Carbohydrate Research, 161 (1987) 305–313
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Structural characterization of sulfated glycosaminoglycans by fast atom bombardment mass spectrometry: application to heparin fragments prepared by chemical synthesis

VERNON N. REINHOLD*[a], STEVEN A. CARR[†], BRIAN N. GREEN[b], MAURICE PETITOU[c], JEAN CHOAY[c], AND PIERRE SINAŸ[d]

[a]*Harvard School of Public Health, Boston, MA (U.S.A.).* [b]*VG Analytical, Manchester (UK).* [c]*Institute Choay, Paris (France).* [d]*Université d'Orleans (France).*

(Received October 5th, 1984; accepted for publication in revised form, October 2nd, 1986)

Heparin was the first therapeutically effective anticoagulant made available for clinical use, and it remains the most widely used drug for the treatment and prophylaxis of a great number of thromboembolic disorders. Commercial preparations of heparin are obtained from porcine intestinal mucosa or bovine lung, and it has been determined that, in the United States alone, several tons are used annually[1]. Physiologically, heparin is stored in mast cells[2] and basophilic granulocytes[3], and the tissue concentration has been correlated with the number of these cells[4]. The macromolecule consists of several polysaccharide chains of molecular weight 40,000 to 100,000, joined by a protein-core structure.

The complete chemical structure of heparin is still unknown; however, it is known that the fundamental unit is a repeating disaccharide, 6-*C*-sulfated, *N*-sulfated D-glucosamine joined α-D-(1→4) to a 2-*C*-sulfated uronic acid residue. These units are attached to each other by β-(1→4)-glycosidic linkages[1,5], and account for ~50% of the heparin chain[6]. The remaining 50% consists of the same repeating units with a lower degree of sulfation, some *N*-acetylation, and some C-5 epimerization in the uronic acid residue.

Oligosaccharide sulfate residues cannot be approached in the usual sequencing manner[7], due to the specific lability of the *O*- and *N*-linked sulfate groups. Further complicating a structural understanding is the fact that glycosaminoglycans are polydisperse, and degradation by chemical or enzymic methods introduces size heterogeneity. Consequently, few structures of sulfated oligosaccharides larger than disaccharide have been unambiguously determined, and

---

*To whom correspondence should be addressed.

[†]Present address: Smith Kline Beckman, Philadelphia, PA, U.S.A

0008-6215/87/$ 03.50

those larger structures which have been presented are often composite or average structures, rather than unique molecular entities.

In view of the chemical lability and heterogeneity of the sulfated glycosaminoglycans, physicochemical methods that would permit detailed structural information to be obtained on these materials without the need for prior, chemical degradation or derivatization would be most beneficial. A number of new, mass-spectrometric techniques have been developed that enable direct analysis of polar and thermally labile molecules[8]. In particular, fast-atom-bombardment–mass spectrometry (f.a.b.-m.s.) has been demonstrated to be of value for structure analysis of neutral and acidic oligosaccharides and glycosphingolipids[9–13].

Earlier work had demonstrated that polysulfated oligosaccharides obtained from chondroitin sulfate may be structurally characterized by[14] positive- and negative-ion f.a.b.-m.s. The spectra show intense, cationized molecular ions and fragment ions, from which, sequence and sites of sulfation can be readily determined. In addition, polymers of 4-*O*-sulfo and 6-*O*-sulfo positional isomers could be distinguished, and structural homologs of chondroitin sulfate that contain more than one covalently bound sulfate per two sugar residues were identified. We have now applied this technique to analyze related oligosaccharide structures obtained by chemical synthesis. The results described here indicate that f.a.b.-m.s. may be a valuable tool for detailing the structural features of heparin fragments.

EXPERIMENTAL

Mass spectrometry experiments were conducted by using two instruments. The lower-molecular-weight oligosaccharides were examined with a Finnigan-MAT double-focussing mass spectrometer (San Jose, CA), and the tetra- and penta-saccharide samples were analyzed in a VG ZAB instrument (VG Analytical Ltd., Manchester, UK). Each instrument was equipped with data-acquisition systems, SS-200 and VG II-250, respectively. For fast-atom bombardment, both instruments were equipped with an Ion Tech (Teddington, Middlesex, UK), B-11 fine-beam, saddle-field, fast-atom source; xenon was used as the reagent gas. The underivatized sulfated oligosaccharides (~100 μg) were dissolved in distilled water (10 to 15 μL), and aliquots of these solutions containing 5–20 μg of sample were loaded by syringe onto a stainless-steel target coated with 1-thioglycerol (3-mercapto-1,2-propanediol), and attached to a direct-insertion probe. The probe was then inserted into the ion source of the mass spectrometer, and bombarded with the xenon beam (operating parameters: source pressure, 1.20 mPa Xe; f.a.b.-tube voltage, 8 kV; and tube current, 2.0 mA).

RESULTS AND DISCUSSION

Heparin disaccharides (HD-I and HD-II). — The f.a.b. mass spectra of such organic anions as the sulfated and carboxylated oligosaccharides from heparin

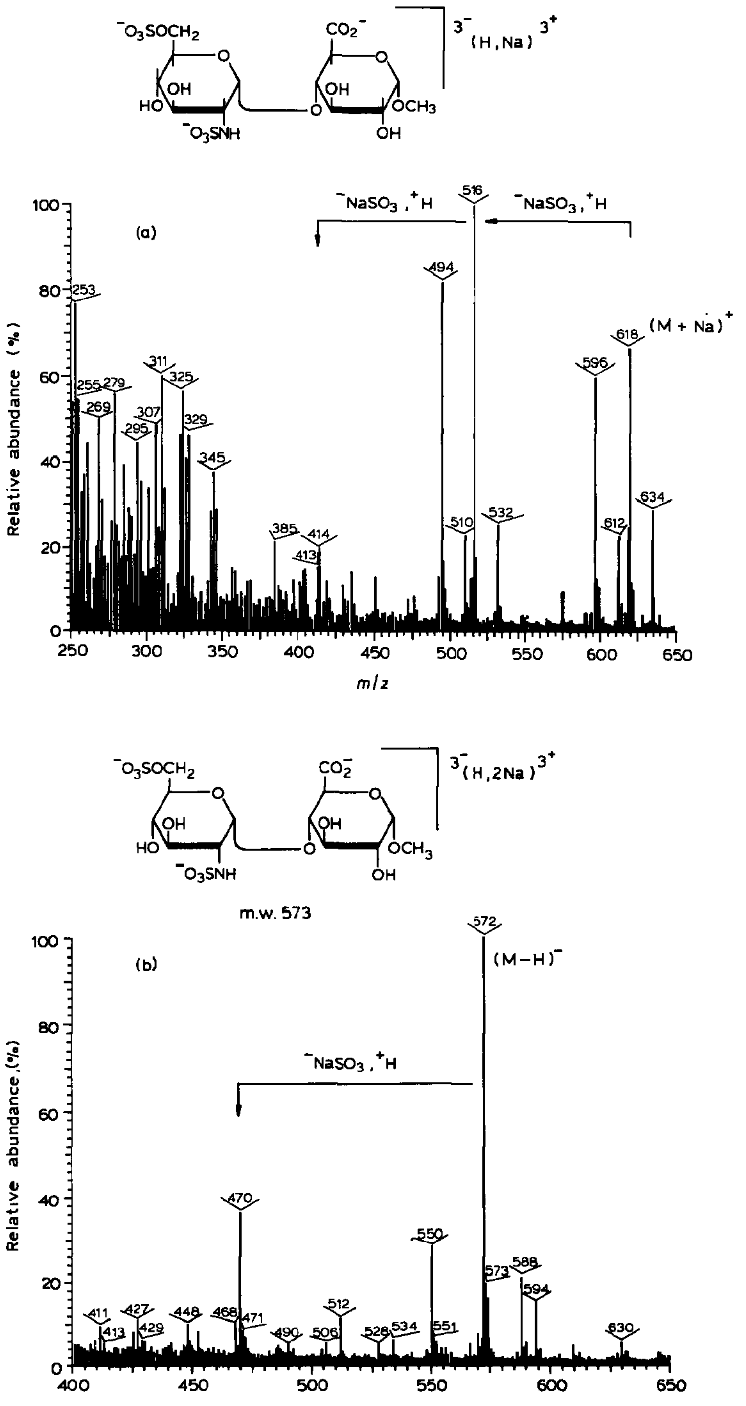

Fig. 1. F.a.b. mass spectra of a heparin-related disaccharide (HD-I), using 1-thioglycerol as a liquid matrix. Positive (Fig. 1a) and negative (Fig. 1b) ion extraction.

TABLE I

COMPOSITION OF MOLECULAR-WEIGHT-RELATED IONS FROM HD-I[a]

Countercation composition	m/z
Positive f.a.b.	
$(4Na)^{4+}$	618
$(H^{1+}, 2Na^{2+}, K^{1+})^{4+}$	612
$(H^{1+}, 3Na^{3+})^{4+}$	596
$(2H^{2+}, 2Na^{2+})^{4+}$	574
$(3H^{3+}, Na^{1+})^{4+}$	552
Negative f.a.b.	
$(2NA)^{2+}$	572
$(H^{1+}, Na^{1+})^{2+}$	550

[a] HD-I = heparin disaccharide with three anionic sites.

exhibit clusters of ions related to the molecular weight. The cluster and its dispersion are due to variations in the resident countercations, as well as to the addition or removal of a proton or alkali-metal cation to generate the singly charged ion. Earlier f.a.b.-m.s. studies of sulfated glycosaminoglycans from chondroitin sulfate[14] provided disaccharide spectra which were dominated by molecular and structurally significant fragment-ions. Three pronounced features could be readily observed that aid in the characterization of these polyionic saccharides: a molecular-ion pattern indicating countercation distribution and molecular weight, the presence of sulfuric esters from the fragment intervals following rupture, and an oligomer sequence as a consequence of glycosidic cleavage.

The positive-ion, f.a.b. mass spectra of heparin disaccharides exhibit similar features that are useful for structural characterization. First are the series of intense molecular-weight-related ions, which are dispersed by their composition of countercations. Because the fundamental structure of these anionic materials remains constant and the countercations cause the dispersion observed on f.a.b. mass-spectral analysis, tables have been established to relate the observed molecular-weight-related ions to cation composition. Separations of 22 units preponderate, and correspond to replacement of a proton by a sodium cation (see, *e.g.*, Fig. 1a, *m/z* 618, 596, and 574; see Table I). Much smaller satellite-peaks 16 units above the corresponding natriated species are due to replacement of a single sodium cation with potassium. For clarity, only the most intense molecular-weight-related ion and associated fragments have been labeled on the mass spectra. Second, the presence and number of sulfuric esters may be readily determined from the f.a.b. spectra by the losses of 102 units which result from facile elimination of sodium sulfite with hydrogen replacement (see, *e.g.*, Fig. 1a, *m/z* 618/516, 596/494, and 516/414). Thus, the spectrum of HD-I indicates the presence of two sulfate groups, and yields the complete cationic ditribution for the three anionic sites, from the highly protonated to the fully natriated species. F.a.b. mass-spectral analysis of

TABLE II

COMPOSITION OF MOLECULAR-WEIGHT-RELATED IONS FROM HD-II[a]

	m/z
Positive f.a.b.	
Countercation composition	
$(3Na)^{3+}$	558
$(Na^{1+}, H^{1+}, K^{1+})^{3+}$	552
$(2Na^{2+}, H^{1+})^{3+}$	536
$(2H^{2+}, K^{1+})^{3+}$	530
$(2H^{2+}, Na^{1+})^{3+}$	514
Negative f.a.b.	
Counterion composition	
$(Na)^{1+}$	512
$(H)^{1+}$	490

[a] HD-II = heparin disaccharide with two anionic sites.

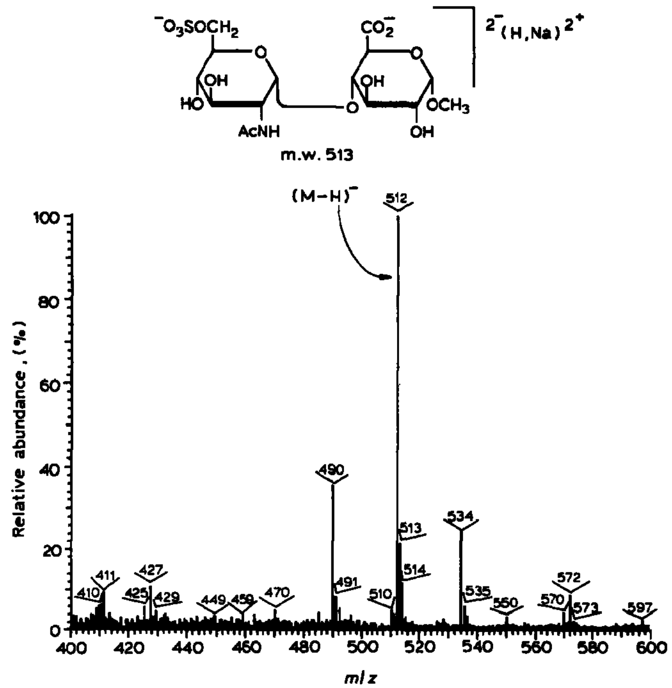

Fig. 2. Negative-ion, f.a.b. mass spectra of a heparin-related disaccharide (HD-II), using 1-thioglycerol as a liquid matrix.

HD-I in the negative-ion extraction mode (see Fig. 1b) provided the same general information of counterion distribution and sodium sulfite loss. The latter process is less pronounced, and only one sulfite loss is readily apparent.

In contrast to the f.a.b. mass spectra of the chondroitin sulfate disaccharides, the spectra of the heparin-related disaccharides do not exhibit sequence-related fragment-ions. The absence of these structurally informative fragments may, in part, be related to the increased lability of the *N*-sulfite moieties during analysis by f.a.b.-m.s. The difference in stability of *C*- *versus* *N*-sulfates is readily apparent upon comparison of the negative-ion spectra of HD-I, which contains both *N*- and *C*-sulfate, and HD-II, which has only a *C*-linked group. The negative-ion f.a.b. spectrum of the former exhibits a significant M-102 peak (see Fig. 1b), whereas ions due to the loss of sulfite are absent from the spectrum of HD-II (see Table II and Fig. 2).

Sulfuric esters are, in general, more stable when analyzed in the negative-ion mode[14]. For example, the fragment represented by sulfite loss for the disaccharide HD-I (*m/z* 470, Fig. 1b, negative mode) was only 38% of its parent ion (*m/z* 572), whereas the related fragment was the base ion when analyzed by positive-ion extraction, (*m/z* 516; see Fig. 1a). It is also interesting that potassium adducts are consistently more abundant in the positive-ion *versus* the negative-ion mode, (compare, *e.g.*, Figs. 1a and 1b). Whether the difference observed is related to molecular stability or to sampling differences during ion extraction is unclear.

Heparin tetrasaccharide (HT-I). — The tetrasaccharide sample was synthesized as a precursor to the pentasaccharide, in order to define the minimal number of residues necessary to mimic biological activity[15]. The material proved to be totally inactive.

The f.a.b. mass spectra confirmed the molecular composition (see Table III) and indicated the presence of at least three sulfite groups. All ions and fragments, in both the positive- and negative-ion mode, could be accounted for as molecular-weight-related, or products of sodium sulfite elimination. The loss of two of the sodium sulfite groups can be readily observed (see Fig. 3; *m/z* 1325, 1223, and

TABLE III

COMPOSITION OF MOLECULAR-WEIGHT-RELATED IONS FROM HT-I[a]

Negative f.a.b. Countercation composition	m/z
$(8Na^{8+}, -H^{1+})^{7+}$	1347
$(7Na)^{7+}$	1325
$(6Na)^{6+}, H^{1+})^{7+}$	1303
$(5Na^{5+}, 2H^{2+})^{7+}$	1281
$(4Na^{4+}, 3H^{3+})^{7+}$	1259
$(3Na^{3+}, 4H^{4+})^{7+}$	1237

[a] HT-I = heparin tetrasaccharide with eight anionic sites.

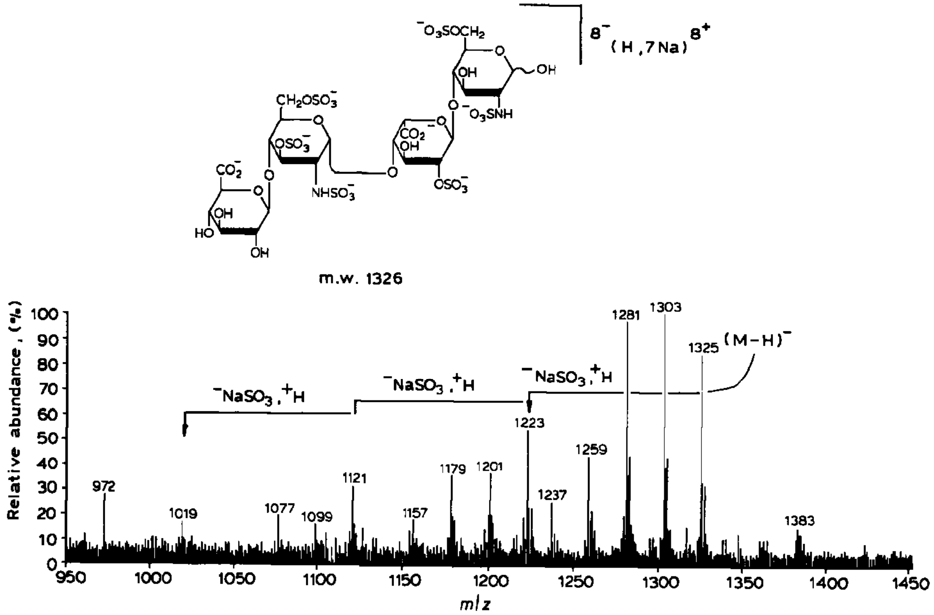

Fig. 3. Negative-ion, f.a.b. mass spectra of a heparin-related tetrasaccharide (HT-I), using 1-thioglycerol as a liquid matrix.

1121). By analogy with the observed dispersal of molecular-weight-related species, each fragment-ion is associated with satellite ions reflecting the composition of associated countercations. The third loss of sodium sulfite is of lower abundance, and no other fragments could be detected (to represent further sulfite loss). Consistent with the disaccharide spectra, ions relating to saccharide sequence are not observed for the heparin tetrasaccharide.

Heparin pentasaccharide (HP-I). — The pentasaccharide investigated in this study represents the minimal sequence that binds to AT-III and elicits anti-Xa activity[15]. This synthetic product was examined under both negative and positive f.a.b.-m.s. The results provided the same general characteristics and spectral features as the other saccharides, with all ions accounted for as either molecular-weight-related or fragment ions representing the products of sodium sulfite elimination. The most abundant, high-mass ion in the negative-ion f.a.b. mass spectrum, *m/z* 1668 (see Fig. 4), can be accounted for by considering the ten anionic sites of the molecule as occupied with one proton and eight sodium countercations, resulting in a net charge of −1; see Table IV. From this molecular ion, a series of three sodium sulfite groups are lost, to yield the fragments *m/z* 1566, 1486, and 1384. Considering the foregoing observation showing enhanced *N*-sulfite lability, and the fact that this sample possesses three such residues, this series may represent those specific losses. Consistant with the other materials, the sample exhibits a somewhat greater stability and better sensitivity when analyzed by f.a.b. in the

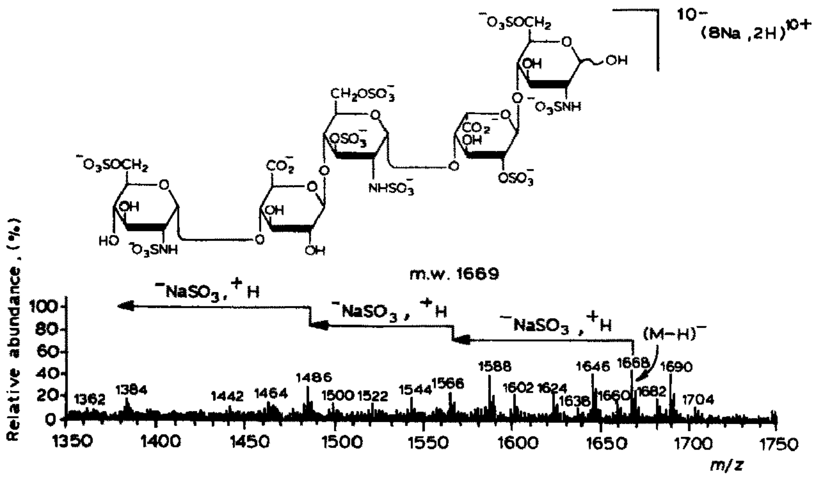

Fig. 4. Negative-ion, f.a.b. mass spectra of a heparin-related pentasaccharide (HP-I), using 1-thioglycerol as a liquid matrix.

TABLE IV

COMPOSITION OF MOLECULAR-WEIGHT-RELATED IONS FROM HP-I[a]

Negative f.a.b. *Countercation composition*	m/z
$(9Na)^{9+}$	1690
$(8Na^{8+}, H^{1+})^{9+}$	1668
$(7Na^{7+}, 2H^{2+})^{9+}$	1646
$(6Na^{6+}, 3H^{3+})^{9+}$	1624
$(5Na^{5+}, 4H^{4+})^{9+}$	1602

[a] HP-I = heparin pentasaccharide with ten anionic sites.

negative mode. The compositions of the molecular-weight-related ions are presented in Table IV.

SUMMARY

We report herein the results of f.a.b.-m.s. experiments conducted on synthetic fragments of glycosaminoglycans, one of them representing the pentasaccharidic sequence present in heparin and responsible for the binding to antithrombin III, and the others being related to this sequence. The results indicate that f.a.b.-m.s. can be very useful for the structural analysis of sulfated glycosaminoglycans. The relatively small amounts of sample required enable molecular characterization at physiologically significant levels. In contrast to the chondroitin sulfates, the heparin saccharides analyzed and reported here do not provide

sequence information. The data indicate that glycosidic rupture is not a process competing with the much more facile loss of *N*-sulfite residues. Dominating the spectra are a series of molecular-weight-related ions (distributed to indiate the associated countercation composition), and fragments related directly to sulfite elimination. This f.a.b.-induced, facile loss of sulfite may impose limitations in molecular-weight analysis for the larger oligomers.

REFERENCES

1 L. B. JAQUES, *Science,* 206 (1979) 528–533.
2 O. WILANDES, *Scand. Arch. Physiol.*, 81 (1938) 1–32.
3 I. OLSSON, B. BERG, AND L. A. FRANSSON, *Scand. J. Haematol.*, 7 (1970) 440–444.
4 G. P. M. CRAWFORD AND A. S. DOUGLAS, *Heparin and Its Current Therapeutic Role,* in L. POLLER (Ed.), *Recent Advances in Blood Coagulation,* Livingstone, Edinburgh, 1977, pp. 337–361.
5 U. LINDAHL, in N. M. MACDUFFIE (Ed.), *Heparin: Structure, Cellular Functions and Clinical Applications,* Academic Press, New York, 1979, pp. 167–179.
6 A. LINKER AND P. HOVINGH, in N. M. MACDUFFIE (Ed.), *Heparin: Structure, Cellular Functions and Clinical Applications,* Academic Press, New York, 1979, pp. 3–24.
7 J. R. TURVEY, *Adv. Carbohydr. Chem.*, 20 (1965) 183–218.
8 K. L. BUSCH AND R. G. COOKS, *Science,* 218 (1982) 247–254.
9 V. N. REINHOLD AND S. A. CARR, *Mass Spectrom. Rev.*, 2 (1983) 153–221.
10 A. DELL, H. R. MORRIS, H. EGGE, H. VONNICOLAI, AND G. STRECKER, *Carbohydr. Res.*, 115 (1983) 41–52.
11 J. P. KAMERLING, W. HEERMA, J. F. G. VLIEGENTHART, B. N. GREEN, I. A. S. LEWIS, G. STRECKER, AND G. SPIK, *Biomed. Mass Spectrom.*, 10 (1983) 420–425.
12 S. HANDA, Y. KUSHI, H. KAMBARA, AND K. SHIZUKUISHI, *J. Biochem. (Tokyo),* 93 (1983) 315–318.
13 M. ARITA, M. IWAMORI, T. HIGUCHI, AND Y. NAGAI, *J. Biochem. (Tokyo),* 94 (1983) 249–256.
14 S. A. CARR AND V. N. REINHOLD, *Carbohydr. Chem.*, 3 (1984) 381–401.
15 J. CHOAY, J. C. LORMEAU, M. PETITOU, P. SINAŸ, B. CASU, AND G. GATTI, *Biochem. Biophys. Res. Commun.*, 116 (1983) 492–499.