

## Oligosaccharide Composition of Heparin and Low-Molecular-Weight Heparins by Capillary Electrophoresis<sup>1</sup>

Umesh R. Desai, Hui-ming Wang, Stephen A. Ampofo, and Robert J. Linhardt<sup>2</sup>

Medicinal and Natural Products Chemistry, College of Pharmacy, University of Iowa, Iowa City, Iowa 52242

Received March 23, 1993

The application of capillary electrophoresis to total compositional analysis of heparin and low-molecular-weight heparin samples has been studied. Optimum resolution of 17 defined oligosaccharides was obtained with the buffer system composed of 10 mM sodium borate and 50 mM sodium dodecyl sulfate at pH 8.81 and at a constant voltage of 20 kV. The ratio of oligosaccharide charge to the number of saccharide residues correlated with the migration time. For oligosaccharides having the same charge to saccharide ratio, the larger of the oligosaccharides eluted earlier. A hexasaccharide, having a 3-O-sulfated glucosamine residue at the reducing end and arising from heparin's antithrombin III binding site, migrated in an unusual fashion. The limit of oligosaccharide detection was from 600 fmol to 1 pmol. Quantitative analysis could conveniently be performed on 10 pmol of an oligosaccharide sample. Oligosaccharide composition using capillary electrophoresis was obtained by nearly complete depolymerization of heparins with a mixture of heparin lyase I, II, and III. The analysis resulted in 95% mass balance for both heparin and low-molecular-weight heparin. Capillary electropherograms of heparin and different low-molecular-weight heparins depolymerized with heparin lyase I alone showed a high level of structural heterogeneity in the products formed. The oligosaccharide maps thus obtained might find use in fingerprinting the heparin and low-molecular-weight samples. © 1993 Academic Press, Inc.

Heparin is a polydisperse [ $M_r$  5000-40,000,  $M_r$  (av) 14,000 (1)], highly sulfated, microheterogeneous, alternating copolymer of 1 → 4 linked 2-deoxy-2-aminoglucofuranose and hexuronic acid (idopyranosyluronic or

glucopyranosyluronic acid) residues. Although heparin has been in use as an anticoagulant for the past five decades (2), its structure has not yet been fully elucidated. While heparin is in widespread clinical use, it has also been cited as the drug most responsible for death in otherwise healthy patients. Attention has been focused on low-molecular-weight (LMW)<sup>3</sup> heparins as heparin substitutes due to their more predictable pharmacological action, reduced side effects, sustained antithrombotic activity, and better bioavailability (3). However, the LMW heparins are derived from heparin and hence are polydisperse and microheterogeneous, with undefined structure. In addition, the numerous synthetic methods for preparing these LMW heparins lead to additional structural complexity (4).

Several techniques have been investigated for the analysis of heparin preparations. Gradient polyacrylamide gel electrophoresis (PAGE) (5) and strong anion exchange-HPLC (6) have been the methods of choice for the qualitative and quantitative analysis of heparin preparations due to the versatility and ease of their application. The gradient PAGE method while useful in determining molecular weight (5) suffers from a lack of resolution particularly of different oligosaccharides having identical size. SAX-HPLC, relying on detection by ultraviolet absorbance, is often insufficiently sensitive for the detection of minuscule amounts of structurally important heparin-derived oligosaccharides.

Recently, capillary electrophoresis (CE) has been introduced as a very sensitive method with high resolving power for the analysis of complex mixtures of peptides

<sup>1</sup> Presented in part at the 204th American Chemical Society National Meeting, Washington, DC, August 23-28, 1992.

<sup>2</sup> To whom correspondence should be addressed.

<sup>3</sup> Abbreviations used: CE, capillary electrophoresis; LMW, low molecular weight; ΔUAp, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; S, sulfate; GlcNp, 2-deoxy-2-aminoglucofuranose; GlcNp2Ac, 2-deoxy-2-acetamidoglucofuranose; SAX, strong anion exchange; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ATIII, antithrombin III.

and nucleotides (7,8). The use of CE in micropreparative mode has also been demonstrated (9). A number of workers have investigated the use of CE in the disaccharide compositional analysis of heparin, heparan sulfate (10,11), and chondroitin sulfate (12,13). The total oligosaccharide compositional analysis of heparins by capillary electrophoresis has the potential of detecting subtle changes in heparin structure that can influence its biological activity. This paper describes the application of CE for qualitative and quantitative oligosaccharide compositional analysis of heparin and low-molecular-weight heparins.

## MATERIALS AND METHODS

### *Materials*

The heparin disaccharides **1–8** were obtained from Grampian Enzymes (Aberdeen, Scotland). The oligosaccharides **10–17** were prepared by enzymatic depolymerization of heparin (4,14–16) followed by their isolation by semipreparative SAX-HPLC (Fig. 1). Heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparitinase II, no assigned EC number), and heparin lyase III (heparitinase I, EC 4.2.2.8) were prepared by heparin induced fermentation of *Flavobacterium heparinum* in our laboratory and purified to homogeneity (17). The enzymatic activity of the three lyases were tested using heparin and heparan sulfate as their primary substrates. Heparin lyase I had an activity of 130 IU/mg against heparin, heparin lyase II had an activity of 19 IU/mg and 36.5 IU/mg against heparin and heparan sulfate, respectively, and heparin lyase III had 63.5 IU/mg activity against heparan sulfate. These enzymes are also commercially available from Seikagaku American (Rockville, MD) and Sigma Chemical (St. Louis, MO). Oligosaccharide mapping studies performed on heparin and heparan sulfate indicate that the various commercial enzymes are catalytically identical to the pure enzymes used in this study (17,25).

Porcine intestinal heparin (sodium salt, 145 USP U/mg) was from Hepar (Franklin, OH). LMW heparins Fraxiparine (CY216) (Choay Laboratories, Paris, France), Fragmin (KABI2165) (KabiVitrum, Stockholm, Sweden), Fluxum (OP2123) (Opocrin, Corlo, Italy), Sandoparin (CH8140) (Sandoz Pharmaceuticals), Logiparin (LHN-1) (Novo Industries, Franklin, OH), and Enoxaparin (PK10169) (Pharmuka Laboratories, Gennevilliers, France) were a gift from Dr. J. Farreed (Loyola University Medical Center, Maywood, IL). Sodium borate (decahydrate, 99%) was from Fisher Scientific (Fair Lawn, NJ), boric acid (electrophoresis grade) was from Mallinckrodt (Paris, KY), and sodium dodecyl sulfate (99%) was from BDH Chemicals (Poole, England). Formamide (99%) was obtained from EM Science (Gibbstown, NJ). Sodium hydroxide pellets, hy-

drochloric acid solution (1 N), and phosphoric acid (85%) were from Mallinckrodt (Paris, KY).

Capillary electrophoresis was performed using a Dionex capillary electrophoresis system with advanced computer interface, Model I, equipped with high-voltage power supply capable of constant or gradient voltage control from Dionex Corporation (Sunnyvale, CA). The uncoated silica capillaries (75 or 50  $\mu\text{m}$  internal diameter, 69 cm total length) were from Dionex. Ultraviolet spectroscopy was performed using a Shimadzu UV-160 spectrophotometer. The pH measurements were obtained on Beckmann  $\Phi 40$  pH meter and calibrated using standard buffer solutions (pH 7.0 and 10.0) from Fisher Scientific (Fair Lawn, NJ).

### *Preparation of Oligosaccharide Standard Solution*

The disaccharide standards **1–8** are available in small amounts not suitable for accurate weighing, which makes the preparation of equimolar solution of these disaccharides difficult. The extinction coefficient of the unsaturated uronic acid ( $\Delta\text{UAp}$ ) chromophore ( $\lambda_{\text{max}} = 232 \text{ nm}$ ) at the nonreducing end in 0.03 M hydrochloric acid solution is  $5500 \text{ mol}^{-1} \text{ cm}^{-1}$  (14). For the purpose of CE analysis, an approximate equimolar solution of disaccharides in deionized, distilled water was prepared using nearly equal absorbance ( $\sim 0.01 \text{ AU}$ ) at 232 nm. The oligosaccharide standard solution was prepared by mixing appropriate oligosaccharides (**1–17**), obtained by enzymatic depolymerization. This standard oligosaccharide solution was stored frozen at  $-70^\circ\text{C}$ .

### *Depolymerization of Heparin and Low-Molecular-Weight Heparins*

A stock solution of the heparin or LMW heparin (20 mg/ml) was prepared in deionized, distilled water. Heparin lyase I, heparin lyase II, and heparin lyase III were each added (25 mU/mg of substrate) to a solution of substrate in 5 mM sodium phosphate buffer (500  $\mu\text{l}$ /mg of substrate) at pH 7.1 containing 200 mM sodium chloride for 16 h at  $30^\circ\text{C}$ . A time of 16 h was found sufficient for the nearly complete depolymerization of heparin to disaccharides. The depolymerized samples were freeze-dried and reconstituted in deionized, distilled water at a concentration of 2  $\mu\text{g}/\mu\text{l}$ .

Heparin lyase I was also used separately under the same conditions in oligosaccharide mapping experiments to prepare product mixtures containing higher oligosaccharides including many of the tetrasaccharides and hexasaccharides contained in the oligosaccharide standard solution (above).

### *CE Analysis of Oligosaccharides*

The resolution of oligosaccharides was carried out using a fused silica (externally coated except where the

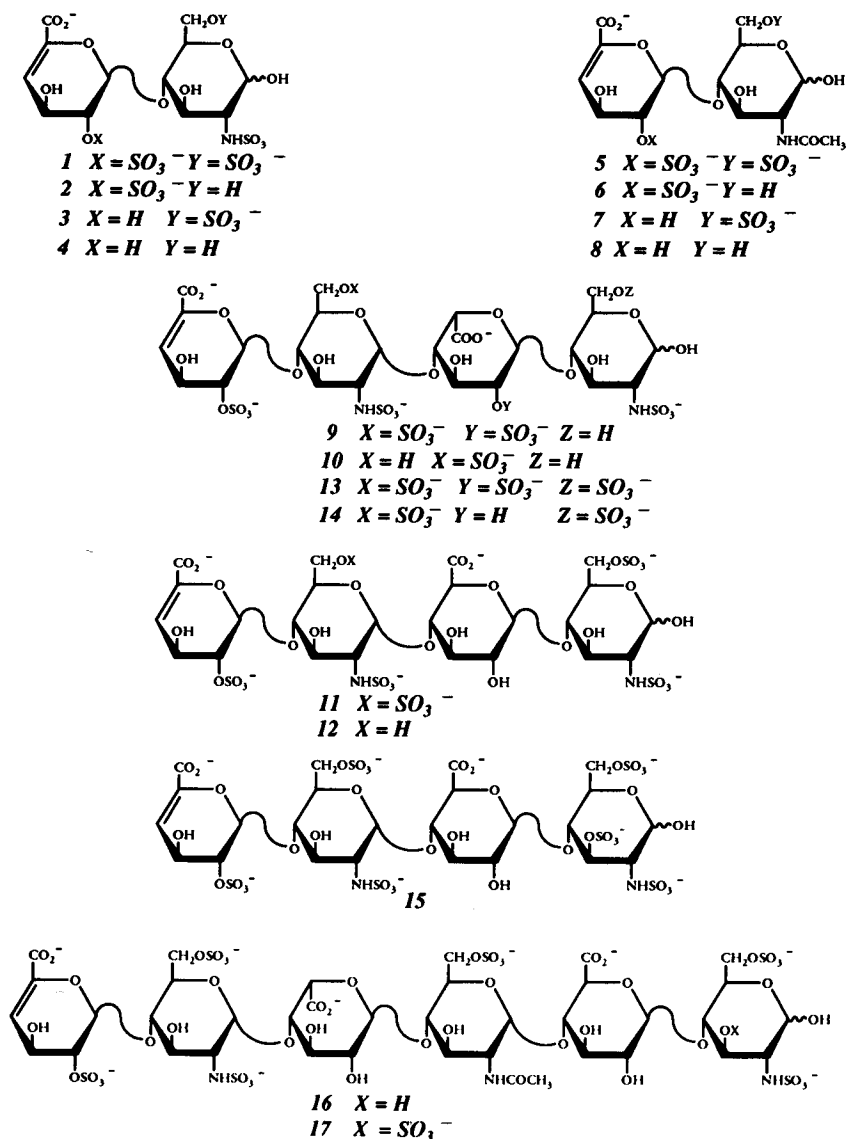


FIG. 1. Structures of heparin-derived disaccharides and oligosaccharides 1-17.

tube passed through the detector) capillary. A new capillary was activated by manually washing with 0.1 M phosphoric acid (500  $\mu\text{l}$ ), 0.5 M sodium hydroxide (1 ml), distilled water (500  $\mu\text{l}$ , pH 5.5), and the buffer (500  $\mu\text{l}$ ) before use. The sample was injected using hydrostatic pressure (45-mm head height, 25-s injection period) to give 14.6 nl total volume of injected solution. The electrophoresis was performed using constant voltage or a linear voltage gradient. Cooling was achieved by nitrogen gas flow from a compressed gas cylinder (100 psi) through a nylon tube (3 mm internal diameter) surrounding the capillary column (375  $\mu\text{m}$  outer diameter). The detection system consisted of a variable-wavelength ultraviolet detector operating at 232 nm. The analysis of data was performed using the software pack-

age from Dionex. For quantitation, baseline was forced at the beginning and at the end of each recognizable peak. The interrun variation in migration time was less than 2%. The interday variation in migration time was approximately 5-10%.

## RESULTS AND DISCUSSION

The disaccharide  $\Delta\text{UAp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$  (1) is obtained from the major disaccharide sequence within the heparin polymer and has one of the highest charge to saccharide ratios ( $c/s = 2$ ) among the various oligosaccharides derived from heparin. The use of 10 mM sodium borate and 50 mM boric acid buffer at pH 8.8 (10) leads to considerable peak tailing and variation in

retention time of this disaccharide. Preliminary experiments suggested that the peak shape of disaccharide 1 (the major disaccharide component in heparin) is crucial for quantitative analysis. By adding sodium dodecyl sulfate to the buffer (12), the tailing of disaccharide peak 1 was reduced. Similarly, tailing of 2, 4, and 8 and fronting of peak 5 were also significantly reduced. Variation in pH of this buffer system did not help eliminate peak broadening of disaccharide 1. With the slow linear voltage gradients (12 to 25 kV in 30 min), peak tailing of all components was observed, whereas steep linear gradients (14 to 20 kV in 20 min) led to loss of resolution for disaccharides 4, 6, and 7. The best resolution was achieved with a gradient of 16 to 20 kV volts in 20 min (data not shown). However, the voltage gradient led to a gradient in current flowing through the capillary, resulting in a steady baseline drift. For quantitative purposes, a constant voltage of 20 kV was found optimum. Organic additives have been reported to increase the resolution of peptides (18). When additives such as methanol (1–10%, v/v), acetonitrile (1–6%, v/v), and ethylene glycol (1–3%, v/v) were evaluated, either the resolution was completely lost or multiple peak shoulders were observed (data not shown). The addition of organic solvents may promote the resolution of various conformational isomers of unsaturated uronic acid residue or of configurational isomers. Formamide is one of the few solvents in which highly charged molecules such as 1–17 are soluble and hence was chosen as a buffer additive. Best resolution was observed at a constant voltage of 20 kV (not shown) at pH 8.8 in the presence of 6% (v/v) formamide. Not only were disaccharides 2, 3, and 5 resolved, but baseline resolution was also observed for disaccharides 4 and 6. While the presence of formamide in the buffer sharpened the peaks, it also increased both the baseline absorbance and noise. In addition, solutions prepared in water gave a sharp, intense peak ascribable to the buffer front peak at about 5 min, although preparation of sample buffer containing formamide partially eliminated this artifact.

#### Identification and Quantification of Oligosaccharides

Since the voltage gradients or the additives did not provide significantly higher resolution, all further experiments were carried out using 10 mM sodium borate buffer containing 50 mM sodium dodecyl sulfate at pH 8.81 and at a constant voltage of 20 kV with forced cooling. The retention time of oligosaccharides is dependent upon the electroosmotic flow through the capillary. The electroosmotic flow varies with the ionic strength of the buffer and the solute concentration. Hence, the identification of oligosaccharides was carried out by sequentially adding each oligosaccharide to the disaccharide mixture 1–8 and noting the appearance of a new peak. Starting with the disaccharide mixture 1–8 (Fig. 2A),

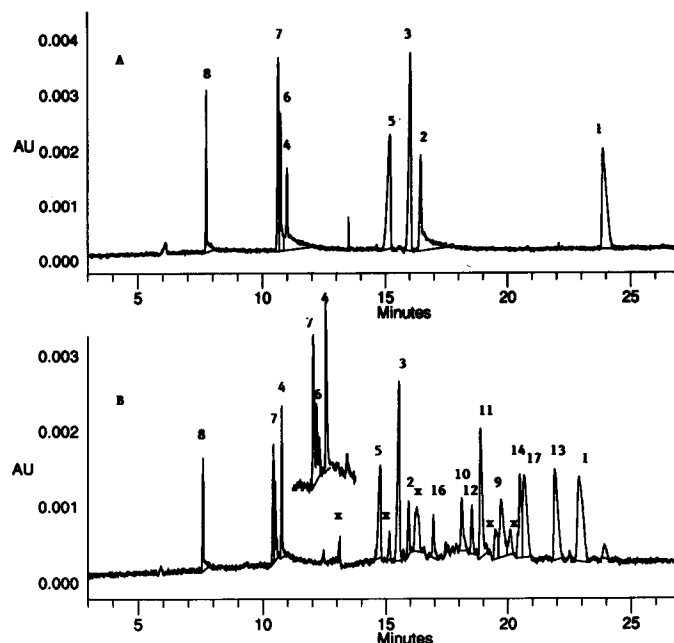


FIG. 2. Optimized capillary electropherograms of disaccharide mixture 1–8 (A) and oligosaccharide mixture 1–17 (B) using 10 mM sodium borate + 50 mM SDS at pH 8.81 under constant voltage of 20,130 V with forced cooling. Inset shows the resolution achieved for disaccharides 4, 6, and 7. Unidentified peaks are marked as "x."

oligosaccharides 13, 16, 11, and 9 could be readily identified (Fig. 2B). Oligosaccharides 10, 12, 14, and 17 were identified in a separate experiment which excluded other oligosaccharides. It should be mentioned that the isolation of higher oligosaccharides at purities greater than 95% is difficult (4,6,14–16) and hence these oligosaccharide samples were typically contaminated. In addition, the anomeric forms ( $\alpha$  and  $\beta$ ) of oligosaccharide-reducing ends, which may be resolvable on CE, might add a further complication. The  $\alpha$ -anomer of heparin oligosaccharides, however, is predominant (>90%) and for quantitation by CE, anomerization is not a major problem. The separation of higher oligosaccharides by capillary electrophoresis had been difficult because of their tendency to comigrate at nearly identical positions in the electropherograms (10). The buffer system described here results in the improved resolution necessary to fractionate these higher oligosaccharides. It should be noted that at pH 8.81 all the acidic groups in these oligosaccharides are ionized. The main mode of separation is zone electrophoresis while micellar electrophoresis, resulting from the addition of sodium dodecyl sulfate, is an auxiliary mode of separation.

The migration times of the oligosaccharides can be correlated with the ratio of total negative charge on the molecule to the number of saccharide residues ( $c/s$ ) (Table 1). Although the uronic acid and glucosamine residues are known to exhibit numerous conformational

TABLE 1  
Compositional Analysis by Capillary Electrophoresis of Heparin and Low-Molecular-Weight Heparins Treated with a Mixture of Heparin Lyase I, II, and III<sup>a</sup>

No.	MT <sup>b</sup>	Oligos. <sup>c</sup>	<i>c/s</i> <sup>d</sup>	Heparin or LMW heparin							
				Heparin	Fraxiparine	Fluxum	Fragmin	Sandoparin	Enoxaparin	Logiparin	
1	7.16	<b>8</b>	0.50	0.4 <sup>e</sup>	<i>nd</i> <sup>f</sup>	nd	nd	nd	nd	nd	0.03
2	9.90	<b>7</b>	1.00	<i>nq</i> <sup>g</sup>	nd	nd	<i>nq</i>	1.4	0.4	0.4	0.2
3	10.02	<b>6</b>	1.00	0.5	0.5	0.5	0.4	0.6	nd	nd	0.2
4	10.29	<b>4</b>	1.00	0.5	0.5	nd	0.3	0.3	0.3	0.3	0.3
5	14.24	<b>5</b>	1.50	1.9	2.0	nd	1.9	1.7	1.5	1.5	1.5
6	15.00	<b>3</b>	1.50	2.3	4.6	0.8	nd	0.8	1.8	1.8	4.0
7	15.45	<b>2</b>	1.50	5.1	9.5	21.7	3.7	2.4	5.6	5.6	0.4
8	16.41	<b>16</b>	1.50	0.7	1.1	nd	<i>nq</i>	1.2	0.3	0.3	nd
9	17.67	<b>10</b>	1.50	nd	0.5	nd	0.4	0.9	0.2	0.2	0.1
10	18.12	<b>12</b>	1.50	nd	0.4	nd	1.1	0.3	0.5	0.5	nd
11	18.74	<b>11</b>	1.75	0.2	0.8	<i>nq</i>	0.8	0.2	0.6	0.6	0.5
12	19.43	<b>9</b>	1.75	0.2	0.3	nd	0.5	0.9	0.4	0.4	0.2
13	20.30	<b>14</b>	1.75	nd	0.4	0.3	0.5	<i>nr</i> <sup>h</sup>	<i>nr</i>	<i>nr</i>	<i>nr</i>
14	20.48	<b>17</b>	1.75	0.2	0.3	0.8	0.1	0.4	0.3	0.3	0.9
15	20.94	<b>15</b>	2.00	nd	1.0	<i>nq</i>	0.2	5.8	0.5	0.5	nd
16	21.89	<b>13</b>	2.00	1.0	2.8	2.3	11.4	1.3	3.5	3.5	3.4
17	23.01	<b>1</b>	2.00	86.8	74.4	73.4	77.4	78.8	84.0	84.0	88.2
18	—	<i>u</i> <sup>i</sup>	—	0.2	0.9	0.3	1.3	3.1	0.1	0.1	0.3

<sup>a</sup> Electrophoresis was performed in 10 mM sodium borate buffer containing 50 mM sodium dodecyl sulfate at pH 8.81 and at 20 kV constant voltage.

<sup>b</sup> Migration time in minutes.

<sup>c</sup> Oligosaccharide (see Fig. 1 for structure).

<sup>d</sup> Ratio of total negative charge (*c*) to number of saccharide residues (*s*).

<sup>e</sup> Percentage of total peak area.

<sup>f</sup> Not detected or determined.

<sup>g</sup> Not quantified.

<sup>h</sup> Not resolved.

<sup>i</sup> Unidentified components.

preferences (19,20), which determine the global three-dimensional conformation of the molecule, it appears that the under the influence of high electric fields the mobility of these species is governed to a major extent by their overall charge density (*c/s*). Hexasaccharide **17**, having a *c/s* ratio of 1.75, is the only oligosaccharide eluting at an unexpected position. The reason for this behavior, although unclear, is probably the result of a distortion in the conformation of the molecule, due to the 3-*O*-sulfate group, allowing greater charge interaction with the capillary wall. Local charge density may also play a role as demonstrated by the differences in retention times of oligosaccharides **5–8**, **11** and **14**, and **13** and **15**. It is this potential of exquisite resolving power of capillary electrophoresis that must be exploited if all the minor biologically active oligosaccharides present in heparin are to be quantified. One additional distinguishing feature of CE is its ability to resolve structures that differ only in the position of a sulfate group (**9** from **14**), and also to resolve stereoisomers (**14** from **11**). Within the family of equivalent charge densities (*c/s*), oligosaccharides with a greater number of saccharide residues tend to migrate faster.

The linearity of peak area to the amount of oligosaccharide analyzed was studied using disaccharides **1** and **8**, tetrasaccharide **13**, and hexasaccharide **17**. Disaccharide **1** is the major product of the heparin lyase catalyzed depolymerization of heparin. Disaccharide **8** is a tailing peak, tetrasaccharide **13** is a highly symmetric peak, and hexasaccharide **17** is in a crowded region of the electropherogram. The detector response was analyzed by serially diluting the standard oligosaccharide mixture (**1–17**) (Fig. 3). For most of the oligosaccharides, peak height gave a poor correlation to oligosaccharide amount due to variable peak width, as well as fronting and tailing. Peak area gave a good correlation for the well-resolved oligosaccharides **1**, **13**, and **8**. Reasonably good correlation was observed for overlapped peak **17**. Other explanations for reduced correlation between peak response and sample size might include variations in introduced volume or slight variations in migration time causing changes in peak width. Thus, quantification might be improved by using an internal standard. The limit of detection for a typical oligosaccharide was in the range of 600 fmol to 1 pmol. The sample required for accurate quantification was 10 pmol.

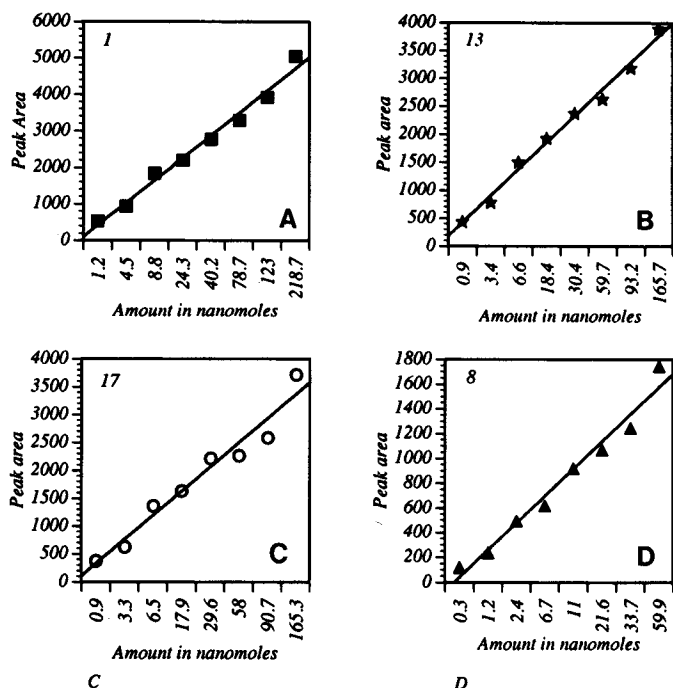


FIG. 3. Quantification of oligosaccharides by peak areas. (A) Disaccharide 1:  $r^2 = 0.983$ . (B) Tetrasaccharide 13:  $r^2 = 0.990$ . (C) Hexasaccharide 17:  $r^2 = 0.955$ . (D) Disaccharide 8:  $r^2 = 0.972$ .

#### Compositional Analysis of LMW Heparins

Since the LMW heparins are prepared using different chemical methods that destroy or alter specific saccharide residues, it may be feasible to elucidate the structural differences between these low-molecular-weight heparins using oligosaccharide compositional analysis. Preliminary attempts to study these differences were made using SAX-HPLC (4). Unfortunately, subtle differences occurring in a single residue in a chain result in minor oligosaccharides that often go undetected by this relatively low-resolution, low-sensitivity technique. The application of CE for the quantitative analysis of enzymatic digests of LMW heparins has the potential of both improved resolution and higher sensitivity. Fraxiparin is obtained by size fractionation of commercial porcine mucosal heparin, while Fragmin is prepared by nitrous acid depolymerization of porcine mucosal heparin (21). Fluxum is obtained by oxidative cleavage of bovine mucosal heparin (22). Since these LMW heparins are prepared by different methods, the CE electropherograms of their oligosaccharide products should be different.

The oligosaccharide composition was obtained by depolymerization of heparin and LMW heparins using a mixture of heparin lyase I, II, and III, followed by electrophoresis under conditions as described above. As expected, the oligosaccharide composition for different LMW heparins varied. The amount of disaccharide 2 in

these LMW heparins is very different (Fig. 4, Table 1). The amount of tetrasaccharides 13 and 9 also reflect the differences in the microheterogeneity of these LMW heparins. Hexasaccharide 17 is obtained from the antithrombin III (ATIII) binding sequence (23,24) of the polysaccharide chain of heparin. The compositional analysis suggests that low-molecular-weight heparins have a significantly different proportion of ATIII binding sequence, which may explain the differences in their biological activity (10). The proportion of unidentified components in various LMW heparins differ considerably (Table 1). These result from the different processing methods used in their preparation. The higher proportion of unidentified components implies greater differences between the LMW heparin and the parent heparin. Although it is not possible to identify and quantify all the unidentified components in various LMW heparins, for the lack of appropriate standards,

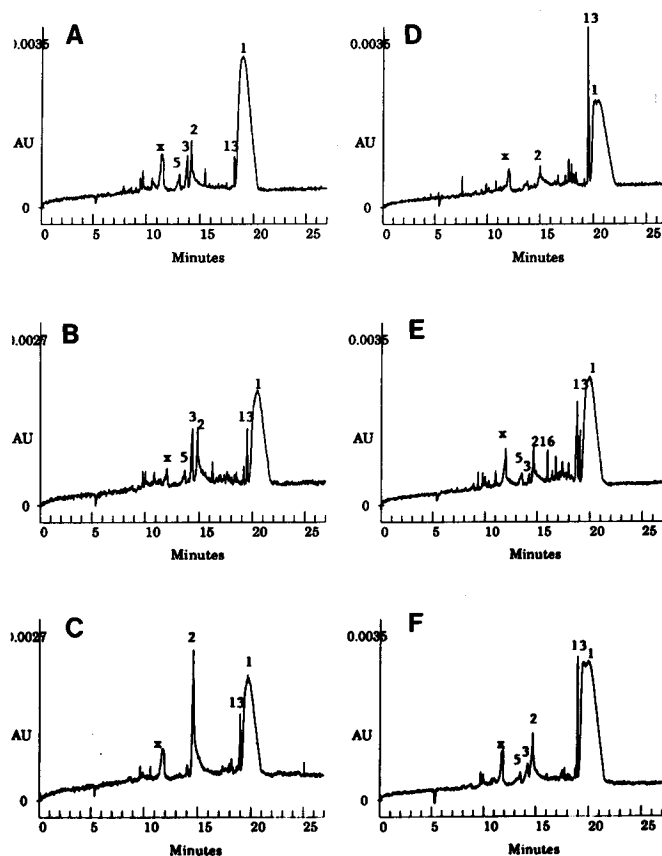


FIG. 4. Capillary electropherograms of heparin lyase I, II, and III depolymerized heparin and low-molecular-weight heparins. The conditions used were 10 mM sodium borate buffer containing 50 mM SDS at pH 8.81 and at 20 kV constant voltage. (A) Porcine intestinal heparin, (B) Fraxiparin, (C) Fluxum, (D) Fragmin, (E) Sandoparin, and (F) Enoxaparin. The broad peak ("x") is due to protein corresponding to the three heparin lyases that were used in high amounts. Note the dissimilarity in the electropherograms for the LMW heparins due to the difference in the methods of their preparation. See Table 1.

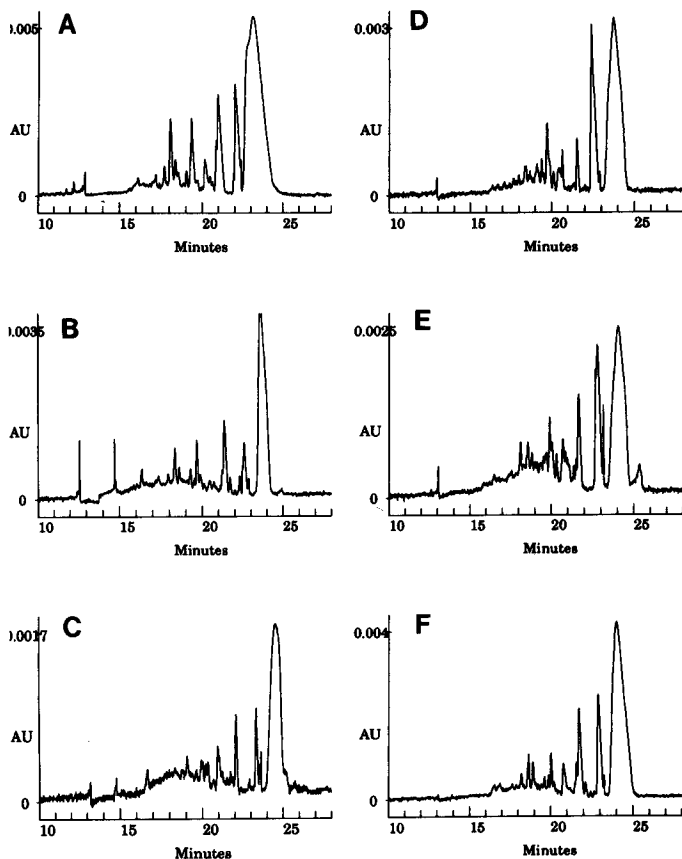


FIG. 5. Capillary electropherograms of heparin lyase I depolymerized heparin and low-molecular-weight heparins. (A) Porcine intestinal heparin, (B) Fraxiparin, (C) Fluxum, (D) Fragmin, (E) Enoxaparin, and (F) Logiparin.

the relative differences in oligosaccharide composition between LMW heparin samples are good indicators of the differences or similarities between these samples. The capillary electropherograms of heparin lyase I depolymerized heparin and LMW heparins (Fig. 5) represent oligosaccharide maps. These also show significant structural differences between these LMW heparins. This figure suggests oligosaccharide mapping by capillary electrophoresis might be useful in fingerprinting the LMW heparin samples, as well as in controlling the quality of these pharmaceutical preparations.

The percentage composition of disaccharide **1** obtained from low-molecular-weight heparins measured in this study (~73–87%) is significantly higher than that measured using SAX-HPLC (6). Earlier studies relying on SAX-HPLC (6,16) use only heparin lyase I while the present study utilizes a mixture of the three heparin lyases for greater depolymerization of heparin or LMW heparins. The substrate specificity study of the heparin lyases demonstrates a broad specificity for linkages within heparin when all three lyases are used in combination (25). This results in the nearly complete

depolymerization of polysaccharide chains to disaccharide components, the major constituent of which is disaccharide **1**. CE can separate all the disaccharides formed using a mixture of the three lyases but SAX-HPLC has insufficient resolving power to separate all the disaccharides formed. The proportion of disaccharide **1** formed on treatment with the three lyases reflects the degree of homogeneity for the heparin sample and hence should also serve as a good indicator of biological differences. The broad peak corresponding to disaccharide **1** dominates the profile because of its high concentration in the sample. If the sample is diluted and a second injection is performed sharp peak obtained can be used to assist in accurate quantification.

In conclusion, we have demonstrated the feasibility of the oligosaccharide compositional analysis of heparin and low-molecular-weight heparins. Most of the oligosaccharides could be resolved on an uncoated, silica capillary using 10 mM sodium borate and 50 mM sodium dodecyl sulfate buffer at pH 8.81 under a constant voltage of 20 kV. The relative compositional analysis of LMW heparins highlights the differences between the different commercially available low-molecular-weight heparins. Although the identification of all the natural and unnatural (those due to processing method for preparing LMW heparin) components in the LMW heparins is currently not feasible, it would certainly aid in elucidating the subtle compositional differences between commercially available LMW heparins as well as lot to lot variation.

#### ACKNOWLEDGMENT

This work was supported by Grant GM38060 from the National Institutes of Health.

#### REFERENCES

1. Laurent, T. C., Tengblad, A., Thunberg, L., Hook, M., and Lindahl, U. (1978) *Biochem. J.* **175**, 691–701.
2. Linhardt, R. J., and Loganathan, D. (1990) in *Biomimetic Polymers* (Gebelein, G., Ed.) pp. 135–175, Plenum, New York.
3. Breddin, H. K., Fareed, J., and Bender, N. (1988) *Haemostasis* **18**(Suppl. 3), 1–87.
4. Linhardt, R. J., Loganathan, D., Al-Hakim, A., Wang, H.-M., Walenga, J. M., Hoppensteadt, D., and Fareed, J. (1990) *J. Med. Chem.* **33**, 1639–1645.
5. Edens, R. E., Al-Hakim, A., Weiler, J. M., Rethwisch, D. G., Fareed, J., and Linhardt, R. J. (1992) *J. Pharm. Sci.* **81**, 823–827.
6. Linhardt, R. J., Rice, K. G., Kim, Y. S., Lohse, D. L., Wang, H.-M., and Loganathan, D. (1988) *Biochem. J.* **254**, 781–787.
7. Jorgensen, J. W. (1984) *Trends Anal. Chem.* **3**, 51–54.
8. Kuhr, W. G. (1990) *Anal. Chem.* **62**, 404R–414R.
9. Albin, M., Chen, S., Louie, A., Pairaud, C., Colburn, J., and Wiktorowicz, J. (1992) *Anal. Biochem.* **206**, 382–388.

10. Ampofo, S. A., Wang, H.-M., and Linhardt, R. J. (1991) *Anal. Biochem.* **199**, 249-255.
11. Damm, J. B. L., Overklift, G. T., Vermeulen, B. W. M., Fluitsma, C. F., and van Dedem, G. W. K. (1992) *J. Chromatogr.* **608**, 297-309.
12. Carney, S. L., and Osborne, D. J. (1991) *Anal. Biochem.* **195**, 132-140.
13. Al-Hakim, A., and Linhardt, R. J. (1991) *Anal. Biochem.* **195**, 68-73.
14. Rice, K. G., and Linhardt, R. J. (1989) *Carbohydr. Res.* **190**, 219-233.
15. Linhardt, R. J., Rice, K. G., Merchant, Z. M., Kim, Y. S., and Lohse, D. L. (1986) *J. Biol. Chem.* **261**, 14448-14454.
16. Linhardt, R. J., Wang, H.-M., Loganathan, D., and Bae, J.-H. (1992) *J. Biol. Chem.* **267**, 2380-2387.
17. Lohse, D. L., and Linhardt, R. J. (1992) *J. Biol. Chem.* **267**, 24347-24355.
18. Zhu, M., Rodriguez, R., Hansen, D., and Wehr, T. (1990) *J. Chromatogr.* **516**, 123-131.
19. Torri, G., Casu, B., Gatti, G., Petitou, M., Choay, J., and Jacquinet, J. C. (1985) *Biochem. Biophys. Res. Commun.* **128**, 134-140.
20. van Boeckel, C. A. A., van Aelst, S. F., Wagenaars, G. N., Mellem, J.-R., Paulsen, H., Peters, T., Pollex, A., and Sinnwell, Y. (1987) *Recueil Trav. Pays-Bas* **106**, 19-29.
21. Shivley, J. E., and Conrad, H. E. (1976) *Biochemistry* **15**, 3932-3942.
22. Fussi, F. (1981) *U.S. Patent* 4,281,108, July 28.
23. Lindahl, U., Backstrom, G., and Thunberg, L. (1983) *J. Biol. Chem.* **258**, 9826-9830.
24. Atha, D., Stephens, A. W., and Rosenberg, R. D. (1984) *Proc. Natl. Acad. Sci.* **81**, 1030-1034.
25. Linhardt, R. J., Turnbull, J. E., Wang, H.-M., Loganathan, D., and Gallagher, J. T. (1990) *Biochemistry* **29**, 2611-2617.