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PROFILING GLYCOL-SPLIT HEPARINS BY HPLC/MS ANALYSIS OF THEIR HEPARINASE-GENERATED OLIGOSACCHARIDES¹

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Abstract

Glycol-split (gs) heparins, obtained by periodate oxidation / borohydride reduction of heparin currently used as anticoagulant and antithrombotic drug, are arousing increasing interest in anti-cancer and anti-inflammation therapies. These new medical uses are favored by the loss of anticoagulant activity associated with glycol-splitting-induced inactivation of the antithrombin III (AT) binding site. The structure of gs-heparins has not been studied yet in detail. In this work, an ion-pair reversed-phase chromatography (IPRP-HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) widely used for unmodified heparin has been adapted to the analysis of oligosaccharides generated by digestion with heparinases of gs-heparins usually prepared from porcine mucosal heparin. The method has been also found very effective in analyzing glycol-split derivatives obtained from heparins of different animal and tissue origin. Besides the major 2-O-sulfated disaccharides, heparinase digests of gs-heparins mainly contain tetra- and hexasaccharides incorporating one or two gs residues, with distribution patterns typical for individual gs-heparins. A heptasulfated, mono-N-acetylated hexasaccharide with two gs residues has been shown to be a marker of the gs-modified AT binding site within heparin chains.

Keywords

Heparin; Glycol-split heparins; Heparin lyases; Oligosaccharide analysis; Ion-pair reversed-phase high performance liquid chromatography; Mass spectrometry

INTRODUCTION

Heparin, a natural sulfated polysaccharide belonging to the family of glycosaminoglycans (GAG)², is widely used in clinics as anticoagulant and – prevalently under the form of low-

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²**Abbreviations used:** AT – antithrombin III, GAG – glycosaminoglycan, LMWH – low molecular weight heparin, TSD – trisulfated disaccharide, ATBR – antithrombin-binding region, PMH – porcine mucosal heparin, HS – heparan sulfate, RO-heparin – reduced oxy-heparin, NRE – non-reducing end, LR – linkage region, RE – reducing region, IPRP-HPLC – ion-pair reversed phase high performance liquid chromatography, BMH – bovine mucosal heparin, BLH – bovine lung heparin, GMH – caprine mucosal heparin, FmPMH – fast-moving porcine mucosal heparin, Mw – weight average mean molecular mass, NA fraction – no affinity fraction, HA fraction – high affinity fraction, TDA – triple detector array, 2D-HSQC – two-dimensional heteronuclear single quantum coherence, HP-SEC – high pressure size-exclusion chromatography, DBA – dibutylamine, RSD – relative standard deviation, LOD – limit of detection, LOQ – limit of quantification, S/N – signal-to-noise, LOL – limit of linearity, DP – degree of polymerization, SD – sulfation degree, UFH – unfractionated heparin.

molecular-weight heparin (LMWH) – as antithrombotic drug. Heparin is constituted by linear chains of 1,4-linked disaccharide repeating units of alternating uronic acids (*L*-iduronic, IdoA, and *D*-glucuronic, GlcA) and a hexosamine (*D*-glucosamine, GlcN). The prevalent repeating unit in heparins is a trisulfated disaccharide (TSD), where the uronic acid is 2-*O*-sulfated IdoA (IdoA2S) and GlcN is *N*- and 6-*O*-sulfated (GlcNS,6S). The TSD sequences constitute the so-called regular, “fully sulfated” regions of heparin, which are separated by undersulfated regions where a low proportion of IdoA residues, and most of the residues of the minor heparin component GlcA, usually preceded by *N*-acetylated GlcN residues (GlcNAc), do not bear sulfate substituents. The specific pentasaccharide sequence *N*-acetyl-*D*-glucosamine-6-*O*-sulfate 1→4 *D*-glucuronic acid →4 *D*-glucosamine *N*,3-*O*,6-*O*-trisulfate 1→4 *L*-iduronic acid 2-*O*-sulfate 1→4 *D*-glucosamine *N*,6-*O*-disulfate (GlcNAc,6S-GlcA-GlcNS,3,6SIdoA2S- GlcNS,6S), with GlcN and IdoA residues in the α configuration and GlcA in the β configuration, constitutes the antithrombin-binding region (ATBR), which is essential for a high anticoagulant and antithrombotic activity of heparins. This sequence is contained in only a portion (about one third) of the chains constituting the heparins most commonly used in therapy (extracted from porcine intestinal mucosa) and even less of those of the corresponding LMWHs (1–3). Typical structural features of a chain of porcine mucosal heparin (PMH) containing the ATBR are shown in Fig. 1A (adapted from ref.4).

Through its ATBR, heparin binds antithrombin (AT) and induces a conformational change of this protein that greatly accelerates inhibition of the serine proteases thrombin and Factor Xa and consequently inhibits blood clotting (1–3,5). Heparin binds also to a number of other proteins (6,7) and modulates some of the important biological functions of its structural analog heparan sulfate (HS), which is an important constituent of cell surfaces and intercellular matrices (8,9). There is growing interest in investigating and exploiting “new” activities of heparin, especially for cancer and inflammation therapy (10,11). However, since the anticoagulant activity of heparin involves risks of bleeding, new pharmaceutical applications require non-anticoagulant heparin species (12).

One effective way of drastically reducing the anticoagulant activity of heparin consists in oxidizing the GAG with periodate, which splits the C(2)-C(3) bond of non-sulfated uronic acids, including the essential glucuronic acid (GlcA) residue within the ATBR. Periodate oxidation is a classical reaction in carbohydrate chemistry and has been widely applied to determine vicinal, unsubstituted hydroxyl groups in polysaccharides (13). Under controlled conditions, periodate oxidation can be performed with limited cleavage of glycosidic bonds, i.e., without significant depolymerization of the polysaccharide. The primary products of the reaction (the polyaldehydic oxy-heparins) are usually stabilized by borohydride reduction, leading to reduced oxy-heparins (RO-heparins) (6,14,15). The designation “RO” is being used for heparins in which the glycol-split residues correspond to the non-sulfated uronic acid residues of the original heparin chains, while the term “glycol-split heparins” (gs-heparins) is currently used to design both heparins partially oxidized/reduced (14) and those in which the number of split residues also includes chemically 2-*O*-desulfated IdoA (originally IdoA2S) oxidized and reduced residues (16). The gs-heparins considered in this work are all of the RO-type with one exception (HI-3). The structure of an RO-heparin chain is depicted in Fig. 1B, where, for the sake of simplicity, the diol in 3,4 position of GlcN residue at the non-reducing end (NRE), also susceptible to glycol-splitting is indicated with a dotted circle. Residues of the linkage region (LR) at the reducing end (RE) of some heparin chains also contain vicinal glycol groups that can be split.

The periodate oxidation/reduction reaction usually does not impair the biological activities of heparin that are not critically dependent on the intact structure of the AT-binding sequence. In fact, most of the literature data (as well as unpublished experiments from our

group) indicate that gs-heparins are more effective than their parent GAGs in interacting with some heparin-binding proteins and eliciting the associated biological activities (16). Potentiation of peculiar heparin activities by glycol splitting has been ascribed to the increased local mobility of glycol-split residues, which act as flexible joints along the polysaccharide chains, thus facilitating interactions with the heparin/HS binding sites of proteins (16,17). A number of non-anticoagulant, gs-heparins differing in molecular size, extent of glycol-splitting, and further derivatization, including hydrolysis, are being considered, *i.e.*, as potential antilipemic (15), platelet interaction-inhibiting (18), antithrombotic (19), antiangiogenic (20–23), antimetastatic (20, 24, 25), antimalarial (26), labor-reducing in obstetrics (27), and anticancer drugs (28–31).

Pharmaceutical development and establishing correlations between structure and biological activities of gs-heparins require setting up appropriate analytical methods. Structural characterization of gs-heparins has largely relied on ^1H and ^{13}C NMR analysis, which permits to determine also the extent of glycol splitting (21,22). However, as with the parent heparins (32), a major problem in the analysis of gs-heparins is associated with structural microheterogeneity, which can be unraveled only through careful cleavage of GAG chains and identification of the generated di- and oligosaccharide fragments. One common method for cleaving heparin exploits the substrate specificity of heparinases (heparin lyases I, II and/or III) (33). To circumvent complications involved in the separation and characterization of higher oligosaccharides, heparins are currently profiled in terms of their disaccharide composition, most often through analysis of exhaustive digests with a cocktail of heparinases. These digests consist mainly of unsaturated disaccharides, which are usually separated by chromatographic or electrophoretic methods and identified through comparison with authentic samples (32–36).

Significant advances in the analysis of heparin/HS oligosaccharides have been achieved by coupling ion pair reversed-phase high performance liquid chromatography (IPRP-HPLC) with electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) (32,35–37). We chose this flexible approach preferentially for profiling the composition of exhaustive heparinase digests of gs-heparins obtained from porcine mucosal heparin (PMH). On consideration of the substrate specificity of heparin lyases, we expected that the heparinases preferentially cleaved glycosidic bonds of unmodified heparin sequences, but should be unable to cleave those including glycol-split residues. Exhaustive digests of gs-heparins with heparin lyases I, II, and III should accordingly consist prevalently of disaccharides, structurally identical to those obtained from the corresponding unmodified heparins. In addition, heparinase digests of gs-heparins were expected to contain several small oligosaccharides containing glycol-split GlcA/IdoA residues. We actually found that the oligosaccharide fractions of heparinase digests of gs-heparins prevalently consisted of tetra- and hexasaccharides that contained glycol-split residues. The exact masses of some of these oligosaccharides found by LC/MS correspond to the structures of fragments derived from the ATBR. We also found that the HPLC/MS profiles are characteristic for each type of gs-heparins, reflecting different distribution of GlcA and IdoA and different sulfation patterns in the parent heparins.

MATERIALS AND METHODS

Reagents and starting materials

Sodium chloride (>99.5%), formic acid (98–100%), hydrochloric acid standard solution 0.1 N, Tris (tris(hydroxymethyl)aminomethane), hydrochloric acid >37% (p.a.), sodium periodate (> 99%), dibutylamine (>99.5%), pentylamine (99%) and hexylamine (99%) were from Sigma-Aldrich. Volumetric solution of sodium hydroxide 0.1 N and sodium borohydride (95%) were from Riedel-de Haën. Solid sodium hydroxide (>99%) and sodium

acetate were from Merck. Calcium acetate (>97%) were from BDH. Methanol (LC-MS grade) and acetic acid (glacial, 99.9%) were from Novachimica. Deionized water of conductivity less than 0.06 μ S was prepared with an osmosis inverse system. Heparin lyases I (EC4.2.2.7), II and III (EC4.2.2.8) were from Iduron or Grampian Enzymes, UK. Heparins were commercial and experimental preparations from: porcine mucosal heparin, PMH, bovine mucosal heparin, BMH (both from Laboratori Derivati Organici, Italy), porcine lung heparin, BLH (Upjohn), caprine mucosal heparin, GMH (Sigma-Aldrich); fast-moving porcine mucosal heparin, FmPMH (Opocrin, Italy), that represents the heparin component of Sulodexide, a mixture of GAGs byproducts of PMH production. Uronate unsaturated (UA) disaccharides standards Δ UA,2S-GlcNS,6S (> 95%), Δ UA,2S-GlcNS (> 95%) Δ UA-GlcNS,6S (> 95%), Δ UA-GlcNS (> 95%), Δ UA-GlcNAc,6S (> 95%) and Δ UA,2S-GlcNAc (> 95%) were from Iduron, UK.

Antithrombin high affinity fraction of PMH

A column of AT-Sepharose gel (10 $\times$ 1.6 cm) prepared as previously reported (38) was used for semi-preparative affinity fractionation. 12 mg of PM heparin was eluted in two steps (Tris-HCl 0.05 M pH 7.4, containing 0.05 M or 2.5 M NaCl): the fraction with no AT affinity (NA) was eluted at lower ionic strength (0.05 M NaCl) while the high affinity fraction (HA) was eluted at higher salt concentration (2.5 M NaCl). The effluent fractions were analyzed for uronic acid content by the carbazole reaction (39), building up the appropriate calibration curve at 530 nm. Fractions HA and NA were desalted first by dialysis with 3500 Da cutoff membranes (Spectrapor), then by size exclusion chromatography with Toyopearl HW-40 resin (TOSOH) column (58 $\times$ 2.6 cm). To remove last traces of Tris buffer a cation-exchange chromatography was finally performed on an Amberlite IR-120 [H⁺] column (12.5 $\times$ 2 cm). Eluents were neutralized with 0.1 N NaOH, and samples recovered after freeze drying.

Partially 2-O-desulfated heparin with conversion of the original IdoA2S into L-GalA residues, and glycol-splitting to derivative HI-3

Partially 2-O-desulfated heparin was prepared by a modification of methods used by Jaseja et al. (40) and Rej and Perlin (41) essentially as described previously (42). PM heparin (~1g) was dissolved in 6.4 ml of water, after addition of 6.4 ml of 2 M NaOH the solution was heated at 60°C for 15 min. After cooling at room temperature, the pH value was adjusted to 7 with 0.1M HCl and the solution was maintained at 70°C for 48h. After cooling, desalting dialysis and freeze drying the GalA derivative was obtained in a 80% yield. HI-3 was obtained after glycol-splitting using conditions previously described for heparins (21, 22).

Glycol-split heparins

Glycol-split heparins were prepared by exhaustive periodate oxidation followed by borohydride reduction of heparin. About 50 mg of each heparin sample were dissolved in 1.5 ml of H₂O, and 1.5 ml of 0.2 M NaIO₄ were added and, then, the reaction mixture was stirred at 4°C overnight in the dark. The excess of periodate was neutralized by adding 150 μ l of ethylene glycol maintaining the stirring for a further hour at 4°C. Solid sodium borohydride (32 mg) was added to the reaction mixture under stirring at room temperature. After 3h the pH value was adjusted to 4 with 1N HCl. After neutralization with 1N NaOH, the gs-heparins were desalted with a column (100x2.5 cm) of Toyopearl HW-40 resin (TOSOH), then, freeze dried as previously described (24).

Yields, average molecular mass (Mw), determined by TDA (43) and % gs residues (determined by integration of 2D-HSQC spectra) of compounds are: RO-PMH yield 86%, Mw 11.4 KDa, % gs 13; RO-BMH yield 79%, Mw 12.1 KDa, %gs 8; RO-GMH yield 73%, Mw 10 KDa, %gs 17; RO BLH RO yield 84%, Mw 13.1 KDa, %gs 1; RO-FmPMH yield

59%, Mw 6.7KDa, %gs 20; RO 2-O-des PMH (HI-3) yield 87%, Mw 10 KDa, %gs 43. RO HA PMH was prepared starting from 4 mg of HA PMH as described above.

NMR analysis

Spectra were recorded at 25°C on a Bruker Avance 500 spectrometer (Karlsruhe, Germany) equipped with 5-mm TCI cryoprobe. Integration of peak volumes in the 2D-HSQC spectra was made using standard Bruker TOPSPIN 2.0 software. The relative content of glycol-split uronic acid residues (gsIdoA and gs-GlcA %) was calculated from the corresponding anomeric cross-peaks identified by HSQC (unpublished) following the procedure previously applied to unmodified heparins (44) and to LMWHs (45).

Molecular weight determination

Molecular weight determinations were performed by HP-SEC-TDA on a Viscotek (Houston, Texas) instrument equipped with a VE1121 pump, Rheodyne valve (100 µl), and triple detector array 302 equipped with refraction index (RI), viscometer, and light-scattering (90° and 7°) systems. A G2500 and G3000 (7.8 mm × 30 cm TSK GMPWxl) Tosoh columns were used with 0.1 M NaNO₃ as eluent (flow, 0.6 ml/min). Samples were dissolved in the eluent to obtain 10 mg/ml solution. Peak integration and data processing were performed by using OMNISEC 4.1 software. All molecular weight parameters (number-average mean molecular mass (Mn), weight-average mean molecular mass (Mw), and polydispersity (Mw/Mn, D)) were determined for each sample. The TDA system does not require use of chromatographic standards. For light scattering and viscometry detectors calibration, Viscotek GPC standards for aqueous applications were used: in particular, poly(ethylene oxide) (PEO) 23K (Mw 22,300, Mn 22,300, 0.373 dL/g) and PEO 100K (Mw 97,900, Mn 31,500, 0.809 dL/g) were used as narrow and broad standard, respectively.

Enzymatic cleavage of original heparins and their gs-derivatives

The substrate (2–3 mg) was dissolved in a 1:1 (v/v) mixture of 100 mM sodium acetate buffer (pH 7.0) and 10 mM calcium acetate to obtain a 7.7 mg/ml solution. To carry out the enzymatic cleavage 144 µl of the mixture of 100 mM sodium acetate and 10 mM calcium acetate (1:1), and 3 µl of heparin lyases mixture (1 µl of each lyase, 2 mU/µl enzyme solution) were added to 13 l of the heparin solution. The reaction was stirred at 37°C (Termo shaker TS-100 Biosan) for 24 h. The reaction was stopped by adding 3 µl of 3% HCOOH. Each sample was diluted two times with water and analyzed by IPRP-HPLC/ESI-TOF.

IPRP-HPLC/ESI-TOF-MS analysis

LC/MS analysis was performed on a LC system (Dionex Ultimate 3000, Dionex) equipped with degasing system (model LPG-3400), pump (model LPG-3400A), autosampler (model WPS-3000TSL) and UV-detector (model VWD-3100) and coupled with an ESI-QTOS mass-spectrometer (microQTOF, Bruker Daltonics).

The chromatographic separation was performed using a Kinetex C18 analytical column (100 × 2.1 mm I.D., 2.6 µm particle size, Phenomenex) with Security Guard Cartridges Gemini C18 (4 × 2.0 mm, Phenomenex). A binary solvent system was used for gradient elution. Solvent A (10 mM DBA, 10 mM CH₃COOH in water:methanol (9:1)) and solvent B (10 mM DBA and 10 mM CH₃COOH in methanol) were delivered at 0.1 ml/min. Oligosaccharides were separated using a multi-step gradient. The solvent composition was held at 17% B for the first ten minutes, then increased to 42% B over 20 min, and to 50 % B over another 20 min; afterwards, the content of the eluent B was increased to 90 % where it was held for 10 min; finally, it was returned to 17% B over 1 min, and held for the last 19 min for equilibrating the chromatographic column before the injection of the next sample.

The injection volume was 5 μ l. The MS spectrometric conditions were as follows: ESI in negative ion mode, drying gas temperature +180°C, drying gas flow-rate 7 ml/min, nebulizer pressure 0.9 bar; and capillary voltage +3.2 kV. The mass spectra of the oligosaccharides were acquired in a scan mode (m/z scan range 200 – 2000).

Inter- and intra-day variations have been evaluated for the twelve di-, tetra-, and hexasaccharides eluted from the chromatographic column with different retention times in the range from 8 up to about 60 min. As shown in the Table S1 in Supplementary Material, the value of intra- and inter-day variability (RSD) does not exceed 1.1 % for each target compound.

The quantitative analysis of unsaturated disaccharides has been performed using calibration curves obtained with the standard solutions that were prepared by diluting the disaccharides stock solutions (10 mg/ml in H₂O). The regression equations, correlation coefficients, limit of detection (LOD), limit of quantification (LOQ) and area variability (RSD) are given in Table S2 of Supplementary Material. LOD and LOQ values were calculated as the concentration of the injected sample (with constant injection volume, 5 μ l) which gave signal-to-noise ratio equal to 3 and 10, respectively, using the calibration curves based on the linear regression between signal-to-noise ratio (S/N) value and analyte concentration. For the major trisulfated disaccharide Δ UA,2S-GlcNS,6S the dynamic range of the calibration curve (range between LOQ and limit of linearity (LOL)), was from 40 to 1500 μ g/ml when using UV detection at 232 nm and from 0.20 up to 550 μ g/ml for MS detection. Varying the disaccharide concentration in digested gs-heparins from 5 to 600 μ g/ml, each sample was diluted two times before LC/UV/MS analysis in order to avoid saturation of chromatographic column. Both UV and MS calibration curves for Δ UA,2S-GlcNS were linear in the whole concentration range typical for non-modified and gs-heparins, namely 3 – 100 μ g/ml. The lower limit of the dynamic range corresponds to LOQ, and it was found equal to 21.0 μ g/ml for the UV detector and 0.24 μ g/ml for the MS detector. Despite of lower LOQ value provided by MS, for quantitative analyses, the calibration curves obtained with UV detection have been used because the area variability is lower than for the MS detection mode (Table S2). The curves obtained with the MS detector cannot be used, especially, when saturated disaccharides, such as the trisulfated disaccharide UA,2S-GlcNS, 6S (2,3,0) which has a retention time similar to its unsaturated analog Δ 2,3,0, are present. While they did not absorb in the UV-light at 232 nm, MS detector is sensitive to these compounds that, consequently, leads to incorrectly high results because of the peaks overlap.

For PMH the Δ UA-GlcNS, Δ UA-GlcNAc,6S were also evaluated. Since the extinction coefficient at 232 nm for the mentioned disaccharides were shown to be equal under the adopted analytical conditions (data not shown), it was possible to quantify the total content of these two compounds, even if eluted together, using the same calibration curve (data not shown). For PMH digest the total content of two positional isomers Δ UA,2S-GlcNS and Δ UA-GlcNS,6S was determined using the calibration curve of the former one.

The first level of oligosaccharide structure assignments were performed using criteria for rationalizing m/z values of HS oligosaccharides (46). The error between theoretical and the experimental m/z values of monoisotopic masses was calculated using a software for microTOF, Bruker Daltonics. Errors less than 5 ppm were considered acceptable. An external calibration method using sodium formate clusters as calibrants was used to achieve an accuracy of better than 5 ppm.

RESULTS

1. Preparation and physico-chemical properties of glycol-split heparins

Glycol-split heparins modified only at the level of pre-existing nonsulfated GlcA and IdoA residues (RO) were prepared by periodate oxidation/borohydride reduction (15,20) of commercial unfractionated (UF) heparins extracted from intestinal mucosa of porcine (PMH), caprine (GMH), bovine (BMH), from bovine lung (BLH) and of a porcine mucosal fast moving fraction (FmPMH). A more extensively glycol-split heparin (HI-3) was obtained starting from a partially (43%) 2-O-desulfated PMH (21,22). Overall yields were from 59% to 86%. The molecular mass (MW, determined by TDA (43) of RO-heparins were from ~10,000 Da to ~13,000 Da, except for the fraction RO-FmPMH (~6.700 Da). Mw values for gs-derivatives are somewhat lower than those of the corresponding parent heparins.

2D NMR spectra provided a more complete characterization of gs-heparins, especially in the $^1\text{H}/^{13}\text{C}$ HSQC approach previously applied to quantificate typical residues and substituent groups of heparins (44) and LMWHs (45). In the HSQC spectra of the present gs heparins (not shown), cross peaks from gs-GlcA and gs-IdoA residues could be observed separately, with different relative % content, e.g., 1.0 / <LOD for RO-BHL and ~8.3 / 2.0 for RO-PMH; RO-FmPMH contains almost equal % amounts of gsGlcA and gsIdoA (4.9 and 5.4), and the extensively 2-O-sulfated and glycol-split HI-3 10.7 and 26.7, respectively.

2 Heparinase-generated fragments

2a. Optimization of HPLC/MS analysis—On the basis of the specificities of heparin lyases (33), we anticipated that heparinase digests of gs-heparins were mainly constituted by the same unsaturated disaccharides generated from the unmodified heparin backbone and by a number of tetra- and higher saccharides containing undigested glycol-split residues. We accordingly focused on the optimization of an IPRP-HPLC method for simultaneous separation of disaccharides and these peculiar oligosaccharides. For this purpose, we used a digest of RO-PMH as a model system. Different aliphatic amines, among which dibutyl-, *n*-pentyl and *n*-hexylamine, have been tested in their acetate salt form, compatible with ESI-MS, as ion pair agents. *n*-Pentyl- and *n*-hexylamines have been chosen, since their very effective use in the IPRP separations of some oligosaccharides has been recently demonstrated (47). Dibutylamine (DBA) has been used for the analysis of HS oligosaccharides (35,46) and for pentasaccharidic and octasaccharidic heparanase substrates and their heparanase-cleaved products (48). However, only DBA acetate salt provided besides the simultaneous separation of di- and oligosaccharides, a good resolution of positional isomers including gs species. Probably, it is more “selective” for the interaction with sulfate groups of different positional isomers than its monoalkyl-analogues due to higher stereochemical hindrance. It should be noted that our results do not contradict some of the published data (47), where the emphasis was on the increasing of the MS detection sensitivity for oligosaccharides with high polymerization degree (up to dp 22) and on the fingerprinting intact heparins, but not on the separation of isomeric oligosaccharides.

In this study we put special emphasis on the separation of oligosaccharides with the same sulfation degree and number of gs residues. For this reason, DBA was chosen for a further gradient optimization. The dependence of the resolution of gs oligosaccharides generated from RO-PMH by enzymatic cleavage, on DBA concentration in eluents has been investigated using a linear gradient changing from 10% B to 90% B in 90 min at flow-rate 100 $\mu\text{L}/\text{min}$ was selected. It has been shown that the change of the ion pair reagent concentration from 5 to 10 mM was crucial for the chromatographic resolution of gs oligosaccharides (Fig. S1), while further increase of its concentration up to 15 mM did not

influence significantly the chromatographic separation (Fig.S1) but led to an essential decrease (~ 2 times) of MS detection sensitivity and to increase of the total analysis time.

Having found the optimal concentration of DBA (10 mM), the gradient has been adapted to achieve better resolution for the simultaneous separation of di-, tetra- and hexasaccharides. The LC/UV chromatograms of heparinase-cleaved porcine mucosal heparin (PMH) and its glycol-split derivative (gs-PMH) obtained under the optimized conditions, using a multi-step gradient as described in Materials and Methods, are shown in Fig.S2. Using MS detector each analytical signal has been identified (Table 1, Fig. 2 and Fig. S2).

The resolution coefficients (R_s) for six oligosaccharide pairs, components of heparinase digests of RO-PMH, were found to be equal to 1.67 for $\Delta 4,4,0,1\text{gs}/\Delta 4,4,1,1\text{gs}$, 1.30 for $\Delta 4,6,0,1\text{gs}/\Delta 6,6,1,1\text{gs}$, 1.33 for $\Delta 6,6,1,2\text{gs}/\Delta 6,6,1,1\text{gs}(1)$, 1.21 for $\Delta 6,6,1,1\text{gs}(1)/\Delta 6,6,1,1\text{gs}(2)$, 1.55 for $\Delta 6,7,1,2\text{gs}/\Delta 6,7,1,1\text{gs}(1)$ and 1.33 for $\Delta 6,7,1,1\text{gs}(1)/\Delta 6,7,1,1\text{gs}(2)$ (See decoding in Table 1). This resolution is sufficient for fingerprinting the enzymatic digests obtained from RO derivatives of different heparin samples through their LC-MS profiles. As usual trend in most chromatographic systems, the order of elution of oligosaccharides increases with the increasing size (degree of polymerization, DP) and sulfation degree (SD) (32). Profiles in Fig. S2 and S2 and data in Table 1 and S3 show that this trend holds also for glycol-split species. However, oligosaccharides with the same DP and DS containing two gs residues elute earlier than those containing only one gs residue. Thus, the order of elution time for hexasulfated hexasaccharides is $\Delta 6,6,1,1\text{gs} > \Delta 6,6,1,2\text{gs}$; for heptasulfated hexasaccharides is: $\Delta 6,7,1\text{gs} > \Delta 6,7,1,2\text{gs}$; for octasulfated hexasaccharides: $\Delta 6,8,0,1\text{gs} > \Delta 6,8,0,2\text{gs}$.

Retention times, molecular formulae, their monoisotopic exact masses and corresponding first level structures (number of monosaccharide residues, sulfation and N-acetylation degree) of the major components of heparinase digests of RO-PMH are reported in Table 1. Data in Table S3 of Supplementary Material indicates in more detail also the major ionic forms of each analyte and the presence or absence of a double bond at the non-reducing uronic acid in their structures. As revealed by the limited number of charged species contributing to each structure, formation of adduct species is clearly minimized under the experimental conditions adopted in the present analyses. Data in Tables 1 and S3 also show that the components with the same “monoisotopic exact mass” values, corresponding to different isomers, are present in the heparinase-digests of gs-heparins. For instance, fractions 4b° (species $\Delta 4,3,1,1\text{gs}$) and 6f° ($\Delta 6,8,0,1\text{gs}$) are both constituted by two species with close retention time values, and fraction 4e ($\Delta 4,5,0$) could consist of four species.

Most of the peaks generated after enzyme treatment by both PMH and RO-PMH are attributable to oligomers initiating with a 2-O-sulfated, 4,5-unsaturated uronic acid residue (Table 1 and S3). However, comparing the LC/MS profile of heparinase-digested PMH and RO-PMH, it is evident that they differ significantly. Mass analysis confirmed that, while heparinase digests of unmodified heparin essentially consisted only of di- and tetrasaccharides, the corresponding digests of gs-heparins contained also hexasaccharides (Fig. S2 and Fig. 2). Notably, most of the tetra- and hexa-saccharides of gs-PMH contain gs residues. This can be detected by the increase of the mass values of two daltons. As illustrated in Fig. 2 and Fig.S2, for porcine mucosal heparin (PMH) and the corresponding RO-type glycol-split heparin (RO-PMH), only a few fragments from this latter have the same retention times as those generated from the original heparin. In fact, though with different relative intensities, only the major unsaturated trisulfated disaccharide TSD ($\Delta \text{UA}, 2\text{S-GlcNS}, 6\text{S}$) and the minor disulfated unsaturated disaccharide $\Delta \text{UA}, 2\text{S-GlcNS}$ are common components of the digestion products of both unmodified and glycol-split species. In the chromatogram of the PMH digest not only sulfated disaccharides but also non-2-O-

sulfated disaccharides (Δ UA-GlcNS, Δ UA-GlcNAc,6S, Δ UA-GlcNS,6S) are present (Fig.S2). All disaccharides observed in heparin digests have been identified, comparing the obtained retention time and corresponding m/z value with those of the individual disaccharide standards. It should be noted that to the same m/z value of Δ UA-GlcNAc,6S (teor. m/z for $[M-H]^-$ 458.0599), another isomer having the same retention time, namely Δ UA,2S-GlcNAc, could be assigned. However, the corresponding peak (peak 1 in the Fig.S2) disappears totally after glycol-splitting. It means that in PMH digest this disaccharide, containing 2-O-sulfated uronic acid and NAc-glucosamine, was not present. The same observation can be done for Δ UA-GlcNS, 6S. While its positional isomer mentioned before (Δ UA,2S-GlcNS) is preserved, this non-2-O-sulfated compound disappears completely in RO-PMH digest. In this case, the isomers are characterized by the different retention time values (Table 2) and can be identified using the corresponding standard solutions independently.

In the oligosaccharide region, heparin digests essentially consist of tetrasaccharide fragments only. In contrast, a number of hexasaccharide fragments are components of the heparinase digests of the gs heparin. The most prominent oligosaccharide peak derived from unmodified porcine mucosal heparin corresponds to the mass of the tetrasulfated, monoacetylated tetrasaccharide Δ 4,4,1 (49). The most prominent oligosaccharide peak of RO-PMH corresponds to pentasulfated Δ 4,5,0,1gs. It is worth noting that among the RO-PMH-derived hexasaccharides at least one (corresponding to species Δ 6,7,1,2gs, Fig 3) is clearly derived from the ATBR. The fact that this heptasulfated, mono-N-acetylated hexasaccharide Δ 6,7,1,2gs is especially evident in the chromatogram of the gs-derivative of HA fraction of PMH proves this hypothesis.

In the case of heparinase digests of gs-heparins the absence of octasaccharides not containing gs residues is a marker of the efficiency of the enzymatic cleavage reaction and the absence of octasaccharides containing 3 gs residues proves that sequences containing more than two consecutive disaccharide units containing non-sulfated uronic acids were not present in the original heparins.

As detailed in Table 1 for RO-PMH, heparinase-digestion of gs-heparins generate also minor, small-size fragments such as monosaccharides A1,2,0 and A1,3,0(A*) (where A is GlcNS and A* is a 3-O-sulfated GlcNS), disaccharide Δ 2,3,0-R (where R is the remnant at the reducing end, formed during the side hydrolytic reaction (6,50), see Discussion), as well as trisaccharide A3,5,0,1gs starting with GlcNS and containing a gsU residue and an A*, trisaccharide Δ 3,4,0+2H (tentatively assigned to a species terminating with a reduced uronic acid) and trisaccharide Δ 3,5,0-R' thought to terminate with the remnant of a glycol-split GlcN residue. All these small fragments are conceivably generated from reducing and non reducing end units of gs-heparins sequences (see Discussion.) Notably, monosaccharide A1,3,0 (A*) is present also, usually in fairly low amount, in non-glycol-split PMH and other heparinasedigested heparins (unpublished observation from our laboratory).

2b. Differentiation of RO-heparins—After having identified all components of the heparinase-digests of PMH and RO-PMH, the developed approach has been applied for profiling RO-heparins of different sources (RO-GMH, RO-BMH, RO-BLH), including RO-derivatives of fast-moving" fraction of porcine mucosal heparin (RO-FmPMH) and a ~40% glycol-split derivative of PMH (HI-3). For comparing these samples, we focused our attention on three aspects: comparison of the disaccharides composition, comparison of tetra- and hexasaccharide fractions and, finally, on the absence or presence of octasaccharidic components.

As opposed to tetra- and hexasaccharides, the whole series of disaccharide standards are commercially available, that permits their quantitative analysis. We analyzed the composition of the major disaccharides in heparinase-digested gs-heparins using UV detection, which is characterized by a higher reproducibility than MS (Table S2), as described in Methods. The contents of disaccharides in digests reported in Table 2 are expressed as a percentage of the digested heparin or gs-heparin samples. Such an expression permits not only to compare the different samples, but also to evaluate the content of the regular highly sulfated sequences within heparin chains. As expected, since the glycol-splitting reaction converts the non-sulfated uronic acids to glycol-split residues, only Δ UA, 2S-GlcNS and Δ UA, 2S-GlcNS, 6S are preserved during gs-reaction and, consequently, gs-heparin digests showed overall content of disaccharides lower than unmodified heparin. Also, with the exception of RO-BLH, the total disaccharide content in digested RO-heparins of different type (43–55%) is consistently lower than for PMH (~92%). The latter sample contains, as opposed to the digests of the RO-heparins, non-2-O-sulfated disaccharides as well as the higher quantity of the 2-O-sulfated ones. The lower content of Δ UA, 2S-GlcNS and Δ UA, 2S-GlcNS, 6S in the RO-heparin digests is explained by their incorporating in tetra- and higher oligosaccharides containing glycol-split residues. The high disaccharide content of RO-BLH (~85%) correlates with the high sulfation degree of BLH reported in the literature (51). The extensively gs-PMH variant (HI-3) shows the lowest content of disaccharides (~35%) among the preparations analyzed in this study.

The second step consisted in the comparison of the tetra- and hexasaccharide content. As illustrated in Fig. 2, the HPLC/MS profiles of tetra- and hexasaccharide regions of the heparinase digests of RO-heparins, obtained from heparin samples of different tissue/species, significantly differ from each other. Even if most of the fragments contain gs residues, the relative proportion of peaks varies for the different heparins and their overall profiles constitute characteristic fingerprints. Chromatograms of digests from RO-bovine lung heparin (RO-BLH) display the largest differences with respect to those of reference gs-porcine mucosal heparin (gs-PMH), especially because of the presence in the former of peak 4e corresponding to non-digested Δ 4,5,0 tetrasaccharides, and the emergence of 6f^{oo} and 6f^o peaks corresponding to hexasaccharides Δ 6,8,0,2gs and Δ 6,8,0,1gs, containing two and one gs residues, respectively. Peak 4e is observable only as a weak shoulder in chromatograms of glycol-split bovine and caprine mucosal heparins (RO-BMH and RO-GMH).

Generally, the signal intensities of the oligosaccharidic region of the chromatogram, where the major gs-compounds are observed, are the lowest for RO-BLH (Fig. 2), indicating that the high sulfation degree of BLH, demonstrated also by quantitative disaccharide analysis (Table 2).

The low acetylation degree of BLH is also reflected in the relative content of tetrasulfated tetrasaccharide Δ 4,4,0,1gs and Δ 4,4,1,1gs, whose intensities are inverted in comparison with RO-PMH and RO-GMH. This observation is more evident in the digest of RO-BMH.

One of the most interesting difference observed for RO-BLH is the significant increase of Δ 4,6,0,1gs, corresponding to the ATBR, typical for this type of heparin (52).

In contrast to RO-BLH, the chromatographic profiles of the RO-FmPMH and HI-3 are characterized by the higher intensities of tetra- and hexasaccharide fraction with respect to porcine mucosal heparin. This result correlates with the quantitative data on the disaccharide content, which had been demonstrated to be lower for these two RO-samples.

Not unexpectedly, the detectable hexasaccharides of HI-3, which is the only heparin derivative in this study not belonging to the RO series and whose uronic acid residues are glycol-split to the extent of about 40%, are only those that contain two gs residues.

Moreover, though as minor components, also some octasaccharides containing 3 gs residues were present in the enzymatic hydrolyzed of HI-3 (the fourth panel of Fig. 2). At such a high percentage of glycol-splitting, generated by the selective 2-O-desulfation, the chance of finding two gs residues in vicinal disaccharide units is actually rather high.

3. Sequence assignments

Although digestion with the mix of heparinases simplifies the analysis of multidisperse heparin and heparin derivative chains by reducing them to di-, tetra-, and hexasaccharides, structure assignments for isomeric fragments cannot be made only on the basis of their mass values. Strictly, elucidation of the structure of isomeric oligosaccharides should require their isolation and individual characterization by NMR spectroscopy, MS spectrometry, and other techniques (32,53). However, in addition to the knowledge that the non-reducing terminal must necessarily be a unsaturated uronic acid 2-sulfate probably followed by a N-sulfated glucosamine, the structure of the major oligosaccharides of heparinase digests of gs-heparin can be inferred from the structure of those isolated from digests of the parent heparin. Such a comparison is especially feasible for GlcA/IdoA-containing heparin oligosaccharides, whose gs derivatives survive digestion with heparinase I but are susceptible of cleavage with heparinase II and are amenable to further analysis through their glycol-split derivatives. The structure of the major GlcA/IdoA-containing tetra- and hexasaccharides identified in porcine mucosal heparin (4,49,52,54,55) is shown on row A of Table 3, while row B shows the structures expected for the corresponding glycol-split oligosaccharides. It should be noted that four of the major six heparin tetrasaccharide fragments ($\Delta 4,4,1\text{-A}$; $\Delta 4,4,1\text{-B}$; $\Delta 4,4,0\text{-A}$, and $\Delta 4,5,0$) obtained from unmodified heparin upon treatment with heparinase I (4, 54, 55), terminate with a GlcNS,6S reducing residue. These tetrasaccharides are expected to be cleaved (to disaccharides) when treated with heparinase II or with the mix of heparinases I, II and III. In contrast, tetrasaccharide $\Delta 4,6,0$, which terminates with a reducing GlcNS,3S, 6S residue, would be resistant to further cleavage by the mix of heparinases because of the well-established inhibiting effect of the 3-O-sulfate substituent of the aminosugar (4,49,52). The same resistance to enzymatic cleavage is expected for the two hexasaccharides $\Delta 6,6,1\text{-C}$ and $\Delta 6,7,1$, containing an IdoA and a GlcA residue and ending with GlcNS,3S,6S. All the other major heparin hexasaccharidic fragments that contain two uronic acid residues and terminate with GlcNS,6S are susceptible of cleavage with heparinase I (4, 54).

As shown in row B of Table 3, the glycol-split species corresponding to all the major heparin tetra- and hexasaccharides containing one or two non-sulfated uronic acid residues were actually found in heparinase digests of our gs-heparins (see legends in Table 1).

Discussion

The assumption at the basis of the present work, i.e., that digestion of glycol-split heparins with a mix of heparin lyases I, II and III would generate small oligosaccharides containing glycol-split residues was correct. As illustrated in Fig. 2 and Table S3 for RO-heparins of different origin, the HPLC/MS analysis indicates that, in addition to “normal” disaccharides as the expected major products, heparinase digests of gs-heparins are largely constituted by tetra- and hexasaccharides containing one or two glycol-split residues. Mono glycol-split fragments are generated by heparin sequences incorporating isolated uronic acid residues (i.e., containing not more than one GlcA or IdoA per tetrasaccharide), and those containing two gs residues from sequences containing two non-sulfated uronic acids, as, e.g., from (I)AGA*IA-containing sequences of the ATBR. The large majority of fragments in the heparinase digests of glycol-split heparins is even-numbered and starts at their non-reducing end with an unsaturated, 2-O-sulfated uronic acid residue. Odd-numbered fragments are only present in very minor amounts.

Analyses based only on mass values do not permit to discriminate among different sequences of isomeric oligosaccharides. However, reasonable assumptions on actual sequences of fragments containing gs residues can be made by comparison with structures established for the major oligosaccharides obtained from lyase digests of unmodified heparin(s). The structure of gs-containing oligosaccharides expected on the basis of major porcine mucosal heparin oligosaccharides isolated and identified thus far (4,49,52,54,55) are compared in Table 3 with major fragments identified in the present work for RO-PMH. Clearly, fragments containing gs residues derive from non-sulfated GlcA and/or IdoA of heparin sequences.

Oligosaccharides containing gs residues can arise either from heparin HA or NA chains, depending on their ATBR content. As evident from comparison in Fig. 2 of the HPLC/MS profile of the heparinase digest of RO-PMH and the corresponding one of RO-HA-PMH, components associated with ATBR in the original heparin are much more evident in the derivative obtained from the HA fraction. Among oligosaccharides containing glycol-split residues, those arising from heparinase cleavage at the level of the ATBR sequence are also easy to recognize because of the established trend of heparin lyases to preferentially cleave linkages between the 3-O-sulfated residue A* and I2S (49) generating “truncated” 3-O-sulfated GlcNS(6S) residues at the reducing end of the fragments. Similarly to heparin, it was expected, and found in the present work, that also the gs-species originally containing the ATBR region terminated with a A* residue at their reducing end. Indeed, on the assumption illustrated in Fig. 1B, that the ATBR of HA PM heparin species currently contain two uronic acid residues (one IdoA and one GlcA) and one N-acetylated, 6-O-sulfated GlcN residue, the only way to accommodate the sulfate groups indicated by MS analysis for A*-terminating gs oligosaccharides is as shown in row B of Table 3. The structure of the major gs hexasaccharide terminating with a truncated A* residue and taken as an indirect marker of the original ATBR is shown in Fig. 3. Also a N-sulfated variant of the ATBR prevalent in the highly sulfated bovine lung heparin (52) is expected to be present in significant amounts in gs species obtained from heparins of bovine lung origin.

Although the main scope of this study was the identification and profiling of the major components of glycol-split heparins, some minor mono- and tri-saccharide components of the enzymatic digests were also identified and listed in Tables 2 and S3. These small fragments may provide information on end-groups in the original heparin and/or on those generated by some fragmentation associated with the glycol splitting reaction. Thus, the finding of monomeric glucosamine fragments 1a (A1,2,0) and 1b (A1,3,0(A*)), where A* indicates that the aminosugar is 3-O-sulfated, raises the question whether they can be taken as a proof of the concept that heparin molecules essentially as used in clinics are the result of cleavage, by an *endo*- β -D-glucuronidase of consistently longer chains of a proteoglycan biosynthetic precursor (“macromolecular heparin”) (56). That enzyme is essentially an heparanase and would cleave the heparin chains at the level of GlcA residues (57) generating shorter heparin chains terminating with an aminosugar at their non-reducing end, as depicted in the representative structure of Fig. 1. Exhaustive cleavage with heparinases of these chains could then release glucosamine monomers as found in the present work. However, an alternative explanation, which holds especially for the 3-O-sulfated glucosamine A1,3,0 (fragment 1b) is that the glycosidic bond between GlcA and GlcNSO₃,3,6SO₃ within the ATBR of heparin is preferentially cleaved as a result of a side-reaction of periodate oxidation/borohydride reduction under basic hydrolytic conditions (50,58). If such a side reaction had a significant impact under our experimental conditions, monosaccharide 1b should be detected in most non-glycol-split heparins as well. In fact, fragment 1b is detectable also in lyase digests of porcine mucosal heparin (Fig. 3A) and in a number of other heparin preparations (unpublished data from our laboratories). Also in view

of implications on biosynthesis of heparin, the problem of the characterization of non-reducing end residues of the original heparin chains deserves further investigation.

To a much smaller extent than partial hydrolysis as revealed by the some decrease in molecular weights observed as a result of the glycol-splitting reaction, for some preparations we observed also some Smith degradation, i.e., cleavage of RO-heparins at the level of glycolsplit residues (50), as revealed by the identification of a couple of small fragments (such as $\Delta 2,3,0\text{-R}$ (2f) and $\Delta 4,5,0,1\text{gs-R}$ (4h°), see Tables 1 and S3 for RO-PMH) terminating with the remnant (R) of a glycol-split and hydrolyzed uronic acid residue. Such a cleavage may occur under relatively mild acid conditions (50). In addition, borohydride reduction could modify some of the residues at the reducing end, converting them into the corresponding alditols (6): the mass of the minor fragment $\Delta 3,4,0+2\text{H}$ could be rationalized by such a reaction.

Conclusions

In conclusion, HPLC/MS analysis of glycol-split heparins, obtained from commercial and chemically modified heparins under the optimized conditions of the present work, permits to profile their di-, tetra-, and hexasaccharide composition in a way that is characteristic of the structure of the starting materials and especially emphasizes the extent of their glycol-splitting. The analytical approach is also suitable to distinguish as glycol split species heparins of different animal and tissue origin and new analogs under development as experimental drugs. It is also attractive to think that, in parallel with a similar analysis of the parent heparins, analysis of their glycol-split derivatives could permit to extend structural information to the original unmodified heparins, especially regarding sequences containing non-sulfated GlcA and IdoA residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Simplified formula of a representative ATBR-containing chain of porcine mucosal heparin PMH (A) and the corresponding glycol-split derivative RO-PMH (B) obtained from PMH by periodate oxidation and borohydride reduction of nonsulfated GlcA (G/G') residues, to generate gsG/gsG', and gsI. LR = linkage region at the "reducing" end (RE). The biochemical formula of ATBR is indicated in red under structure A, starting with a residue IdoA that is not part of the pentasaccharidic AGA*IA active site, but most often precedes it. Structure A is adapted from Ref. 4; a major modification involves the GlcNS,6S residue at the non-reducing end (NRE), assumed to be the consequence of cleavage (by an endo- β -D-glucosidase) of a GlcA-GlcN,6S glycosidic bond in the carbohydrate chains of the heparin proteoglycan precursor ("macromolecular heparin").

FIG. 3.

Formula of the gs hexasaccharide derived from the ATBR sequence of PMH

TABLE 1

Major fragments in heparinase digests of RO-PMH (including minor mono- and trisaccharide fragments). Isomers eluted with different retention times are numbered in parenthesis.

Abbreviation	Monoglucosamines		Disaccharides			Trisaccharides		
	1a	1b	2c	2e	2f	3b°	3c,red	3d
Species #	A1,2,0	A1,3,0 (A*)	A2,2,0	A2,3,0	A2,3,0-R	A3,5,0,1gs	A3,4,0+ 2H	A3,5,0-R'
Formula	C ₆ H ₁₃ N ₁ O ₁₁ S ₂	C ₆ H ₁₃ N ₁ O ₁₄ S ₃	C ₁₂ H ₁₉ N ₁ O ₁₆ S ₂	C ₁₂ H ₁₉ N ₁ O ₁₉ S ₃	C ₁₆ H ₂₅ N ₁ O ₂₃ S ₃	C ₁₈ H ₂₄ N ₂ O ₃₀ S ₅	C ₁₈ H ₂₉ N ₁ O ₂₈ S ₄	C ₂₂ H ₃₅ N ₁ O ₃₄ S ₅
Exact mass	338.9930	418.9498	497.0145	576.9713	694.9979	917.9800	834.9759	1016.9644
Retention time, min	4.4	9.9	9.1	25.9	35.5	38.8	40.8	43.8
Tetrasaccharides								
Abbreviation	4b°	4b	4c°	4d°	4e	4e°	4g°	4h°
Species #	A4,3,1,1gs	A4,3,1	A4,4,1,1gs	A4,4,0,1gs	A4,5,0	A4,5,0,1gs	A4,5,0,1gs-R	A4,6,0,1gs
Formula	C ₂₆ H ₄₂ N ₂ O ₃₀ S ₃	C ₂₆ H ₄₀ N ₂ O ₃₀ S ₃	C ₂₆ H ₄₂ N ₂ O ₃₃ S ₄	C ₂₄ H ₄₀ N ₂ O ₃₂ S ₄	C ₂₄ H ₃₈ N ₂ O ₃₅ S ₅	C ₂₄ H ₄₀ N ₂ O ₃₅ S ₅	C ₂₈ H ₄₆ N ₂ O ₃₉ S ₅	C ₂₄ H ₄₀ N ₂ O ₃₈ S ₆
Exact mass	958.0985	956.0828	1038.0553	996.0447	1073.9859	1076.0015	1194.0281	1155.9583
Retention time, min	(1) 33.0 (2) 34.0	34.0	38.6	37.7	(1) 39.8 (2) 42.2 (3) 42.9 (4) 43.8	42.3	46.0	47.8
Hexasaccharides								
Abbreviation	6b°	6c°	6c°°	6d°°	6e°	6e°°	6f°	6f°°
Species #	A6,5,1,1gs	A6,6,1,1gs	A6,6,1,2gs	A6,7,0,2gs	A6,7,1,1gs	A6,7,1,2gs	A6,8,0,1gs	A6,8,0,2gs
Formula	C ₃₈ H ₆₁ N ₃ O ₄₆ S ₅	C ₃₈ H ₆₁ N ₃ O ₄₉ S ₆	C ₃₈ H ₆₃ N ₃ O ₄₉ S ₆	C ₃₆ H ₆₁ N ₃ O ₅₁ S ₇	C ₃₈ H ₆₁ N ₃ O ₅₂ S ₇	C ₃₈ H ₆₃ N ₃ O ₅₂ S ₇	C ₃₆ H ₅₉ N ₃ O ₅₄ S ₈	C ₃₆ H ₆₁ N ₃ O ₅₄ S ₈
Exact mass	1455.1130	1535.0698	1537.0854	1575.0317	1615.0266	1617.0423	1652.9729	1654.9885
Retention time, min	(1) 43.8 (2) 44.5 (3) 45.3	(1) 48.4 (2) 49.1 (3) 49.9	47.4	(1) 47.9 (2) 51.8	(1) 53.6 (2) 54.8	52.4	(1) 59.3 (2) 60.3	57.9

Structures are expressed using a code consisting of three numbers (the number of: monosaccharide residues, sulfate groups, and N-acetyl groups, respectively), preceded by the symbol Δ to indicate that the first residue is 4,5-unsaturated uronic acid (as expected by lyases action), or by symbols A or A* for the fragments starting with an aminosugar residue. These designations are followed by 1gs or 2gs symbols to indicate that one of two uronic acid residues of the oligosaccharide are glycol-split.

Table 2

Disaccharide compositional analysis determined by HPLC/UV, of PMH and different RO-heparins (normalized to quantity of digested material)*

	$\bar{x} \pm \Delta x$, % mass.				
	$\Delta U A-GlcNS$ $t_R = 4.3$ min	$\Delta U A-GlcNAc,6S$ $t_R = 4.4$ min	$\Delta U A-GlcNS,6S$ $t_R = 8.2$ min	$\Delta U A,2S-GlcNS$ $t_R = 8.6$ min	$\Delta U A,2S-GlcNS,6S$ $t_R = 25.6$ min
PMH	7.7 ± 0.3		17.5 ± 0.3 #		66.7 ± 0.9
RO-PMH	< LOD	< LOD	< LOD	4.8 ± 0.2	49.2 ± 0.9
RO-GMH	< LOD	< LOD	< LOD	2.1	42.3
RO-BMH	< LOD	< LOD	< LOD	9.5	45.9
RO-BLH	< LOD	< LOD	< LOD	3.9	80.4
RO-FmPMH	< LOD	< LOD	< LOD	4.8	38.1
HI-3	< LOD	< LOD	< LOD	2.7	32.0

* Analyses of digests of PMH and RO-PMH were performed in duplicate (n=2, P=0.95), for other samples single analyses were performed. The t_R values shown in this table were obtained using UV detector.

LOD = limit of detection.

The total amount of these two isomers was determined using the calibration curve obtained for $\Delta U A,2S-GlcNS$, the isomer that is preserved in gs-heparins. Even if $\Delta U A-GlcNS,6S$ is characterized by an extinction coefficient somewhat higher than that of its isomer leading to an overrated value, the percentage found for the sum of the two isomers is in agreement with the results obtained by other authors. [36]

TABLE 3

Structure of the major GlcA- and IdoA-containing tetra- and hexasaccharide fragments obtained from PMH (literature data) A and proposed corresponding glycol-split oligosaccharides (this work) B. Isomers are identified by letters.

A*	B
Δ4,4,1-A	ΔU2S-GlcNAc6S-GlcA-GlcNS6S
Δ4,4,1-B	ΔU2S-GlcNAc6S-IdoA-GlcNS6S
Δ4,4,0-A	ΔU2S-GlcNS-GlcA-GlcNS6S
Δ4,4,0-B	ΔU2S-GlcNS6S-GlcA-GlcNS
Δ4,5,0	ΔU2S-GlcNS,6S-GlcA-GlcNS,6S
Δ4,6,0	ΔU2S-GlcNS,6S-GlcA-GlcNS,3,6S
Δ6,4,1	ΔU2S-GlcNS-IdoA2S-GlcNAc-GlcA-GlcNS
Δ6,5,1-A	ΔU2S-GlcNS-IdoA2S-GlcNAc-GlcA-GlcNS,6S
Δ6,5,1-B	ΔU2S-GlcNS,6S-IdoA-GlcNAc6S-GlcA-GlcN,6S
Δ6,6,1	ΔU2S-GlcNS,6S-IdoA2S-GlcNAc,6S-GlcA-GlcNS,6S
Δ6,6,1-A	ΔU2S-GlcNS,6S-IdoA-GlcNAc6S-GlcA-GlcNS,6S
Δ6,6,1-B	ΔU2S-GlcNS,6S-IdoA2S-GlcNAc-GlcA-GlcNS,6S
Δ6,6,1-C	ΔU2S-GlcNS,6S-IdoA-GlcNAc6S-GlcA-GlcNS,3S
Δ6,7,0	ΔU2S-GlcNS,6S-GlcA-GlcNS,6S-GlcA-GlcNS,6S
Δ6,7,1	ΔU2S-GlcNS,6S-IdoA-GlcNAc6S-GlcA-GlcNS,3,6,S
Δ6,8,0	ΔU2S-GlcNS,6S-IdoA2S-GlcNS,6S-GlcA-GlcNS,6S

* The heparin oligosaccharides were identified from Linhardt's group upon exhaustive digestion with heparinase I, II, or III (4) and partial digestion only with heparinase I (53). Hexasaccharides were obtained from pig mucosal heparin by Sugahara group (54) from a "hexasaccharidic fraction" isolated by gel-filtration on Bio-Gel P-10 and sub-fractionated on a column of amine-bound silica under a sodium phosphate gradient. Tetrasaccharide ΔU2S-GlcNS,6S-GlcA-GlcNS,3S,6S was isolated from bovine lung heparin (52).