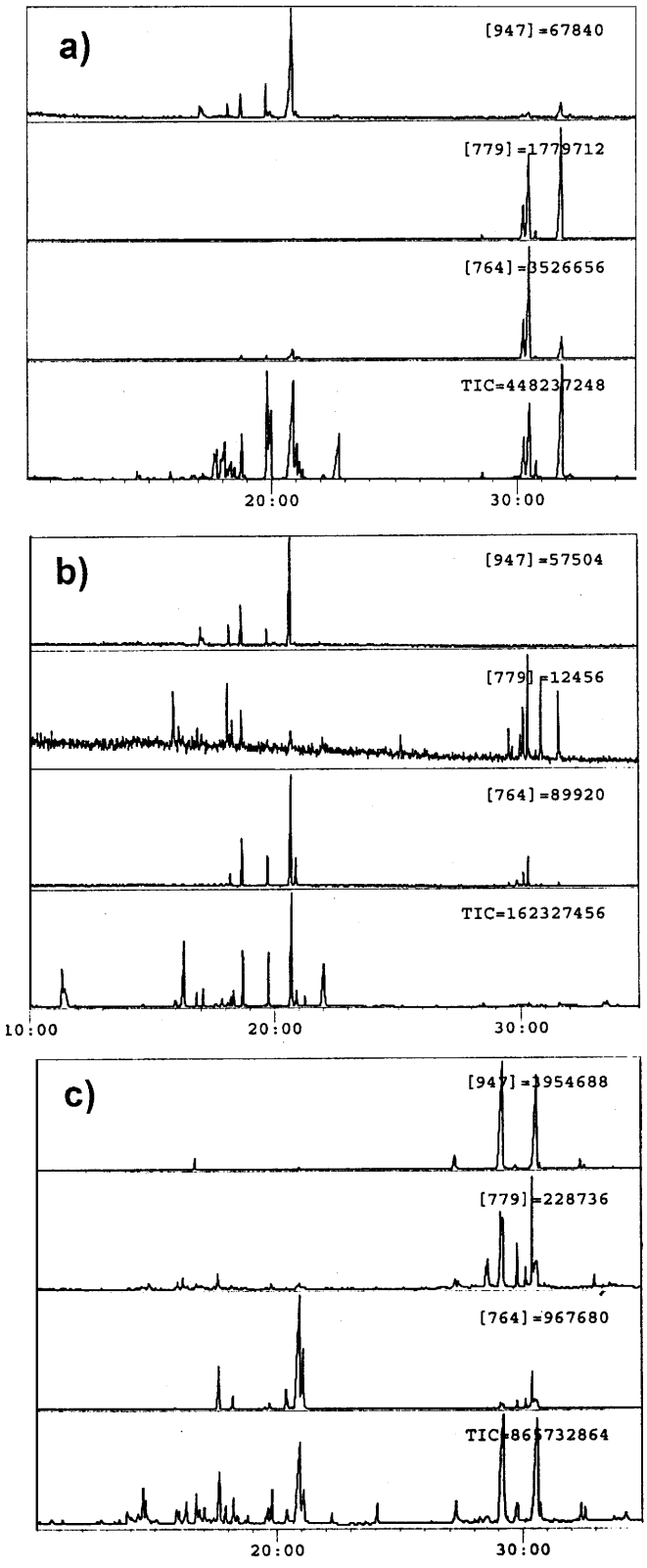

Figure 6. GC/MS in the electron impact mode of the methanolysis products from: a) chondroitin sulphate C, b) chondroitin sulphate B, and c) heparin. Data are presented for the total ion counts (TIC) and for the search of specific ions of disaccharides (764 and 779 specific for CSA and CSC and 947 specific for heparin). Note in b) that in the TIC profile, disaccharides (Rt around 30 min) are almost absent in CSB. But the search of specific ions of CSA and CSB disaccharides indicated that similar disaccharides are present at a level about 0.1%. For heparin, minor ions at 764 and 779 indicated the presence of a contamination of heparin by CSA or CSC. Enlargement of the area of disaccharides (not shown) indicated that the peaks containing these ions are not superimposed with the peaks of the major ion at 947 representative of heparin. The two minor peaks observed in the area of the disaccharides of CSA and CSC are representative of the presence of compounds containing IdoA. Since their Rt were different from those of heparin and since CSB is completely cleaved in the same conditions, it was suggested that they corresponded to another undefined class of glycosaminoglycan.

the quantitation of the different constituents obtained by GC could be determined based on the quantity of mannose and on the quantity of the GlcNAc residue involved in the N-glycosidic bond. Indeed, this GlcNAc residue is recovered as the heptafluorobutyrate derivative of GlcN and not as the derivative of the O-methyl glycoside of GlcN resulting from all other GlcNAc residues [22]. The quantity of O-linked GAG could be determined from the quantity of xylose present in the sample. Therefore, this technique provides important improvements compared to other techniques, the composition of the sulphated GAG being possible starting from 0.1 µg amount of such compounds. It should be stressed that quantitation could be also performed using the TIC response of the MS apparatus for all liberated monosaccharides from sulphated GAGs. The RMRs are the same as those determined for the FID response [11] with the single difference that the response of hexosamines is lowered from an additional 1.300 relative to the response of hexoses. Unfortunately, due to the high mass of disaccharides, the TIC response is reduced by about 50% since high mass ions are not integrated.

Acknowledgements

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