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High-resolution capillary electrophoresis and polyacrylamide gel electrophoresis of heparins

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Abstract

Heparin-oligosaccharides, low-molecular-mass heparins (LMMHs) and heparins were determined using high-resolution capillary electrophoresis (HPCE) and polyacrylamide gel electrophoresis (PAGE). The conditions for HPCE were 20 m*M* phosphate buffer (pH 3.5) and 20°C. The method was equivalent to that used previously with a borate–sodium dodecyl sulfate buffer (pH 8.8). Six LMMHs were determined using PAGE. The method showed a standard deviation of the average molecular mass from 6.4 to 19.8% and of their polydispersity from 0.7 to 10.2%. HPCE and PAGE revealed different important structural and compositional differences of heparins.

1. Introduction

Heparin is an endogenously occurring glycosaminoglycan with anticoagulant properties which has been applied for over half a century in clinical use. The major disaccharide sequences of heparin are established as an $\alpha(1\rightarrow4)$-linked L-iduronic acid 2-sulfate → D-glucosamine N,6 disulfate (IdoA-2S → GlcNS-6S) [1]. The highly sulfated glycosaminoglycan (DS = 2.0–2.5) is commonly determined by different electrophoretic methods; traditionally, heparin electrophoresis is performed by agarose gel electrophoresis using barium trimethylenediamines [2], which resolves a fast- and slow-moving heparin fractions. More information about the primary sequence of heparin is obtained by compositional analysis with high resolution and specificity using high-performance capillary electrophoresis (HPCE). The method is as efficient as the traditional SAX HPLC analysis [3,4]. More detailed information about the secondary structure of heparin can be obtained by polyacrylamide gel electrophoresis (PAGE). This method gives detailed information about the degree of polymerization (D_p), the average molecular mass and the distribution of molecular mass of heparin [5,6].

Recently, it has been demonstrated that the molecular mass of heparin is a crucial parameter for its pharmacodynamics. The anti-Xa activity is associated with the pentasaccharide sequence and the following structure: –GlcNAc(6-OSO_3)–GlcA–$GlcNSO_3$(3,6-di-OSO_3)–IdoA(2-OSO_3)–$GlcNSO_3$(6-OSO_3 [7,8]. The anti-IIa activity, which seems to be of importance for the antithrombotic activity of heparin, requires a saccharide chain of at least 18 saccharide units ($M > 5800$) [9,10].

This paper describes and compares different electrophoretic methods for determining heparins. An HPCE method for the determination of heparins and heparin oligosaccharides is described. PAGE analysis with a new staining

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method was developed and shows a better resolution of oligosaccharides resulting in a different molecular mass distribution and polydispersity for each heparin. The microheterogeneity of heparin macromolecules can be best performed using a combination of HPCE and PAGE.

2. Experimental

2.1. Heparins

The following unfractionated and low-molecular-mass heparins (LMMHs) were used: unfractionated sodium heparin (analytical-reagent grade), prepared from bovine lung, was obtained by Braun Melsungen (Germany), Braun-LMMH containing sodium chloride and water for injection from Braun Melsungen (Germany), Clexane containing water for injection from Nattermann (Cologne, Germany), Fragmin containing sodium chloride and water for injection from Kabi Pfrimmer (Erlangen, Germany), Fraxiparin containing calcium hydroxide and water for injection from Sanofi-Wintrop (Munich, Germany), Merckle LMMH (analytical-reagent grade) from Merckle (Ulm, Germany) Reviparin from Nordmark (Germany) and Mono Embolex was generously provided by Sandoz (Nürnberg, Germany).

Heparin-oligosaccharides prepared by synthesis, ranging from di- to dodecasaccharide, were generously provided by Dr. M. Petitou and Dr. L. Lormeau (Institut Choay, Paris, France).

Heparin-disaccharide standards (H-0895, H-8642, H-1145, H-9517, H-1020, H-9392 and H-9267) were obtained from Sigma (St. Louis, MO, USA). Heparinase I was generously supplied by Baxter Diagnostics (Deerfield, IL, USA) and chondroitinase ABC purified from *Proteus vulgaris* was obtained from Seikagaku (Tokyo, Japan).

2.2. Chemicals

Tris and Sephadex G-25 F were of research grade and were obtained from Sigma (Deisenhofen, Germany), glycerol from Merck (Darmstadt, Germany), 3-dimethylaminopropionitrile (DNPN) from Fluka (Neu-Ulm, Germany) and acrylamide, N,N-methylenebisacrylamide, ethylenediaminetetraacetic acid and glycine of research grade from Serva (Heidelberg, Germany).

2.3. HPCE of heparin and heparin-oligosaccharides

The experiments were performed on a PACE System 2050 from Beckman Instruments (Fullerton, CA, USA), equipped with a variable-wavelength ultraviolet detector. System operation and data management were controlled using System Gold software (Beckman Instruments) running on an IBM personal computer. In both modes the samples were analysed using a 50 cm × 50 μm I.D. capillary cartridge (No. 727604) from Beckman Instruments.

The concentrations of the stock standard solutions were 1 mg/ml for heparin-disaccharides and 10 mg/ml for heparin-oligosaccharides and heparin preparations.

2.4. Normal polarity

The following conditions were used: data rate, 5 Hz; rise time, 1 s; range (AU), 0.05; polarity, direct; wavelength, 200 and 230 nm; time, 30 min; voltage, 18 kV; temperature, 25°C; injection, high pressure for 10 or 15 s; buffer system, 10 m*M* sodium tetraborate and 50 m*M* boric acid (pH 8.5 and 8.8).

2.5. Reversed polarity

The following conditions were used: data rate, 5 Hz; rise time, 1 s; range (AU), 0.2; polarity, indirect; wavelength, 200 and 230 nm; time, 60 min; voltage, 12 kV; temperature, 25°C; injection, high pressure for 15 s; buffer system, 20 m*M* sodium phosphate adjusted to pH 3.5 with hydrochloric acid.

2.6. Preparation of gradient polyacrylamide gels and electrophoresis

Gradient polyacrylamide resolving gels, 20–30% total acrylamide and 2–5% total N,N-

bisacrylamide (pH = 8.3), were prepared using a linear gradient maker from Hoefer Instruments (San Francisco, CA, USA). A stacking gel of 5% total acrylamide (pH 8.0) was prepared. Preconcentration of the samples was performed in the stacking gel at 20 V. Electrophoresis was performed for 4 h at 80 V. The gels were fixed and stained with 1% aqueous acridine orange solution. Destaining was carried out 24 h with gentle shaking using ethanol–water (20:80). The gels were scanned by a densitometer from Hirschmann Instruments (Traufkirchen, Germany) at 548 nm.

2.7. Determination of the average molecular mass by PAGE

Each gel was scanned at 548 nm with a densitometer and the data were transferred as an ASCII file to a Excel program. The oligosaccharides did not differ in resolution and absorbance. The molecular mass and polydispersity were calculated and displayed as mean ± standard deviation.

3. Results

The electrophoretic methods for heparins are dependent on their structure, sequence, charge and mass of the molecule. HPCE, however, is a powerful method for the characterization of different heparins and low-molecular-mass heparins. PAGE analysis is suitable for determining the molecular mass distribution, the average molecular mass and the polydispersity, which are of importance for the effects of heparins.

3.1. HPCE analysis of heparin-disaccharides

Using enzymatic depolymerization, heparin was cleaved to heparin-disaccharides. These commercially available pure heparin-disaccharides with the structure ΔUA 2X (1→4) α-D-GlcNY6X were resolved and detected selectively at 230 nm. The migration time of the heparin-disaccharides was about 30–50% shorter than a 50 μm than with a 75 μm I.D. capillary.

Decreasing the voltage from 20 to 5 kV increased the separation between mono- and disulfated isomers. Consequently, the migration time increased. Increasing the temperature to 40°C resulted in a loss of resolution of the mono- and disulfated compounds.

3.2. Normal polarity

The heparin-disaccharides used were resolved within 8 min and migrated according to their sulfation. In the normal polarity mode the trisulfated heparin-disaccharide ΔUA 2S (1→4) α-D-GlcNHS6S was the slowest and the non-sulfated ΔUA (1→4) α-D-GlcNHAc the fastest component (Fig. 1). At concentrations between 20 μg/ml and 1 mg/ml there was a linear correlation between the peak area and concentration, with correlation coefficient in the range $r^2 = 0.98–0.99$.

3.3. Reverse polarity

On reversing the polarity, the order of migration changed completely. The analysis was performed in 44 min and resolved the heparin-disaccharides from each other. The migration order was reversed: the trisulfated heparin-disaccharide ΔUA 2S (1→4) α-D-GlcNS6S was the fastest and the non-sulfated ΔUA (1→4) α-D-GlcNHAc the slowest component (Fig. 2).

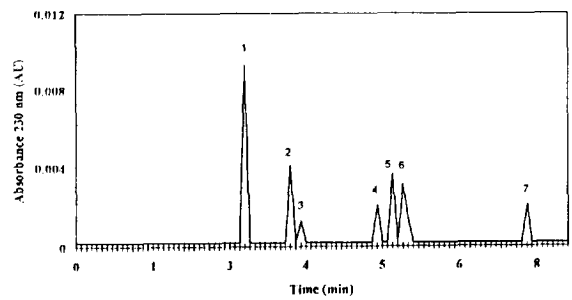

Fig. 1. HPCE separation of seven heparin-disaccharides using normal polarity. Peaks: 1 = ΔUA (1→4) α-D-GlcNAc; 2 = monosulfated ΔUA (1→4) α-D-GlcNAc6X; 3 = ΔUA 2S (1→4) α-D-GlcNAc; 4 = disulfated ΔUA 2S (1→4) α-D-GlcNAc6S; 5 = ΔUA (1→4) α-D-GlcNS6S; 6 = ΔUA 2S (1→4) α-D-GlcNS; 7 = trisulfated disaccharide ΔUA 2S (1→4) α-D-GlcNS6S. The buffer system contained 10 m*M* borate and 50 m*M* SDS.

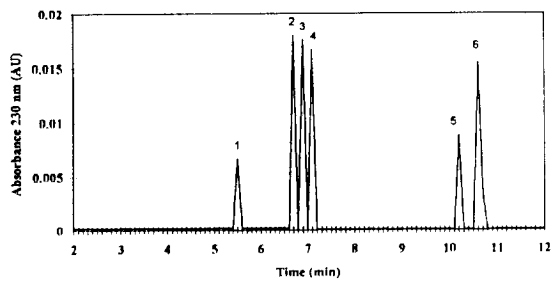

Fig. 2. HPCE separation of seven heparin-disaccharides using reverse polarity. Peaks: 1 = trisulfated ΔUA 2S (1→4) α-D-GlcNS6S; 2 = disulfated ΔUA 2S (1→4) α-D-GlcNS; 3 = ΔUA (1→4) α-D-GlcNS6S; 4 = disulfated ΔUA 2S (1→4) α-D-GlcNAc6S; 5 = monosulfated ΔUA 2S (1→4) α-D-GlcNAc; 6 = ΔUA (1→4) α-D-GlcNAc6X; 7 = non-sulfated disaccharide ΔUA (1→4) α-D-GlcNAc (44.4 min). The buffer contained 20 m*M* phosphate (pH 3.5). Rise time, 1 s; data rate, 5 Hz; range (AU), 0.02; polarity, direct; wavelength, 230 nm; time, 60 min; voltage, 12 kV; temperature, 25°C; injection, high pressure for 15 s.

At concentrations between 20 μg/ml and 1 mg/ml there was a linear correlation between the peak area and concentration, with $r^2 = 0.98–0.99$.

3.4. HPCE analysis of heparin-oligosaccharides

Heparin-oligosaccharides with the general structure IdoA2 (1→4) α-D-GlcNY6X were analysed; they were detected at 200 nm using normal and reverse polarity. Their purity was confirmed using HPSEC and a peak purity check of the components [11]. The oligosaccharides migrated due to their mass and charge. The heparin-disaccharide (Fig. 3a) using was monosulfated, as was shown by co-injection experiments. Using reverse polarity, the heparin-disaccharides could be separated from tetra- and hexasaccharides. The hexasaccharides showed a shorter migration time than the heparin-tetrasaccharides.

An increase in the molecular mass of the heparin-oligosaccharides also increased the possibility of the simultaneous detection of heparin-oligosaccharides of different size and sulfation. Thus the large peak between 9 and 10 min of the heparin-oligosaccharides contains overlapped heparin-oligosaccharides. The octasaccharide was better resolved than the decasaccharide, which also illustrates the loss of resolution of longer heparin-oligosaccharides (Fig. 3). For higher oligosaccharides and a mixture of these, complete resolution was not possible.

In the normal mode, the migration of heparin-disaccharides interfered with higher oligosaccharides. Hence they could not be separated from tetrasacharides and higher heparin-oligosaccharides (Table 1).

3.5. HPCE analysis of heparins and low-molecular-mass heparins

Different heparin preparations differ in their structure, molecular mass and sulfation [12]. All heparins and low-molecular-mass compounds used have similar degrees of sulfation, ranging from 2.1 to 2.3. The higher the sulfation of the compound, the shorter was the migration time. Chondroitin sulfates with a considerably lower degree of sulfation (1.1) showed longer migration times than heparins.

Changing the voltage or the pH of the buffer did not increase the resolution of heparin-disaccharides or those of heparins and of low-molecular-mass heparins. Heparins were detected at 200 nm. Modified heparins, produced by enzymatic cleavage or β-elimination, have an unsaturated uronic acid residue (ΔUA) at their non-reducing end. Hence they exhibit an absorbance maximum at 230 nm and permit selective detection.

Heparin (**4a**, calcium salt) and heparin (**4b**, sodium salt) showed only minor differences in their resolution because their degree of sulfation and molecular mass were similar. The samples were of high purity and dissolved in water. The large peaks from 5.8 to 5.9 min could be attributed to the simultaneous detection of disulfated heparin-disaccharides, higher charged heparin chains and heparin-oligosaccharides of different lengths, whereas the peaks from 7.0 to 7.4 min co-migrated with monosulfated heparin-disacharides. The peak areas, peak widths and migration times of the two heparin preparations differed.

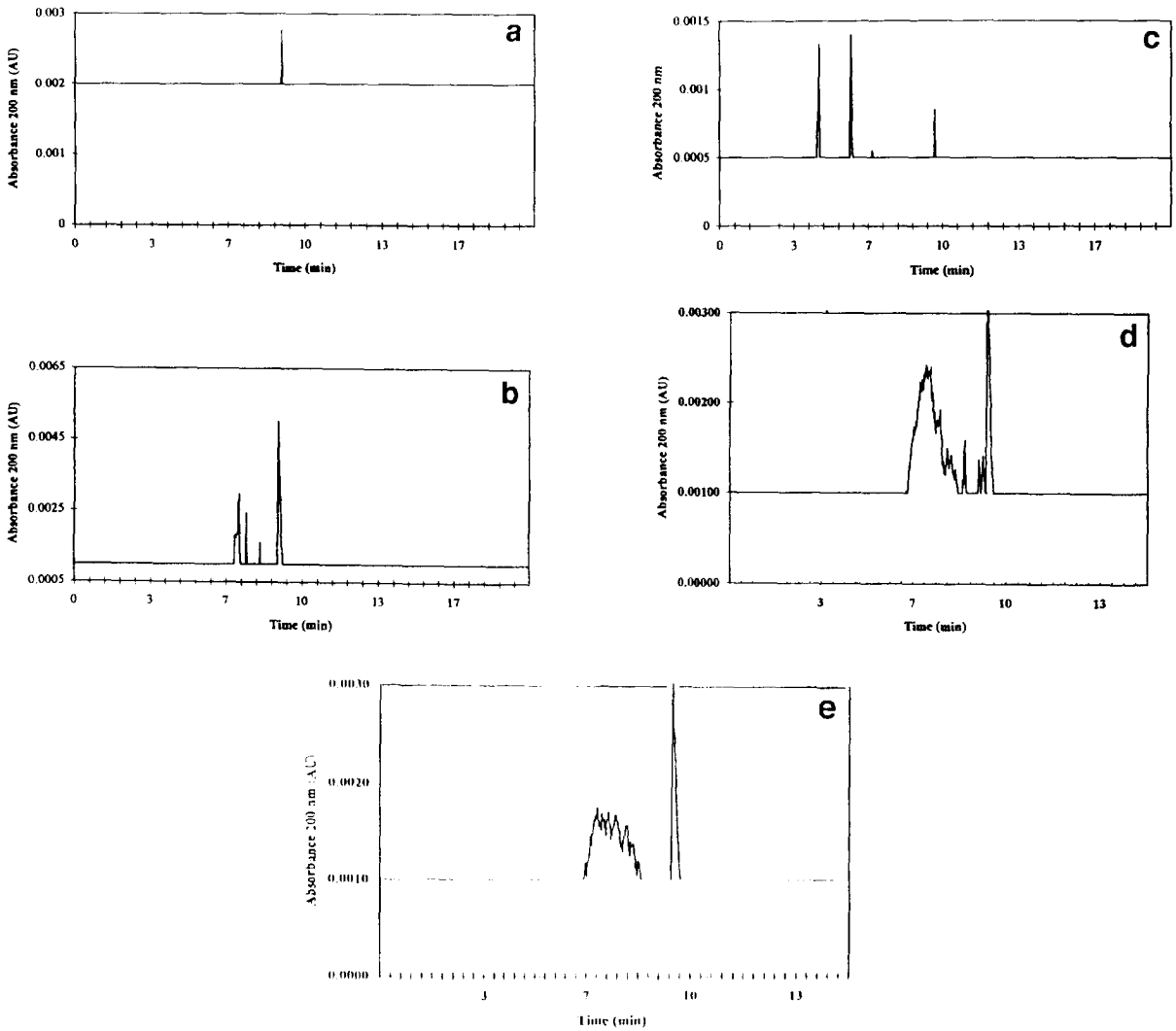

Fig. 3. Electropherograms of heparin-oligosaccharides of different chain length using reverse polarity. (a) Heparin-disaccharide; (b) heparin-tetrasaccharide; (c) heparin-hexasaccharide; (d) heparin-octasaccharide; (e) heparin-decasaccharide. The oligosaccharides migrate differently depending on their mass and charge. Experimental conditions: 20 m*M* phosphate buffer (pH 3.5), range (AU), 0.02; polarity, indirect; wavelength, 200 nm. For further conditions, see Experimental.

The simultaneous detection of heparin-oligosaccharides of different sizes and sulfation results in a characteristic electropherogram for each heparin preparation.

Fraxiparin (**4c**), Clexane (**4d**) and Fragmin (**4e**) reflect typical electropherograms of low-molecular-mass heparins. Compounds **4c** and **4e** have an end-terminal 2,5-anhydromannitol residue and similar molecular masses. The large peak from 5.8 to 5.9 min could be assigned to the simultaneous detection of disulfated heparin-disaccharides and higher charged heparin chains and heparin-oligosaccharides of different lengths, whereas the peaks from 7.0 to 7.4 min co-migrated with monosulfated heparin-disaccharides. The peaks between 6.0 and 7.0 min are smaller heparin-oligosaccharides (tetrasaccharides). The peak areas, peak widths and

Table 1
HPCE of heparin-oligosaccharides using normal polarity

Heparin-oligosaccharide	Migration time (min)	Peak area (AU)
Disaccharide	3.22	0.16
	3.89	0.56
	5.31	0.50
	5.73	0.14
Tetrasaccharide	3.37	0.19
	3.97	0.19
	4.32	0.09
	5.44	0.21
	5.72	0.10
	6.72	0.04
Hexasaccharide	3.12	0.18
	3.76	0.66
	4.04	0.59
	4.10	0.30
	5.10	0.97
Octasaccharide	3.01	0.26
	3.74	0.60
	4.16	0.18
	5.00	0.48
Decasaccharide	3.07	0.37
	3.72	0.91
	4.04	0.29
	5.00	0.73

The buffer system was 10 m*M* sodium tetraborate and 50 m*M* SDS (pH 8.8). Higher oligosaccharides were separated according to their charge and overlapped with lower oligosaccharides.

migration times of the two low-molecular-mass heparin preparations differed, however. Clexane (**4d**) is produced by β-elimination of a benzyl ester of heparin and contains an unsaturated uronic acid residue. The profile of this compound differs strongly from those of heparin and the other low-molecular-mass heparins.

Using reverse polarity for each heparin, a typical electropherogram was obtained (Fig. 4). This is due to the different structure, molecular mass and sulfation. Glycosaminoglycans structurally related to heparin show different migration profiles.

Using the normal polarity mode, no significant differences in the resolution were obtained for LMMH and heparins. The different migration times of the heparins did not correlate with their molecular mass or with the degree of sulfation.

3.6. PAGE– analysis of heparin-oligosaccharides

Heparins and LMMHs from different commercial sources were analysed by gradient PAGE using a modified Laemli system. The gels were stained using acridine orange. The sensitivity of the staining method ranged from 0.2 to 1.0 μg. Defined heparin oligosaccharides ranging from di- to dodecasaccharide and heparin standards of molecular mass ranging from 11 000 to 21 000, which were prepared by HPSEC, were run by co-migration with LMMHs to standardize the PAGE. The equation obtained on plotting the R_F value (y) versus the logarithm of the molecular mass (x) was $y = -0.30x + 1.81$ with $r^2 = 0.99$. These data indicated that the migration distance of the oligosaccharides in the gel was dependent on the molecular mass of the saccharide chains (Fig. 5).

3.7. PAGE analysis of heparins and low-molecular-mass heparins

An LMMH produced by nitrous acid showed defined bands ranging from tetra- to dodecasaccharide and could be used as an internal ladder of the molecular mass for PAGE [14]. The migration of their oligosaccharides corresponded to their molecular mass ($r^2 > 0.98$). The bands showed a resolution of oligosaccharides of the LMMH that could be identified better than by staining with alcian blue or toluidine blue. Scanning the intensity of the bands at 548 nm, the average molecular mass M_m and the polydispersity (M_m/M_n) of the LMMH were calculated.

The glycosaminoglycans samples were analysed by PAGE on five different days (Fig. 6). They showed a standard deviation of the average molecular mass M_m from 6.4 to 19.8% and of their polydispersity P from 0.7 to 10.2%. Table 2 shows the inequivalence of these heparins. For low-molecular-mass preparations M_n ranged from 3256 to 5107, M_m from 4629 to 9084 and M_z from 5291 to 11 354. The standard deviations of the LMMHs also differed significantly from 1.23 to 1.57 (Table 2). The polydispersity of the

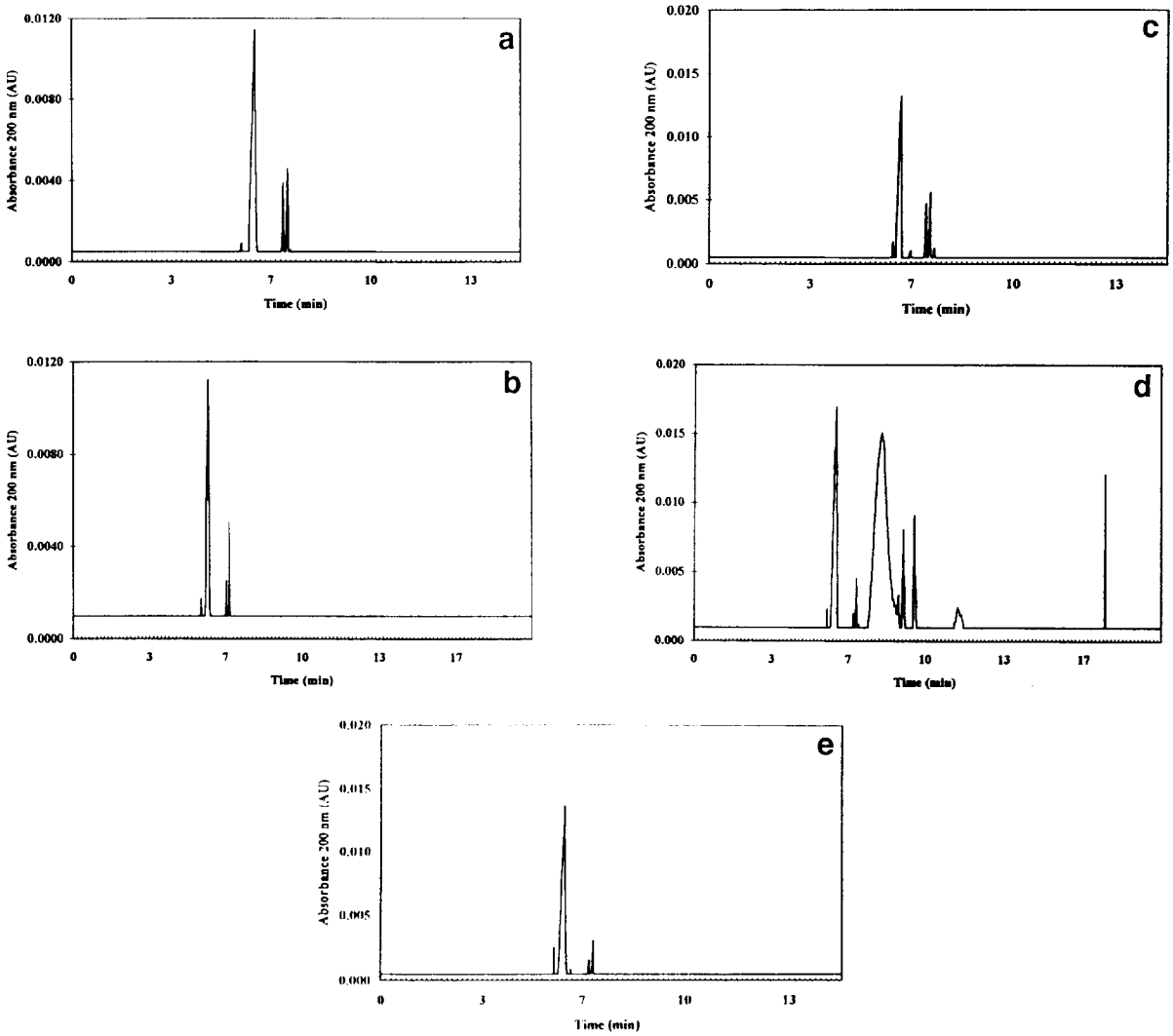

Fig. 4. Electropherograms of heparins and low-molecular-mass heparins. (a) Heparin (calcium) and (b) heparin (sodium) show only minor differences in their resolution because their sulfation and molecular mass were similar. (c) Fraxiparin; (d) Clexane and (e) Fragmin show electropherograms of low-molecular-mass heparins. Experimental conditions: 20 m*M* phosphate buffer (pH 3.5); range (AU), 0.02; polarity, indirect; wavelength, 200 nm. For further conditions, see Experimental.

compounds was always higher than 1.0, which represents an ideal homogeneous polymer.

4. Discussion

Capillary zone electrophoresis of heparin-disaccharides was first described by Ampofo et al. [5]. The composition of heparin and heparin sulfate was determined with heparin-disaccharides which were standardized by fast atom bombardment mass spectrometry. Two disaccharides, ΔUA (1→4) GlcNS and ΔUA 2S (1→4) GlcNAc, had very similar migration times and co-migrated on CZE analysis. The introduction of 50 m*M* SDS in 10 m*M* sodium borate without boric acid gave optimum resolution of the disaccharides used. The buffers described and the heparin-disaccharides were of the highest purity and were used to determine heparin-oligosac-

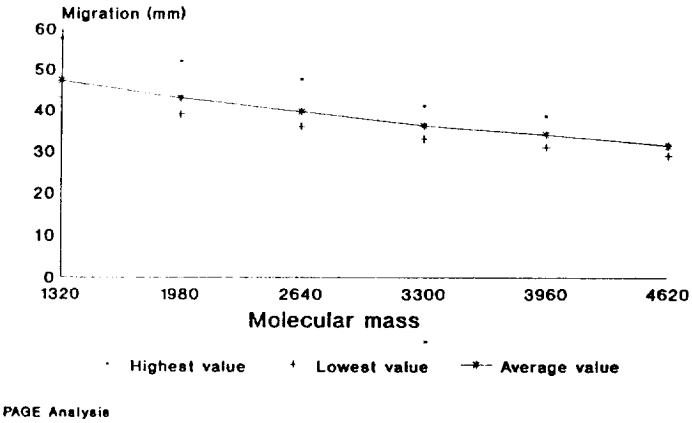

Fig. 5. Migration of heparin-oligosaccharides in polyacrylamide gels. The migration in the gel was dependent on the molecular mass of the oligosaccharide. The gels contained 20–30% total acrylamide and 2–5% total N,N-bisacrylamide (pH 8.3). The electrophoresis was performed for 4 h at 80 V.

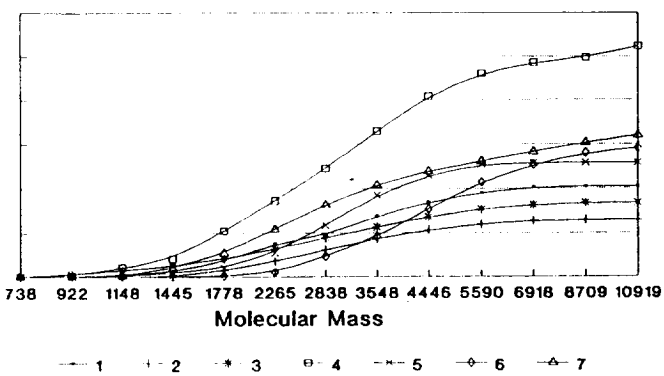

Fig. 6. Different molecular distributions of seven low-molecular-mass heparins using PAGE. For conditions, see Experimental.

Table 2
Inequivalence of the average molecular mass and polydispersity of heparins

Compound:	M_m	Polydispersity (P)
Heparin	11272 ± 837	1.48 ± 0.043
Clexane	4629 ± 208	1.27 ± 0.034
Fragmin	6441 ± 403	1.35 ± 0.094
Fraxiparin	5453 ± 1106	1.23 ± 0.019
LMMH-Braun	4774 ± 442	1.40 ± 0.027
LMMH-Merckle	9084 ± 186	1.37 ± 0.018
LMMH-Sandoz	5558 ± 186	1.57 ± 0.091
Reviparin	4903 ± 111	1.30 ± 0.033

PAGE was performed using a 20–30% total acrylamide and 2–5% total N,N-bisacrylamide (pH 8.3) linear gradient gel. Results are means of five runs ± S.D.

charides and heparins (see Experimental). Several standardized heparin-disaccharides also commercially available.

Reversing the polarity of the system gave a better resolution of longer oligosaccharides [13]. Tetrasaccharides of different structures were examined by Dam et al. [4] in the context of heparin that was cleaved by heparinases. The longer time of analysis, however, requires optimization, which was achieved by using a thinner capillary. Varying the applied voltage from 20 to 5 kV or the pH of buffer from 2.0 to 4.0 did not improve the resolution of heparin-oligosaccharides and heparins. The heparins used were of research quality and contained small amounts of ethanol (0.5–1%) and dermatan sulfate

(<1%). Contaminants in low-molecular-mass heparin preparations most likely represent synthetic precursors, although their identity is not known. It should be realized that, in general, detection based on UV absorption is much more sensitive for the synthetic substituents than for the end products, which may lead to an overestimation of contaminants.

Gradient PAGE has already been used for the determination of glycosaminoglycans [6]. Heparin and chondroitin sulfates in aqueous and endogenous fluids have been determined [7]. However, the method has not been run using smaller slab gels. This method needs a shorter run time, less material and buffer. Further, several slab gel units can be run in parallel. The method requires a molecular ladder to characterize the migration times.

Calculating the electrophoretic mobility may be a valuable parameter for characterizing various heparin-oligosaccharide fractions, because it is independent of voltage and the length of the gel. The mobility is dependent only on the buffer type, pH and temperature. This mobility can only be calculated for the resolving gel because the samples were first preconcentrated in the stacking gel. The difference in the voltage applied implies that the mobility would not be a better parameter than the migration time for characterizing the PAGE of the heparin oligosaccharides. The standard used (Reviparin) was an LMMH, which was produced by nitrous acid and showed clearly resolved bands that ranged from di- to tetradodecasaccharide.

A new staining procedure for glycosaminoglycans with acridine orange allowed a better resolution of the oligosaccharide bands compared with alcian blue (more defined bands could be detected). This difference may be due to the different molecular mass of alcian blue and acridine orange forming cation–anion complexes. Smaller molecules might be bound even by weaker interactions. The LMMHs were scanned five times on different days with a densitometer and their molecular mass was calculated as described previously [14].

The PAGE method presented could be automated to obtain more information about the oligosaccharide composition of each heparin preparation.

A combination of PAGE and HPCE methods may be the best for the characterization of the different heparin preparations. PAGE analysis provides information about the composition of oligosaccharides fractions, which is most important with regard to the anticoagulant activity because the antifactor Xa, anti-thrombin activity and the neutralization of heparin require oligosaccharide chains of different chain length.

Acknowledgements

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Abbreviations

GAGs, glycosaminoglycans; CZE, capillary zone electrophoresis; HPCE, high-performance capillary electrophoresis; HPSEC, high-performance size-exclusion chromatography; LMMH, low-molecular-mass heparin; D_p, degree of polymerization; P, polydispersity; M, average molecular mass; M_m, mass-average molecular mass; M_n, number-average molecular mass; M_z, z-average molecular mass; L-IdoA, L-iduronic acid; $GlcNSO_3$, glucosamine N-sulfate; ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid; GlcNAc, 2-deoxy-2-acetamidoglycopyranose; SAX, strong anion exchange; Am, anhydromannose; SDS, sodium dodecylsulfate.

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