Oligosaccharide Mapping of Low Molecular Weight Heparins: Structure and Activity Differences

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Low molecular weight heparins from a variety of commercial sources were examined. These had been prepared by several methods including peroxidative cleavage, nitrous acid cleavage, chemical β-elimination, enzymatic β-elimination, and chromatographic fractionation. The molecular weight and polydispersity of these low molecular weight heparins showed greater differences than were observed for typical commercial heparin preparations. Considerable differences were also observed in the antithrombin III mediated anti factor Xa activity, the heparin cofactor II mediated antifactor IIa activity, and the USP activity of these low molecular weight heparins. An oligosaccharide-mapping technique (comparable to the peptide mapping of proteins) was applied to these low molecular weight heparins in an effort to understand the structural features responsible for their activity differences. Heparin lyase from *Flavobacterium heparinum* was first used to depolymerize the low molecular weight heparin into its constituent oligosaccharides. The oligosaccharides present in the resultant mixture were identified and quantitated by using standard oligosaccharides of defined structure on gradient polyacrylamide gel electrophoresis and strong anion exchange high pressure liquid chromatography. Six of the oligosaccharide products have been identified and represent nearly 90 wt % of heparin's mass. Even though all the low molecular weight heparins showed these six oligosaccharide components, their content in each varied greatly, accounting for 20 to over 90% of their mass. The antithrombin III mediated anti factor Xa activities of the low molecular weight heparins correlated only poorly to the concentration of a hexasaccharide containing a portion of heparin's antithrombin III binding site. The heparin cofactor II mediated antifactor IIa activity, however, could not be correlated to these six oligosaccharides of known structure nor to the molecular weight or charge density of these low molecular weight heparins. The low molecular weight heparins prepared by different methods each showed a new distinctive oligosaccharide in their maps. Their isolation and structural characterization, which included two-dimensional NMR and fast atom bombardment mass spectrometry, indicated that these unusual oligosaccharides result from end-sugar modification during chemical depolymerization. Both gel electrophoresis and high-pressure liquid chromatography mapping techniques showed a greater structural diversity between low molecular weight heparins than had previously been observed between similarly analyzed commercial heparins.

Heparin is a polydisperse (M_r 5000–40 000, M_r (av) 14 000[1]), highly sulfated, alternating copolymer of 1→4 linked glucosamine and hexuronic acid and has been used over the last half-century as an anticoagulant.[2] Heparin exhibits undesirable side effects, such as hemorrhagic complications,[3] and thus while heparin is in widespread clinical use it has been cited as the drug most responsible for death in otherwise healthy patients.[4] Low molecular weight (LMW) heparins have recently been introduced as heparin substitutes and are claimed to have reduced side effects (a higher safety/efficacy ratio), more predictable pharmacological action, sustained antithrombotic activity, and better bioavailability.[5] These potential advantages have led to the development of several commercial LMW heparin preparations.

LMW heparin is prepared, in low yield, by size fractionation or in higher yield by the partial, controlled depolymerization of heparin. Fraxiparin (CY216) is obtained by the fractionation of commercial porcine mucosal heparin. CY222 and Fragmin (KABI 2165) are both prepared by partial nitrous acid depolymerization of porcine mucosal heparin.[6] RD heparin and Fluxum (OP 2123) are prepared from the oxidative cleavage of porcine mucosal and bovine mucosal heparins, respectively.[7] Enoxaparin (PK 10169) is obtained by benzylation followed by β-elimination and alkaline hydrolysis.[8] Logiparin (LHN-1) is obtained by heparin lyase catalyzed partial β-eliminative depolymerization of porcine heparin.[9] A LMW heparin standard, prepared by partial nitrous acid depolymerization, has been introduced by the National Institute of Biological Standards and Control (NIBSC, London, England) under the auspices of the World Health Organization (WHO, Geneva, Switzerland).[10]

Table I. Ash Content and Degree of Sulfation of LMW Heparins

sample	percent ash	degree of sulfation[a]
tetrasaccharide standard **7**	39.1	3.00
Hep A Porc hep	31.4	2.41
Hep B CY216	22.9	1.75
Hep C CY222	29.3	2.29
Hep D Kabi 2165	28.9	2.22
Hep E OP2123	20.3	1.55
Hep F RD heparin	34.7	2.66
Hep G PK10169	29.5	2.26
Hep H LHN-1	12.9	0.99
Hep J NIBSC	33.9	2.60

[a] Degree of sulfation is the number of sulfate groups per disaccharide unit. The heparin tetrasaccharide (oligosaccharide **7** in Table IV) is salt-free and has six sulfate groups. It was used to calculate the degree of sulfation of the LMW heparins. The salt content of the LMW heparins was <7 wt % by conductivity with F, G, and C having the highest conductivity. This could result in our overestimating the degree of sulfation by 9–10% for these three LMW heparins.

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Table II. Molecular Weight and Dispersity of LMW Heparins

sample	molecular weight by GPC-HPLC[a] wt av	molecular weight by GPC-HPLC[a] no. av	polydispersity by GPC-HPLC[a]	molecular weight by gradient page[b]
Hep B CY216	5506	4626	1.190	4470
Hep C CY222	3410	2529	1.348	3160
Hep D Kabi2165	6370	5393	1.181	6100
Hep E OP2123	6511	4922	1.323	6610
Hep F RDhep	6221	4567	1.362	6460
Hep G PK10169	3789	2823	1.342	4170
Hep H LHN1	4850	3561	1.362	6100

[a] Molecular weight data was obtained from ref 12. [b] Molecular weight was estimated from a plot of log M_r vs migration distance with use of the partially depolymerized bovine heparin ladder of bands (Figure 2, lane I). A correlation of $r = 0.99$ was obtained between $M_r = 1000$ and 10 000. Porcine heparin and bovine heparin (a) (Figure 1, lanes A and I) had molecular weights estimated at 17 380 and 11 220, respectively.

Recent comparative biochemical and pharmacological studies of these LMW heparins have clearly demonstrated that these are not biologically equivalent products.[11,12] This study probes the chemical differences between LMW heparins by using the recently developed technique of oligosaccharide mapping.[13,14] Two oligosaccharides with unique sequences are isolated from these LMW heparins and their structures characterized. These unusual sequences represent artifacts introduced in the depolymerization process and may play a role in the biological activity of a particular LMW heparin.[15]

Results

The LMW heparins studied represented bulk drug preparations and thus were initially assessed for ash content (Table I). The ash content of the LMW heparins ranged from 12.9 to 34.7 wt %. In addition, the ash content of a pure, salt-free, hexasulfated tetrasaccharide having a molecular formula $C_{24}H_{31}O_{38}N_2S_6Na_8$ was determined to be 39.1%. On the basis of the ash content of this standard and its three sulfates per disaccharide unit, the degree of sulfation of each LMW heparin was estimated. The LMW heparins all contained <7 wt % salt as measured by conductivity using a potassium chloride standard curve, with LMW heparins F, G, and C having the greatest conductivity.

The molecular weight and dispersity of each LMW was measured by using gel permeation HPLC using UV detection and are presented in Table II. Molecular weights were verified by viscometry[16] and by gradient polyacrylamide gel electrophoresis (PAGE) (Figure 1). Gradient PAGE not only provides both an estimate of molecular weight and dispersity (Table II) but also, because it is a high-resolution technique, gives information on the presence and distribution of oligomer and polymer chains of defined length or degree of polymerization (dp).

Figure 1. Stained gradient polyacrylamide gel of intact heparins and LMW heparins. Lanes contain the following (100 μg): A, Hep A porcine mucosal PM16885; B, LMW-Hep B, CY216; C, LMW-Hep C, CY222; D, LMW-Hep D, Kabi2165; E, LMW-Hep E, OP2123; F, LMW-Hep F, RD heparin; G, LMW-Hep G, PK10169; H, LMW-Hep H, LHN-1; and I, Hep-I bovine lung 16F0188.

Table III. Activity of LMW Heparins

sample	HCII-mediated antifactor IIa: IC_{50}, μg/mL	ATIII-mediated[a] antifactor Xa, units/mg	USP activity,[b] units/mg
Hep A porc	1.5	172	148
Hep B CY216	44.6	95	45
Hep C CY222	50.0	58	28
Hep D Kabi 2165	17.0	142	75
Hep E OP2123	19.0	82	80
Hep F RD heparin	23.7	60	72
Hep G PK10169	50.0	95	55
Hep H LHN-1	19.0	85	78
Hep I bov (b)	nd	155	160

[a] Antifactor Xa activity was measured against NIBSC LMW heparin standard. Data from ref 12. [b] USP activity was measured against the USP K-1 standard heparin. Data from ref 12.

The antithrombin III (ATIII) mediated antifactor Xa activity of each LMW heparin (measured on a relative basis against the NIBSC LMW heparin standard) and the USP activity (measured against USP K-1 heparin standard) have been previously reported[11,12] and are presented in Table III along with the their heparin cofactor II (HCII) mediated antifactor IIa activities. Since a heparin (or LMW heparin) standard having defined HCII mediated antifactor IIa unitage is not available, we have chosen instead to express this activity in terms of an IC_{50} value[17] (Table III).

The LMW heparins were each depolymerized with heparin lyase and oligosaccharide maps were constructed by using gradient PAGE and strong anion exchange (SAX) HPLC (Figures 2 and 3). Components within each map were identified and their concentrations were measured

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Table IV. Oligosaccharide Components of LMW Heparins

sample	μg of oligosaccharide/100 μg of heparin[a-c]							total of oligosaccharides in μg/100 μg of heparin[b]
	1	2	4	5	7	8	9	
Hep A porc hep	1.12	33.39	3.20	8.93	10.60	–	3.83	61.07
Hep B CY216	1.00	22.51	1.26	8.69	5.61	–	3.27	45.34
Hep C CY222	0.55	10.88	2.78	2.51	2.64	–	2.24	21.26
Hep D KABI2165	0.95	29.27	4.60	7.09	11.01	–	7.30	60.21
Hep E OP2123	1.95	33.06	5.42	7.80	6.36	1.50	1.04	56.95
Hep F RD heparin	1.60	50.90	9.82	15.33	19.91	–	4.76	102.31
Hep G PK10169	1.52	40.14	2.06	24.96	12.00	2.60	5.27	88.55
Hep H LHN-1	2.50	55.03	6.39	16.36	17.62	2.08	5.18	105.12

[a] Oligosaccharides structures are as follows: **1**, ΔUA2S(1→4)-α-D-GlcN2S; **2**, ΔUA2S(1→4)-α-D-GlcN2S6S; **4**, ΔUA2S(1→4)-α-D-GlcN2S(1→4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S; **5**, ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S6S; **7**, ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA2S(1→4)-α-D-GlcN2S6S; **8**, ΔUA2S(1→4)-α-D-GlcN2S6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S3S6S; and **9**, ΔUA2S-(1→4)-α-D-GlcN2S6S(1→4)-α-L-IdoA(1→4)-α-D-GlcNAc6S(1→4)-β-D-GlcA(1→4)-α-D-GlcN2S3S6S. [b] Heparin content determined by carbazole assay; ref 29. [c] The assignments of oligosaccharide **8** in Hep G and Hep H and **9** in Hep F are based on coelution of the peak with standard samples.

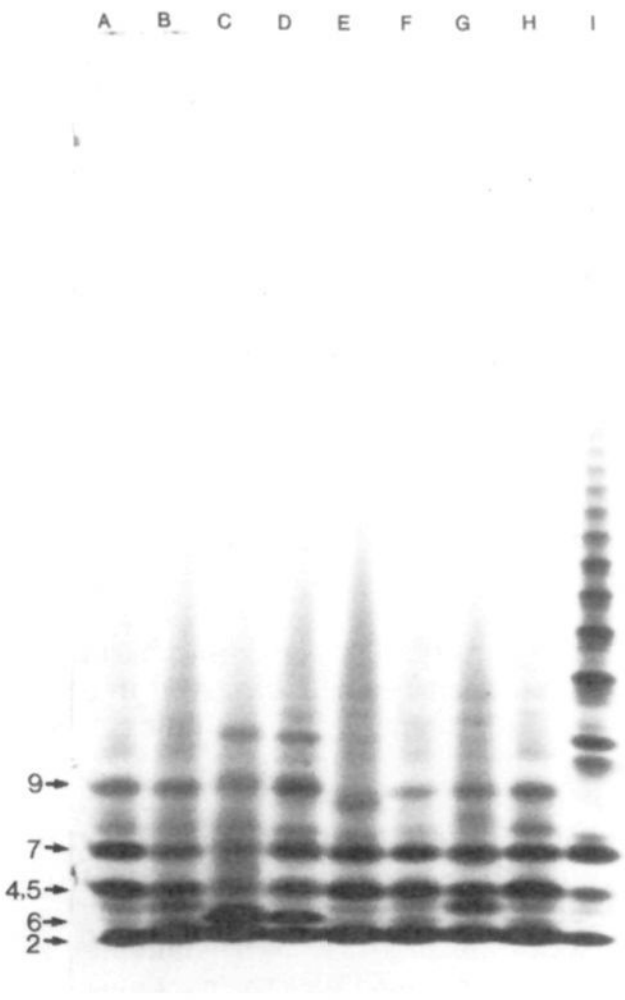

Figure 2. Stained gradient polyacrylamide gel showing oligosaccharide maps of heparin lyase depolymerized heparins and LMW heparins. Lanes contain the following (100 μg): A, Hep A porcine mucosal PM16885; B, LMW-Hep B, CY216; C, LMW-Hep C, CY222; D, LMW-Hep D, Kabi2165; E, LMW-Hep E, OP2123; F, LMW-Hep F, RD heparin; G, LMW-Hep G, PK10169; and H, LMW-Hep H, LHN-1. Lane I contains partially (80%) depolymerized Hep-I bovine lung (a) 16F0188, the major bands in this lane correspond to repeating trisulfated disaccharide oligomers ranging from dp 2 (M_r 665) to dp 30 (M_r 9975).

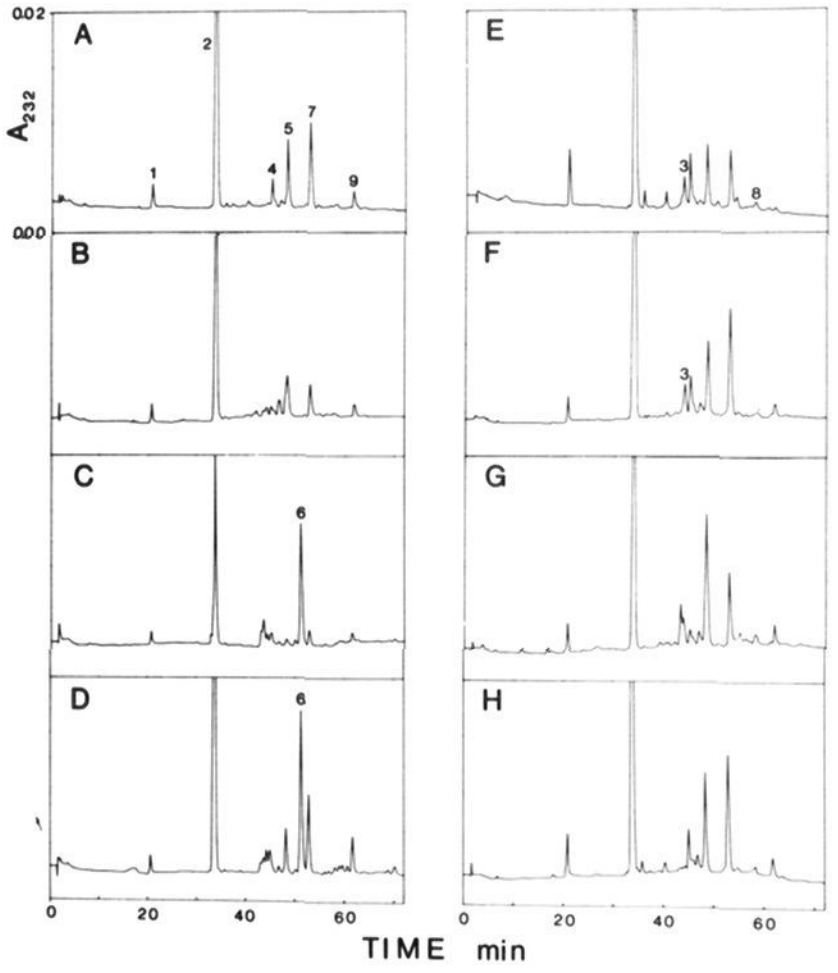

Figure 3. SAX-HPLC oligosaccharide maps of heparin lyase depolymerized heparin and LMW heparins: A, Hep A porcine mucosal PM16885; B, LMW-Hep B, CY216; C, LMW-Hep C, CY222; D, LMW-Hep D, Kabi2165; E, LMW-Hep E, OP2123; F, LMW-Hep F, RD heparin; G, LMW-Hep G, PK10169; and H, LMW-Hep H, LHN-1. Peaks are labeled which correspond to oligosaccharides 1–8.

by SAX-HPLC against standard curves generated by using defined oligosaccharide standards (Table IV). Major and previously unidentified peaks were observed in the SAX-HPLC chromatograms of heparin lyase depolymerized LMW heparins prepared with use of nitrous acid and LMW heparins prepared by oxidative depolymerization (Figures 2 and 3). These components were isolated and purified, and their structures were examined by using 600-MHz ^{1}H NMR and fast atom bombardment (FAB) mass spectral methods (see Experimental Section). Together with the known specificity of heparin lyase[18] and the known chemistry of the nitrous acid[6] and oxidative[7] depolymerization reactions this spectral data establishes structure **6** for the unusual product resulting from nitrous acid cleavage. The oligosaccharide arising from oxidative cleavage could not be rigorously established owing to its instability, and a tentative structure **3** for the oligosaccharide is proposed.

The ATIII-mediated antifactor Xa activity of each LMW heparin correlated only poorly with the number of AT III binding sites in each preparation calculated from the oligosaccharides containing the characteristic[19,20] 2-deoxy-2-sulfamino-α-D-glucopyranose 3,6-disulfate residue.

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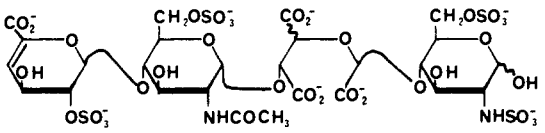
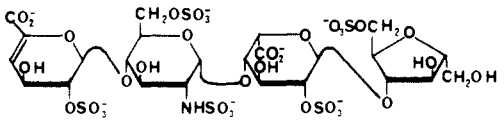


3



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The HCII-mediated activity of the LMW heparins failed to correlate to the concentration of any of the six oligosaccharides of known structure seen in the SAX-HPLC maps (Figure 3). Neither the molecular weight (Table II) nor the degree of sulfation (Table I) of the LMW heparins correlated to the HCII-mediated anti factor IIa activity (Table III).

Discussion

LMW heparin was recently introduced as a heparin substitute.[5] Clinical studies using LMW heparin in a variety of medical applications continue to demonstrate both its efficacy as an antithrombotic agent and its decreased toxic side effects compared to the parent drug, unfractionated heparin.[5] Size fractionation of commercial (glycosaminoglycan) heparin (M_r 5000–40000, M_r (av) 14000) results in a LMW heparin having the characteristics of heparin with reduced molecular weight. This LMW heparin (M_r 5000–8000) represents less than 15 wt % of the commercial product,[17] making this approach to preparing LMW heparin unprofitable. A more practical approach to preparing LMW heparin is to reduce the average molecular weight of heparin by partial chemical or enzymatic depolymerization. Manufacturers have approached this depolymerization by using several different methods including (1) deaminative cleavage at N-sulfated glucosamine residues using nitrous acid followed by reduction of the resulting anhydromannose reducing end sugar;[6] (2) oxidative cleavage of heparin at the free vicinal diol functionality present in unsulfated glucuronic and iduronic acid residues;[7] and (3) eliminative cleavage either enzymatically at the glycosidic linkage between a 2-sulfated glucosamine residue and 2-sulfated iduronic acid,[18] or chemically by first forming the benzyl ester of iduronic acid followed by base-catalyzed β-elimination and hydrolysis.[8] Recent studies have demonstrated the bioinequivalence of LMW heparin preparations.[11,12]

Analysis of the intact LMW heparins (Table I) indicates that there are substantial differences in their residual ash content. Since the samples showed similar conductivities and were each dried under uniform conditions to obtain a consistent water content, the difference in their ash content is primarily attributable to the different relative degree of sulfation of these LMW heparins. The weight and number average molecular weights and dispersity of the LMW heparins also showed marked variability (Table II). The use of high-resolution gradient PAGE revealed a remarkable level of variation in the presence and distribution of polymer chains of defined dp (Figure 1). These data confirm the differences in the molecular weight and polydispersity of LMW heparins and clearly demonstrate the chemical inequivalence of commercial LMW heparins. While the data shown in Tables I and II and Figure 1 measure the physical-chemical properties of the LMW heparins, they do not permit the correlation of differences in any specific structural feature or features to the bioinequivalence of these preparations (Table III).

Oligosaccharide mapping of these LMW heparins was undertaken to obtain the structural information necessary to begin to establish a structure–activity relationship. Oligosaccharide mapping of acidic polysaccharides is an approach comparable to the peptide mapping of proteins.[13,14] LMW heparin is first depolymerized enzymatically and then fractionated by either using gradient PAGE or SAX-HPLC. By use of standard oligosaccharides, specific sequences within the polymeric substrate can be identified and quantitated. The results of mapping these LMW heparins are given in Figures 2 and 3 and Table IV. We had previously reported that for commercial heparins, ATIII affinity fractionated heparins, and charge-fractionated heparins the concentrations of ATIII binding sites, present within a heparin preparation, correlates to its ATIII mediated antifactor Xa activity.[14,17] Although a similar relationship was observed in this study for LMW heparins, a poor correlation was observed, possibly due to the narrow range of the activities.[14,17] We had previously reported that the HCII-mediated antifactor IIa activity of heparin fractionated by ion-exchange chromatography or HCII-affinity chromatography, correlated well with their degree of sulfation, their molecular weight, and their content of fully sulfated oligosaccharides **2**, **7**, and **8**.[17] The HCII-mediated antifactor IIa activity of LMW heparins, however, only poorly correlated to the relative concentration of oligosaccharide sequences having a high level of sulfation (RS = [2 + 7 + 8]/[1 + 4 + 5 + 8 + 9]). LMW heparins D, E, F, and H have an RS >0.67 while LMW heparins B, C, and G have an RS <0.63. Neither the degree of sulfation, as determined from ash content (Table I), nor the molecular weight (Table II) of the LMW heparins correlated to the HCII mediated antifactor IIa activity.

One unusual feature of the oligosaccharide maps of LMW heparins (not previously observed in commercial heparins[14]) is the presence of major unidentified oligosaccharides (Figures 2 and 3). We postulated that these might be the result of a chemical modification in the heparin introduced during the depolymerization reaction. The unidentified oligosaccharide formed in the nitrous acid depolymerized products accounted for 120 nmol/mg of the total oligosaccharide mixture. The nanomoles of this product is approximately 40% of the nanomoles of LMW heparin C, consistent with this unidentified oligosaccharide arising from the reducing end of each polymer chain formed during nitrous acid depolymerization. Fast atom bombardment mass spectral analysis (FAB-MS) of this oligosaccharide showed a distinct molecular ion at m/z 1190 consistent with a pentasulfated tetrasaccharide.

Analysis of the 600-MHz ^{1}H NMR spectrum revealed that the nonreducing end sugar residue is ΔUA2S (4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid) as shown by the olefinic proton signal at 5.99 ppm and an anomeric proton signal at 5.51 ppm. The other two anomeric proton signals seen at 5.44 and 5.19 ppm could be assigned to the internal disaccharide, →4)-α-D-GlcN2S6S(1→4)-α-L-IdoA2S (1→ (IdoA, iduronic acid).[21] The presence of only three anomeric proton resonances and only one H-2 resonance at 3.31 ppm, assignable to the internal 2,6-disulfated glucosamine, suggested that the reducing end sugar is ring contracted. Structure **6** was proposed on the basis of these NMR and mass spectral data and consistent with both the specificity of heparin

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lyase[18] and the known chemistry of nitrous acid depolymerization of heparin.[6,22] Two-dimensional homonuclear correlated spectroscopy (COSY) (not shown) confirmed the structure 6. Starting from the olefinic proton signal (5.99 ppm) of the nonreducing end, ΔUA2S, all proton resonances of this residue are easily identified as follows: 4.32 (H-3), 4.62 (H-2), and 5.51 (H-1) ppm. These data are consistent with those reported for ΔUA2S residues of oligosaccharides prepared by heparin lyase depolymerization.[21,23] The anomeric proton resonance (5.44 ppm) of the internal GlcN2S6S residue showed connectivity to the H-2 resonance at 3.31 ppm which in turn showed cross-peaks with the H-3 resonance at 3.65 ppm, confirming that the C-3 position is unsulfated. The coupling network of the internal IdoA2S residue was also easily identifiable and led to the following assignment: 5.19 (H-1), 4.30 (H-2), 4.21 (H-3), 4.16 (H-4), and 4.61 (H-5) ppm. Finally the signals of the reducing end were identified beginning with the H-1 and H'-1 signals seen at 3.71 and 3.77 ppm, respectively. Both of these signals showed cross-peaks with the H-2 resonance at 4.00 ppm that in turn showed connectivity to the H-3 resonance at 4.13 ppm, confirming that the C-3 position bears a free hydroxyl group. These data are comparable to those reported for a similar ring-contracted tetrasaccharide prepared by nitrous acid depolymerization of bovine lung heparin.[22] It is, however, unusual that the ring-contracted anhydromannose reducing end is found primarily in a single oligosaccharide component. This result suggests selectivity of nitrous acid toward certain 2,6-disulfated glucosamine residues within the heparin polymer. The unexpected selectivity of this reagent requires additional study on structurally defined substrates.

The unidentified oligosaccharide formed in the oxidative depolymerization of heparin occurred in 18% of the chains, representing a somewhat lower amount than anticipated from the molar concentration of the LMW heparin from which it was derived. This suggests that less than half of the LMW heparin chains carry the sequence contained in this unidentified oligosaccharide. Several attempts to unambiguously establish the structure of this oligosaccharide failed. An oligosaccharide having four to five sulfate groups was isolated by SAX-HPLC in 1.3% yield. The expected yield was 4% based on analytical SAX-HPLC. This component ran true when reanalyzed by SAX-HPLC and gave a band on gradient PAGE analysis runing slightly ahead of a pentasulfated tetrasaccharide standard (4 in Table IV). High-field NMR analysis on 800 μg of sample required that it be held at room temperature for 1 h. The spectrum showed the sample to be a complex mixture. Appearance of signals of a major component at 5.99 and 5.51 ppm indicated that its nonreducing end sugar was ΔUA2S and a signal at 2.05 ppm was attributed to the protons of the methyl group of an N-acetylated glucosamine residue. Reanalysis of this sample by SAX-HPLC confirmed that it had decomposed. Many minor products were observed in addition to a predominant, unsaturated disulfated disaccharide. The low recovery of 3 by SAX-HPLC had also suggested that decomposition was taking place. A tentative structure for oligosaccharide 3 is proposed on the basis of the NMR data which is consistent with the specificity of heparin lyase,[24] the mechanism of oxidative cleavage of uronic acid residues present in heparin,[14] the instability of this oligosaccharide and its breakdown to an unsaturated disulfated disaccharide.

LMW heparins prepared by either enzymatic or chemical β-eliminative cleavage contain unsaturated uronic acid at their reducing end as demonstrated by an absorbance maxima at 232 nm in the intact LMW heparin (Table I). The presence of this unusual sugar residue, in the drug, may act to block its biotransformation in vivo (as has been reported for oligosaccharides having this same structural feature[15]) slowing its rate of clearance. Similarly, the effect of altered reducing ends, now established to be present in LMW heparins prepared through nitrous acid depolymerization, on in vivo biotransformation needs to be examined.

In conclusion, this study demonstrates that commercial LMW heparins are not chemically equivalent. The differences are primarily the result of the chemical or enzymatic process used in their depolymerization. The chemical inequivalence of these preparations results in different molecular weights, degrees of sulfation, and oligosaccharide maps. These altered physical–chemical properties affect the anticoagulant activities of LMW heparins making them bioinequivalent. Finally, all the LMW heparins prepared by chemical or enzymatic depolymerization contain certain modified sugar residues, at either their reducing or nonreducing ends. The effect of these modified sugars on the biological activities of the various LMW heparins requires additional investigation.

Experimental Section

Materials. Low molecular weight heparins obtained as bulk drug from the manufacturers included Fraxiparin, CY216 (lot no. CY 216A) and CY222 (lot no. PII WH), Choay Laboratories, Paris, France, Fragmin, KABI 2165 (lot no. DXN 75), KabiVitrum, Stockholm, Sweden, Fluxum OP 2123 (lot. no. LMW 2123), Opocrin, Corlo, Italy, RD heparin (lot. no. 11885), Hepar Industries, Franklin, OH, Logiparin, LHN-1 (lot. no. F 85030), Novo Industries, Copenhagen, Denmark, Enoxaparin, PK 10169 (lot no. 781), Pharmuka Laboratories, Gennevilliers, France, and the first international LMW heparin standard 85/600 (1000 IU, freeze-dried in an ampule), NIBSC, London, England. Porcine mucosal heparin, sodium salt (160 units/mg, lot no. PM16885) was from Hepar Industries. Bovine lung heparin, sodium salt (a) (149 units/mg, lot no. 16F0188) and (b) (140 units/mg, lot no. 0324B) were from Sigma Chemical Co. and Upjohn Co., Kalamazoo, MI, respectively. Standard USP (Bethesda, MD) K1 heparin (355 units/mL) was used. Homogeneous, structurally defined heparin–oligosaccharide standards were prepared from porcine mucosal heparin[18] and bovine lung heparin.[23] Antithrombin III and heparin cofactor II were either prepared from human plasma and purified to homogeneity by our laboratory[25] or purchased from KabiVitrum and Diagnostica Stago, Asnieres, France, respectively. Factor IIa (thrombin) was from Ortho Diagnostics, Raritan, NJ, factor Xa was from Enzyme Research Laboratories, South Bend, IN, and chromogenic substrates were from American Diagnostica, Greenwich, CT. Heparin lyase (heparinase, EC 4.2.2.7) was prepared from *Flavobacterium heparinum*[24] and purified [5 units (μmol/min)/mg] to catalytic purity.[26] All regents used in electrophoresis were from Fisher Chemical Co., Fairlawn, NJ. Carbazole was from Sigma Chemical Co.

Electrophoresis was performed on a Hoefer (San Francisco, CA) SE600 vertical-slab-gel unit equipped with a Bio-Rad (Richmond, CA) Model 1420B power source. Desalting was performed with Spectropore dialysis tubing (M_r cut-off 1000) from Spectrum Medical, Los Angeles, CA, or with a P-2 gel desalting column from Bio-Rad. Gel permeation HPLC was performed with a TSK 3000

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column (30 × 0.75 cm) on a Waters 840 liquid chromatography system equipped with a model 490 multiwavelength UV detector and a Digital 300 series minicomputer running Waters 840 software specifically designed for applications in the molecular weight determination of polymers. Strong anion exchange HPLC was performed with a Phase Separations (Norwalk, CT) Spherisorb (analytical column, 4.6 mm × 25 cm and semipreparative column, 2 cm × 25 cm, both were 5 μm particle size) column on dual LDC (Riviera Beach, CA) Constametric II pumps and a variable-wavelength UV-5 detector from ISCO, Lincoln, NE. Gradient control and data collection used an Apple IIe microcomputer running software from Interactive Microware, State College, PA. Conductivity was measured with a solution analyzer 4530A from Amber Scientific, San Diego, CA. Residual ash analysis was performed by Desert Analytics, Tucson, AZ.

NMR spectroscopy was performed in ^2H_2O (99.996 atom %) with TSP-D_4 (99+ atom %) as internal standard both from Aldrich Chemical Co., Milwaukee, WI, on a Bruker AM600 (^1H at 600 MHz) spectrometer. The two-dimensional COSY-45 experiment[27] was run in the absolute magnitude mode with standard Bruker software on a Bruker WM 360 spectrometer equipped with ASPECT 2000 computer. A spectral width of 1404 Hz in both dimensions was used to collect a 512 × 2048 data matrix with 16 transients for each of the 512 experiments. The matrix was transformed by using sine-bell window functions in both dimensions. The sample concentration was about 5 mM. Mass spectrometry was performed on a VG Analytical ZAB-HF spectrometer operating under FAB mode.[28]

Preparation of Low Molecular Weight Heparin Samples. Stock solutions of LMW heparins were prepared by dissolving bulk drug at 20 mg/mL in distilled water. Porcine and bovine heparins were dialyzed exhaustively against 100 volumes of distilled water, freeze-dried, and prepared at 20 mg/mL.

Analysis of Intact LMW Heparins. Stock solution of each LMW heparin (150 μL) was transferred to a sample container, freeze-dried, and analyzed for residual ash. A heparin sample (and a salt-free sample of hexasulfated tetrasaccharide) was similarly treated and was analyzed for ash content. A determination of the uronic acid content of each stock solution was made with carbazole.[29] Each stock solution (100 μL) was diluted 100-fold with distilled water, and the conductivity was determined at 25 °C.

Molecular Weight Profiling of LMW Heparins. Prior to their use, each gel permeation column was calibrated with a collection of anionic polymers serving as molecular weight standards.[16] LMW heparin (20 μL, 10 mg/mL) was injected on the column and eluted with 0.5 M sodium sulfate at a flow rate of 1 mL/min and eluant was monitored at 205 nm. Values for retention time (t_R) and polymer dispersity (D) were determined and used along with the known molecular weights of the standards to calculate a calibration curve by the third polynomial regression with coefficients D_0, D_1, D_2, and D_3. Molecular weight was calculated with the use of eq 1. The total area under the elution

$$MW = D_0 + D_1(t_R) + D_2(t_R)^2 + D_3(t_R)^3 \quad (1)$$

curve was determined by integration. The computer also cut the curve into 50 equal time slices resulting in 50 MW and t_R values which could be used to determine D. The molecuar weights were verified by viscometry.[16] The molecular weight and dispersity of intact LMW heparin samples were also examined with gradient polyacrylamide gel electrophoresis (PAGE).[13] LMW heparin (140 μL, 40 mg/mL) was analyzed on a gradient gel (12–22% linear gradient of total acrylamide) stained with alcian blue.[13] Average molecular weight was estimated from an oligosaccharide ladder of bands (degree of polymerization (dp) = 2–30) of partially (80% completion) depolymerized bovine heparin.[13]

Activity of LMW Heparins. The antithrombin III mediated antifactor Xa activities of LMW heparins were measured against a standard curve constructed with use of the NIBSC standard as previously reported.[11] The heparin cofactor II mediated antifactor IIa activities of the LMW heparin samples were determined with use of an amidolytic assay. Heparin or LMW heparin (0, 3, 6, and 9 μL of 1 mg of heparin/mL of distilled water) was added to 50 mM Tris–hydrochloride, 7.5 mM EDTA, and 150 mM sodium chloride, pH 8.4 buffer (340, 337, 334, and 331 μL, respectively). Heparin cofactor II (10 μL, 500 μg/mL in saline) and factor IIa (50 μL, 6.67 NIH units) were then added to each tube. Following incubation for 1 min at 37 °C, 200 μL of chromogenic substrate (0.75 mM Chromozym Th or Spectrozyme Th in distilled water) was added. The change in optical density at 405 nm was monitored for 1 min. Heparin (in micrograms) vs log A_{405} was plotted. The IC_{50} for each heparin sample was determined from its own standard curve by eq 2.

$$0.5 = [\Delta A_{405} \text{ with } 0\ \mu g/mL \text{ heparin} - \Delta A_{405} \text{ with } (IC_{50})\ \mu g/mL \text{ heparin}]/[\Delta A_{405} \text{ with } 0\ \mu g/mL \text{ heparin} - \Delta A_{405} \text{ without factor IIa}] \quad (2)$$

Oligosaccharide Mapping of LMW Heparins. LMW heparin (50 μL of 20 mg/mL) was added to 425 μL of 0.2 M sodium chloride and 5 mM sodium phosphate, pH 7.0. Heparin lyase (25 μL, 0.015 IU) was added to make the total solution volume 500 μL. The reactions were run at 30 °C to completion in 8 h and terminated by heating to 100 °C for 1 min. Depolymerized LMW heparin was analyzed by strong anion exchange HPLC by injecting two concentrations, 40 μg/40 μL and 4 μg/mL, onto the Spherisorb column equilibrated with 200 mM sodium chloride at pH 3.5. The sample was eluted with use of a linear salt gradient from 0.2 M to 1.4 M over 80 min at a flow rate of 1.5 mL/min. The elution profile was monitored at 232 nm, 0.02 AUFS. Peaks were tentatively identified by either coelution with an authentic sample or retention time. Depolymerized LMW heparin was analyzed with use of gradient PAGE by adding sample (40 μL of 40 μg/mL) to a stacking gel of 5% (total acrylamide) with a 12–22% linear gradient resolving gel.[13] After 400 V was applied for 16 h (running a bromophenol blue marker 20 cm into the resolving gel), the gel was fixed and stained in alcian blue and destained.[13] The bands were tentatively identified by comigration with authentic standards.

Isolation and Characterization of Unusual, Modified Oligosaccharides Present in LMW Heparins Prepared with the Use of Nitrous Acid and Oxidative Depolymerizations. Twenty five milligrams of LMW heparin CY 222 and 100 mg of RD heparin were depolymerized as described above with use of 375 and 1500 mIU of heparin lyase, respectively. The resulting oligosaccharide products were desalted with use of a 2.5 × 45 cm P-2 column eluted with water at a flow rate of 2 mL/min. After freeze-drying, each sample was reconstituted in 100 mg/mL of water and applied to a semipreparative strong anion exchange HPLC column[18] to obtain the oligosaccharides of interest. The recovered oligosaccharide (**3** (1.3 mg), **6** (2.5 mg)) was desalted, freeze-dried, and reapplied to the same HPLC column. Following final desalting and freeze-drying, oligosaccharides **3** and **6** were analyzed to be >85% and >95% pure, respectively, by analytical strong anion exchange HPLC and gradient polyacrylamide gel electrophoresis. After each sample was thoroughly exchanged with ^2H_2O, ^1H NMR was obtained on each sample at 600 MHz with TSP as an internal standard. The FAB mass spectrum of oligosaccharide **6** was obtained with use of a triethanolamine matrix.[28]

Oligosaccharide **6** gave a molecular ion at m/z 1190 $[M - Na^+]^-$ when analyzed by FAB-MS corresponding to $C_{24}H_{30}O_{35}NS_5Na_6$. ^1H NMR (D_2O) (sugar residues of this tetrasaccharide are designated as A→B→C→D): residue A, δ 5.51 (d, J = 2.2 Hz, 1 H, H-1), 4.62 (m, 1 H, H-2), 4.32 (m, 1 H, H-3), 5.99 (dd, $J_{3,4}$ = 4.8 Hz, $J_{2,4}$ = 1.2 Hz, 1 H, H-4); residue B, 5.44 (d, $J_{1,2}$ = 3.6 Hz, 1 H, H-1), 3.31 (dd, $J_{2,1}$ = 3.6 Hz, $J_{2,3}$ = 10.6 Hz, 1 H, H-2), 3.65 (m, 1 H, H-3), 3.80–4.40 (m, 4 H, H-4, H-5, H-6, and H′-6); residue C, 5.19 (d, $J_{1,2}$ = 3.1 Hz), 4.30 (m, 1 H, H-2), 4.21 (m, 1 H, H-3), 4.16 (m, 1 H, H-4), 4.61 (m, 1 H, H-5); residue D, 3.77 (m, 1 H, H-1), 3.71 (m, 1 H, H′-1), 4.00 (m, 1 H, H-2), 4.13 (m, 1 H, H-3), 4.16 (m, 1 H, H-4), 4.10–4.30 (m, 3 H, H-5, H-6, and H′-6).

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Registry No. 1, 122863-71-2; 2, 98797-50-3; 3, 126580-50-5; 4, 126580-51-6; 5, 126580-52-7; 6, 126580-53-8; 7, 119370-32-0; 8, 126613-94-3; 9, 126720-81-8; heparin, 9005-49-6; antithrombin, 9000-94-6; blood coagulation factor Xa, 9002-05-5; blood coagulation factor IIa, 9002-04-4.

Supplementary Material Available: The SAX-HPLC chromatogram and gradient PAGE analysis (both glossy photo and a scan) of oligosaccharide 6 demonstrating its purity (1 page). Ordering information is given on any current masthead page.

Synthesis and Structure–Activity Relationships of 5-Substituted 6,8-Difluoroquinolones, Including Sparfloxacin, a New Quinolone Antibacterial Agent with Improved Potency[1]

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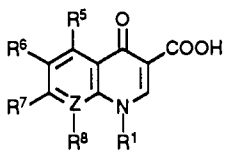
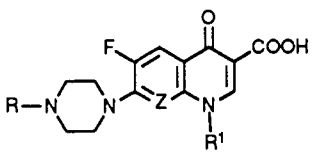
A series of 5,7-disubstituted 1-cyclopropyl-6,8-difluoro-4(1*H*)-oxoquinoline-3-carboxylic acids (**10–36**) were prepared; the C-5 substituent in these compounds comprised halo, hydroxy, mercapto, and amino groups and the C-7 functional group included variously substituted piperazines. In vitro antibacterial screening results indicated that the amino group was optimal among the C-5 substituents. A combination of the C-5 amino group and the C-7 3,5-dimethylpiperazinyl appendage in this series conferred the best overall antibacterial property with lack of adverse drug interactions. Compound **36k** [named sparfloxacin, originally AT-4140, 5-amino-1-cyclopropyl-6,8-difluoro-7-(*cis*-3,5-dimethyl-1-piperazinyl)-4(1*H*)-oxoquinoline-3-carboxylic acid] was superior to ciprofloxacin in both in vitro and in vivo potency and hence was selected as a promising candidate for an improved therapeutic agent.

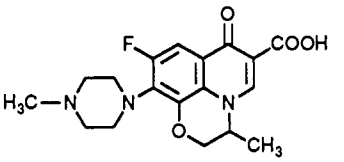
During recent years, much attention has increasingly been given to the synthesis of quinolone antibacterials as a source of new agents.[2] Successful chemical modifications[3] in this area to date are realized especially at positions C-1 (ethyl, fluoroethyl, cyclopropyl, fluorophenyl, and methylamino), C-6 (fluoro), C-7 (4-pyridyl, piperazinyl, and aminopyrrolidinyl), C-8 (fluoro and chloro), and N-1–C-8 ($-CH(CH_3)CH_2CH_2-$ and $-CH(CH_3)CH_2O-$) of the quinolones represented generically by **1**. However, little attention has been paid to the role of C-5 substituents as a possible contributor to the antibacterial property of this class of agents; although several examples of C-5 variants, including alkyl,[4] halo,[4a,5] nitro,[6a] and amino[6] groups, have been reported, neither extensive structure–activity relationships (SARs) of the C-5 function nor clinically useful agents (or even promising candidates) appended with a C-5 substituent have been developed thus far. It seemed to us that the substitution at C-5 might influence activity, because the C-5 position neighbors on the C-4 oxo group,



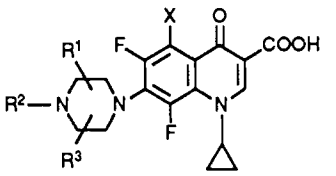
1: Z – R^8 = N or C – R^8



2: Z = N, R^1 = C_2H_5, R = H (enoxacin)
3: Z = CH, R^1 = *c*-C_3H_5, R = H (ciprofloxacin)
4a: Z = CF, R^1 = *c*-C_3H_5, R = H
4b: Z = CF, R^1 = *c*-C_3H_5, R = CH_3



5 (ofloxacin)



6: R^1, R^3 = *cis*-3,5-dimethyl, R^2 = X = H
7: X = halogen, OH, SH, NH_2, etc.

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