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Capillary electrophoretic separation of heparin oligosaccharides under conditions amenable to mass spectrometric detection

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Abstract

A capillary electrophoresis method for the separation of high-molecular-mass heparin oligosaccharides compatible with mass spectral detection was developed. Structurally defined heparin oligosaccharides ranging in size from tetrasaccharide to tetradecasaccharide were used to optimize the conditions. Applying normal and reversed polarity modes, these oligosaccharides were separated by CE under various conditions. Ammonium hydrogencarbonate (30 m*M* at pH 8.50) used as the running electrolyte system gave good separation efficiency and resolution in the normal polarity mode. Application of this method to the separation of complicated heparin oligosaccharide mixtures required the addition of electrolyte additives. Ammonium hydrogencarbonate (30 m*M*), containing triethylamine (10 m*M*), was useful for the separation of complex oligosaccharide mixtures. Run-to-run and day-to-day precision and limits of detection were established for these separations.

Keywords: Buffer composition; Heparin; Oligosaccharides

1. Introduction

Heparin is a polydisperse (M_r 5000–40 000; average M_r 14 000) highly sulfated, linear polysaccharide that has a wide range of important biological activities [1–3]. These activities result from the ability of heparin to interact with various proteins [4]. Some of these interactions, such as the binding of heparin to antithrombin III, are known to take place at specific oligosaccharide sequences present within the heparin polymer. Other heparin–protein interactions are also suspected of being highly specific [4–7] making it very important to improve our understanding of heparin's structure. The incomplete biosynthetic modification of this molecule results in substantial structural complexity. The major disaccharide unit of heparin is composed of α (1→4) linked L-iduronic acid substituted with *O*-sulfo groups at the C2 position and D-glucosamine with sulfo groups at the C2 and C6 positions. In minor disaccharide sequences, the uronic acid may be either L-iduronic acid or D-glucuronic acid. The amino sugar, D-glucosamine, may be either *N*-sulfonated or *N*-acetylated. The amino sugar residues can be substituted with *O*-sulfo groups at C6 and/or C3, as can be the C2 of the uronic acid (Fig. 1). Because of heparin's complex structure, relatively high molecular mass and polydispersity, direct determination of its structure is not possible, so heparin is broken down into smaller oligosaccharides using chemical or enzymatic methods [8]. In our laboratory, heparin-derived oligosaccharides are prepared by partial depolymerization of bovine lung (BL) or porcine

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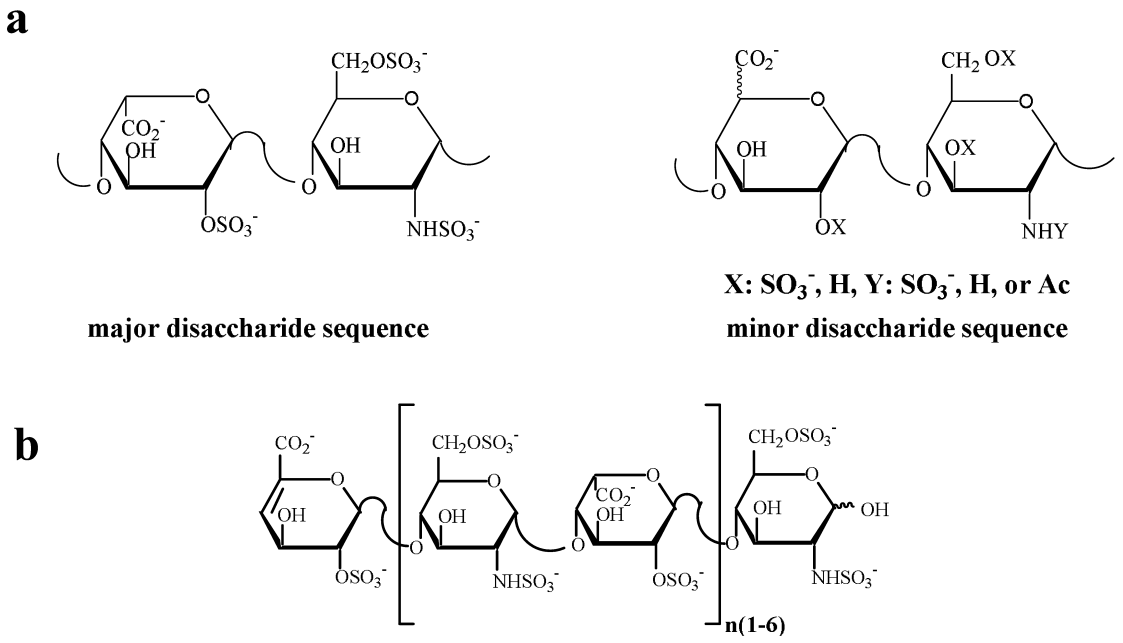

Fig. 1. Chemical structures of (a) heparin and (b) fully sulfated oligosaccharides obtained by heparinase depolymerization of BL heparin, $n=1$ tetrasaccharide, $n=2$ hexasaccharide, $n=3$ octasaccharide, $n=4$ decasaccharide, $n=5$ dodecasaccharide, $n=6$ tetradecasaccharide.

intestinal (PI) heparin using heparin lyase I, which cleaves heparin at the →4)-α-D-GlcNpS6S (1→4)-α-L-IdoAp2S (1→ linkage (where GlcNp is 2-deoxy-2-aminoglucopyranose, IdoAp is idopyranosyluronic acid and S is sulfate) that most frequently occurs in heparin [9]. A partial (30%) digestion of BL heparin provides an oligosaccharide mixture that contains a high percentage of oligosaccharides with sizes between 4 and 14 saccharide units [10]. The oligosaccharide mixture is purified combination of low-pressure gel permeation chromatography (LP-GPC), semi-preparative and analytical strong anion-exchange high-performance liquid chromatography (SAX-HPLC). The assessment of oligosaccharide purity and structure is made using polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE) and one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy [10–12].

CE has recently emerged, as a promising analytical technique that consumes an extremely small amount of sample and that is capable of rapid, high-resolution separation, characterization and reproducible quantification of analytes [13]. CE is widely used as a valuable technique for the analysis of acidic oligosaccharides [14–17], however, in the literature regarding the analysis of heparin oligosaccharides by on-line CE–mass spectrometry (MS), there are a few examples and this work is focused on the analysis of heparin-derived disaccharides.

Ruiz-Calero et al. [18] developed a CE method with reversed polarity mode for the analysis of heparin-derived disaccharides. The same research group in subsequent studies [19] investigated the application of the electrophoretic conditions of CE–UV method to the electrospray ionization (ESI) MS coupling for the analysis of disaccharides. Duteil et al. [20] explored the direct coupling between CE and ESI-MS using both normal and reversed polarity modes. Conditions were optimized using heparin-derived disaccharide standards. They found positive polarity CE with negative MS ionization seemed to be the best approach. This methodology was also applied to characterize enzymatically depolymerized PI heparin. That product was 86% depolymerized into disaccharides and CE–ESI-MS analysis identified eight heparin-derived disaccharides and two heparin-derived tetrasaccharides.

Since in the past, the major work focused on the analysis of heparin-derived disaccharides, we under-

took to develop a CE method for the separation of high-molecular-mass heparin-derived oligosaccharides that could be coupled to CE–MS for direct detection.

2. Experimental

2.1. Chemicals

Unless otherwise specified, all chemicals were of analytical grade. Acetic acid glacial, formic acid 88%, ammonium hydroxide, ammonium hydrogencarbonate, glycine, disodium ethylenediaminetetraacetic acid (EDTA), boric acid, sucrose, *N*,*N*′-methylenebisacrylamide and *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Alcian blue, ammonium persulfate were purchased from Boerhringer Mannheim (Indianapolis, IN, USA). Triethylamine, dibutylamine, tributylamine were obtained from Fluka (Milwaukee, WI, USA). Purified water used throughout was obtained from a Milli-Q system (Bedford, MA, USA). PI and BL heparin and heparin lyase I (EC 4.2.2.7) were obtained from Sigma (St. Louis, MO, USA).

2.2. Capillary electrophoresis conditions

The experiments were performed on a P/ACE system 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with a photodiode array detector at a constant capillary temperature of 25 °C with detection by absorbance at 232 nm.

Running electrolytes were filtered through a 0.22 μm membrane filter, and degassed before use. In the buffer additive studies in 30 m*M* ammonium hydrogencarbonate, the measured pH values of the running electrolytes were: 8.50 for 5 m*M* triethylamine; 9.10 for 10 m*M* triethylamine; 9.05 for 10 m*M* dibutylamine; and 8.40 for 10 m*M* tributylamine. The electrophoretic separations were carried out using uncoated fused-silica capillaries of 57 cm (separation length 50 cm)×75 μm I.D. Prior to use, the new capillary was pre-treated with 0.5 *M* sodium hydroxide for 30 min, then rinsed with water for 10 min. The capillary was conditioned with the running electrolytes for 20 min before the first run and 1 min in between runs. Samples were loaded with pressure injection for 5 s at $3.45 \cdot 10^{-3}$ MPa (0.5 p.s.i.).

2.3. Polyacrylamide gel electrophoresis analysis

Buffer solutions were prepared as follows: resolving gel buffer and lower chamber buffer contain 0.1 *M* boric acid, 0.1 *M* Tris, 0.01 *M* disodium EDTA, pH 8.3. Stacking gel buffer, 0.1 *M* boric acid, 0.01 *M* disodium EDTA, 0.1 *M* Tris–HCl, pH 6.3. Upper chamber buffer, 1.24 *M* glycine, 0.2 *M* Tris. Gradient polyacrylamide resolving gel was prepared from two resolving gel buffer solutions, one containing 11.52% (w/v) acrylamide and the other containing 20% (w/v) acrylamide, with 0.48 and 2% (w/v) *N*,*N*′-methylenebisacrylamide and 1 and 15% (w/v) sucrose, respectively. Gels were poured vertically between glass plates (32×16 cm) separated by 1.5-mm spacers [10]. Electrophoresis was performed at 400 V for 3.5 h, and the bands were visualized by Alcian blue staining.

3. Results and discussion

Six structurally defined, homogenous, fully sulfated, heparin oligosaccharide standards (Fig. 1) [10], ranging in size from tetrasaccharide [degree of polymerization (DP) 4] to tetradecasaccharide (DP 14), were each individually injected under all CE conditions tested to confirm the migration order of these compounds. Next, co-injection experiments were performed on these six compounds to optimize the separation conditions. Oligosaccharides, prepared using heparin lyase, have an unsaturated uronic acid residue (ΔUA) at their non-reducing end and, thus, exhibit absorbance maxima at 232 nm (ε 5000–6000 M^{-1} cm^{-1}) [21]. These six fully sulfated oligosaccharides were analyzed by CE under various conditions by applying normal and reversed polarity modes. In CE, mobility is proportional to analyte charge and inversely proportional to molecular size (associated with a drag force), which correlates to molecular mass. All six fully sulfated oligosaccharide standards have the same calculated charge-to-mass ratio (number of sulfo and carboxyl groups/molecular mass) and thus should show little if any

separation on CE. The actual molecular charge of larger oligosaccharides is reduced because ionization of one group suppresses the ionization of adjacent groups on the same molecule (a polyelectrolyte effect). Thus, the smaller the oligosaccharide, the higher the actual charge-to-mass ratio and the greater the mobility observed under electrophoresis, permitting their separation by CE.

In the reversed polarity mode, the sample is applied at the cathode and detected at the anode, the low pH electrolyte used ensures that silanol residues on the capillary are uncharged. At acidic pH, the electroosmotic flow (EOF) is too weak to overcome the electrophoretic mobility. The major force in the separation is the mobility of ions under electrophoresis. CE separation of heparin-derived oligosaccharides in the reversed polarity has been primarily carried out using sodium phosphate at pH 3.50 [15,17,22], a buffer system that is not compatible with on-line mass detection.

In the reversed polarity mode, volatile buffers ammonium formate and ammonium acetate were selected as running electrolytes. The pH of formic acid and acetic acid solutions were adjusted with ammonium hydroxide (4%, w/v). Ammonium formate, ammonium acetate, pH 3.50 and pH 5.0 buffers at concentrations of 30 and 60 m*M* were tested using an applied voltage of −15 kV at a temperature of 25 °C. None of the electrolyte systems gave good chromatographic separation of these six heparin-derived oligosaccharides (data not shown). The effect of temperature on the separation was also tested, but even by reducing the temperature no improvement of the separation was observed. Pervin et al. [17] reported that the resolution in reverse polarity separations decreased in sodium phosphate buffers with increasing heparin oligosaccharide size. It was suggested that the reason for poor resolution was that low pH reduces EOF towards the cathode and separation of these species having similar charge characteristics mainly results from electrophoresis.

In the normal polarity mode, sample is injected at the anode and detected at the cathode. Negatively charged species are prevented from migrating out of the capillary by the dominant force associated with EOF. Separation of these anions is achieved by both electrophoresis, pushing negative analyte towards the anode, and the dominant EOF, moving all species towards the cathode. The order of migration of oligosaccharides was, as expected under normal polarity, with the tetradecasaccharide having the lowest electrophoretic mobility (counter to the EOF), eluting first and the tetrasaccharide having the highest electrophoretic mobility, eluting last [15–17,25]. Ammonium formate, ammonium acetate and ammonium hydrogencarbonate, pH 8.50 were used as running electrolytes and separations were carried out at 22 kV. The order of migration of oligosaccharides was as expected under normal polarity, with the most highly charged tetradecasaccharide eluting first and the least highly charged tetrasaccharide eluting last. Baseline separation of higher size oligosaccharides (DP 12 and 14) was not obtained with 30 m*M* ammonium formate. Ammonium acetate (30 m*M*) and ammonium hydrogencarbonate (30 m*M*) running electrolytes afforded baseline separation of all six heparin-derived oligosaccharides (Fig. 2). Separation efficiency and resolution were calculated using both ammonium acetate and ammonium hydrogencarbonate buffer systems. Ammonium hydrogencarbonate (30 m*M*, pH 8.50) showed better results than ammonium acetate. The separation efficiency and resolution between oligosaccharides in ammonium acetate ranged from $8.9\cdot10^3$–$6.2\cdot10^4$ theoretical plates/m and resolution of 0.85–2.55, respectively. Ammonium hydrogencarbonate gave values ranging from $1.4\cdot10^4$–$6.5\cdot10^4$ theoretical plates/m and resolution of 0.95–3.51, respectively.

Next, this separation was applied to complex oligosaccharide mixtures. A hexasaccharide mixture prepared from PI heparin was first analyzed. In our laboratory, heparin-derived oligosaccharides are prepared by partial (30%) depolymerization BL or PI heparin using heparin lyase I followed by sizing on LP-GPC [10]. This heparin digest is routinely used as a standard banding ladder for oligosaccharide analysis by high-resolution PAGE. This mixture contains oligosaccharides of different sequences and of sizes ranging from disaccharides to oligosaccharides having over 28 sugar units. The complexity of this mixture is further increased because of the different concentrations of each component present. CE is a very high-resolution analytical technique capable of separating different oligosaccharide isomers, even those that are not resolvable on PAGE.

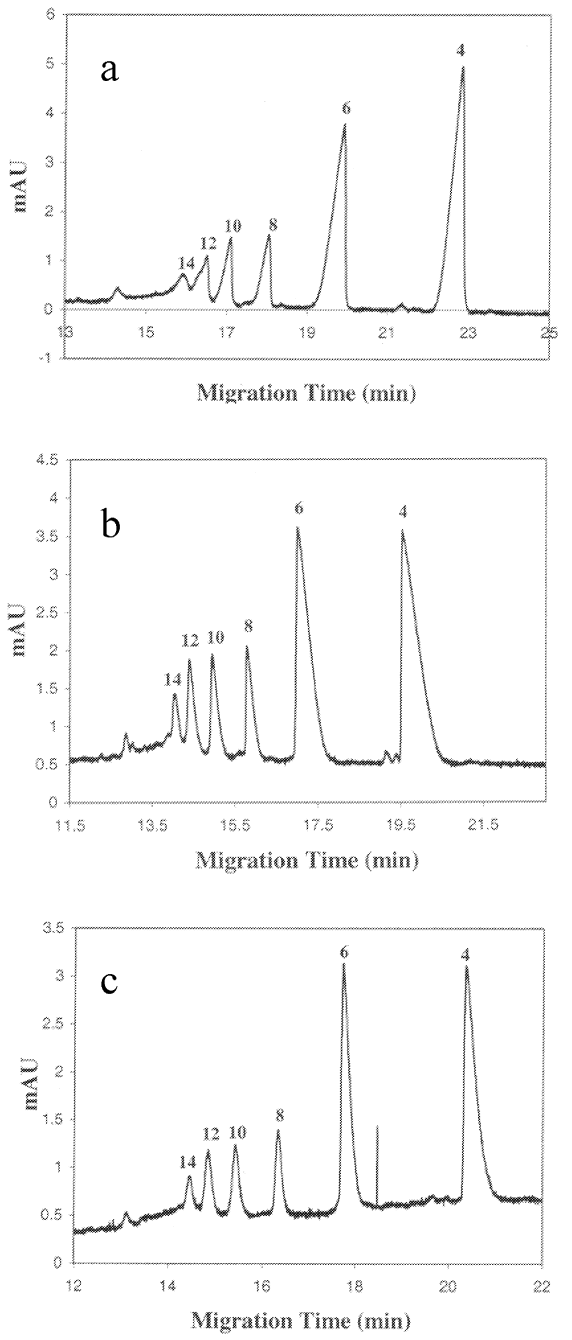

Fig. 2. Portions of electropherograms of fully sulfated heparin-derived oligosaccharides (DP 4–14) under normal polarity mode. Applied voltage: 22 kV, pH 8.50 (a) 30 m*M* ammonium formate, (b) 30 m*M* ammonium acetate, (c) 30 m*M* ammonium hydrogencarbonate.

Ammonium hydrogencarbonate (30 m*M*, pH 8.50) was next used as running electrolyte in the separation of this hexasaccharide mixture. While some separation was obtained, substantial peak overlap was also observed (Fig. 3b). Next, volatile electrolyte additives were added to the running electrolyte system to improve the separation. The pH values of the electrolytes were only slightly altered (varying by no more than 0.6 units) on addition of these volatile agents. Initially, low-molecular-mass volatile agents including triethylamine, dibutylamine and tributylamine were examined. Running buffer system containing 10 m*M* triethylamine afforded additional peaks, and baseline separation was obtained (Fig. 3d). Since 10 m*M* triethylamine as an electrolyte additive provided the best chromatographic resolution, dibutylamine and tributylamine were examined at the same concentration (Fig. 4). The addition of 10 m*M* tributylamine (Fig. 4c) improved the separation slightly compared to 10 m*M* triethylamine (Fig. 4a), the addition of 10 m*M* dibutylamine into running electrolyte (Fig. 4b) afforded no improvement.

Electrolyte additives have a relevant role in affording the best performance, influencing the separation mechanism [23–26]. Electrolyte additives reduce the EOF toward the cathode probably by masking the negatively charged silanol groups on the capillary wall. The electroosmotic velocity differs from additive to additive [24]. Variation in the chemical nature of the amines influences their interaction with capillary wall, thus giving a different EOF for each additive. They also play a role as ion-pairing reagent for negatively charged analytes. The electrophoretic mobilities of negatively charged analytes are decreased towards by partially neutralizing the negative charge on the analytes. Combination of these two factors may be responsible for improvements in the separation. Triethylamine (10 m*M*) and ammonium hydrogencarbonate (30 m*M*) shows the best combination of the additives used for the separation of these hexasaccharides.

Hexa-, octa-, deca- and dodecasaccharide mixtures enzymatically prepared from BL heparin by LP-GPC were next used under this optimized running electrolyte system. PAGE analysis showed that each oligosaccharide mixture contained mainly fully sulfated hexa- (nine sulfo groups), octa- (12 sulfo

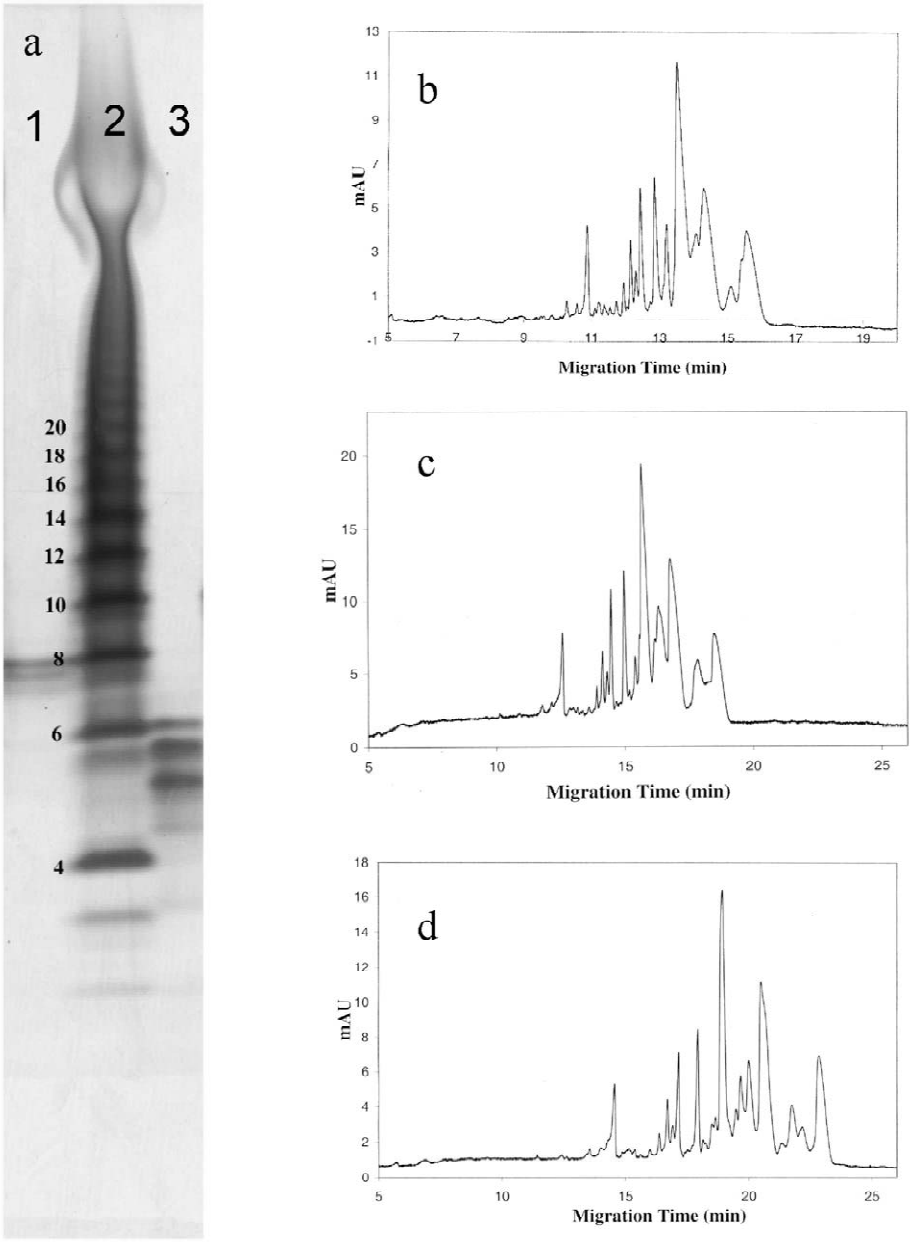

Fig. 3. Gradient PAGE and CE analysis of PI heparin-derived hexasaccharide mixture. (a) PAGE analysis of (lane 1) fully sulfated octasaccharide standard, (lane 2) 30% depolymerized BL heparin, (lane 3) PI hexasaccharide mixture. Panels (b), (c) and (d) CE analysis with applied voltage: 22 kV, 30 m*M* ammonium hydrogencarbonate containing (b) 0 m*M*, (c) 5 m*M*, (d) 10 m*M* triethylamine.

groups), deca- (15 sulfo groups), and dodecasaccharide (18 sulfo groups), respectively, but also other undersulfated components (Fig. 5). The number of expected peaks on CE correlated well with PAGE results of each oligosaccharide mixture. For example, the electrophoregram of BL hexasaccharide mixture shows contaminating tetrasaccharide structures [four components in both PAGE (Fig. 5a, lane 7) and CE

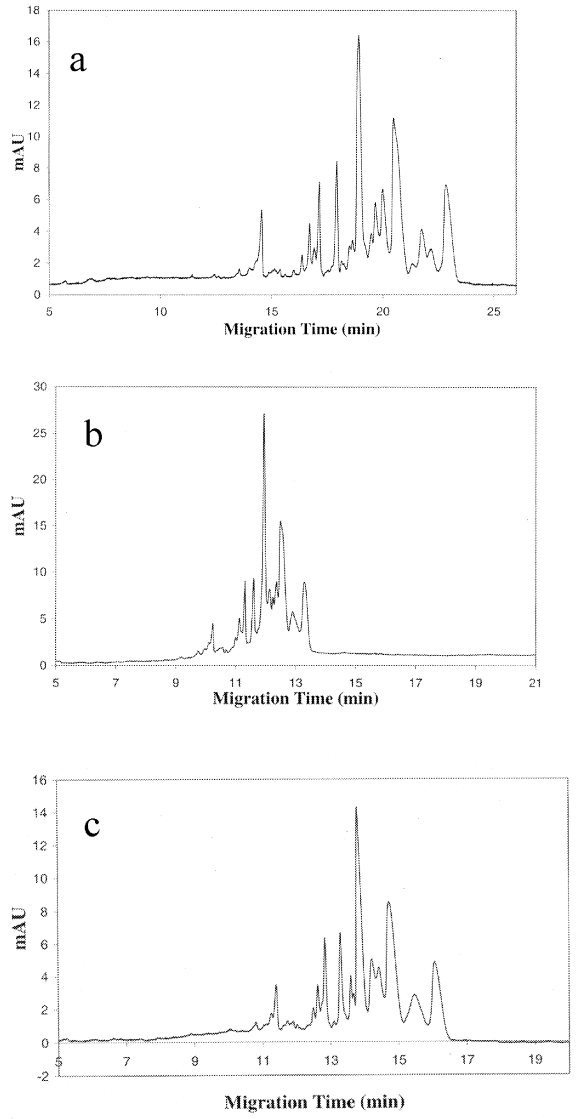

Fig. 4. Sections of the electropherograms of PI heparin-derived hexasaccharide mixture in normal polarity mode, applied voltage: 22 kV, 30 m*M* ammonium hydrogencarbonate, (a) 10 m*M* triethylamine, (b) 10 m*M* dibutylamine, (c) 10 m*M* tributylamine.

(Fig. 5b, migrating between 25 and 32 min], fully and undersulfated hexasaccharide components (five components in PAGE and in CE, migrating between 20 and 24 min) and contaminating fully and undersulfated octasaccharides (four major components in PAGE and in CE, migrating at <20 min). Co-injection of hexasaccharide (Fig. 5b) and octasaccharide mixture (Fig. 5c) was used to identify the fully and undersulfated octasaccharide structures in hexasaccharide mixture (data not shown). It should be pointed out that counting the exact number of bands on gradient PAGE is complicated as some are very faint and some bands are overlapped.

Run-to-run, day-to-day precision and limits of detection with ammonium hydrogencarbonate (30 m*M*), triethylamine (10 m*M*) running electrolyte were established using the standard mixture of the structurally defined homogenous fully sulfated BL-derived heparin oligosaccharides (DP 4–10). Run-to-run precision of migration times and peak areas obtained from four replicates of this standard mixture carried out the same day, and day-to-day precision of migration times and peak areas obtained from four replicates on 4 different days (Table 1). While the run-to-run and day-to-day migration times and run-to-run areas were satisfactory (RSD<2.7%), day-to-day area repeatability was not as good (RSD> 10.4%). These results suggest that daily calibration is required in quantitative applications. A comparison, utilizing identical electrolyte and voltage on the same sample, clearly illustrate the expected slight day-to-day variations observed in electropherograms (Figs. 3d and 4a). The limits of detection with UV detection at 232 nm based on a 3:1 signal-to-background noise ratio ranged from sample concentrations of 0.9 to 6.9 μ*M*. These values are consistent to those in the literature for direct injection and UV detection of heparin-derived oligosaccharides.

4. Conclusions

A CE method for the separation of high-molecular-mass heparin oligosaccharides compatible with mass spectral detection was developed. To optimize the conditions, structurally defined heparin-derived oligosaccharides ranging in size from tetrasaccharide to tetradecasaccharide were used to perform these studies. Ammonium hydrogencarbonate (30 m*M*, pH 8.50) running electrolyte, gave good separation efficiency and resolution in the normal polarity mode. Application of this method for the separation of complicated heparin oligosaccharide mixtures required the addition of volatile electrolyte additives such as triethylamine. Good day-to-day and run-to-

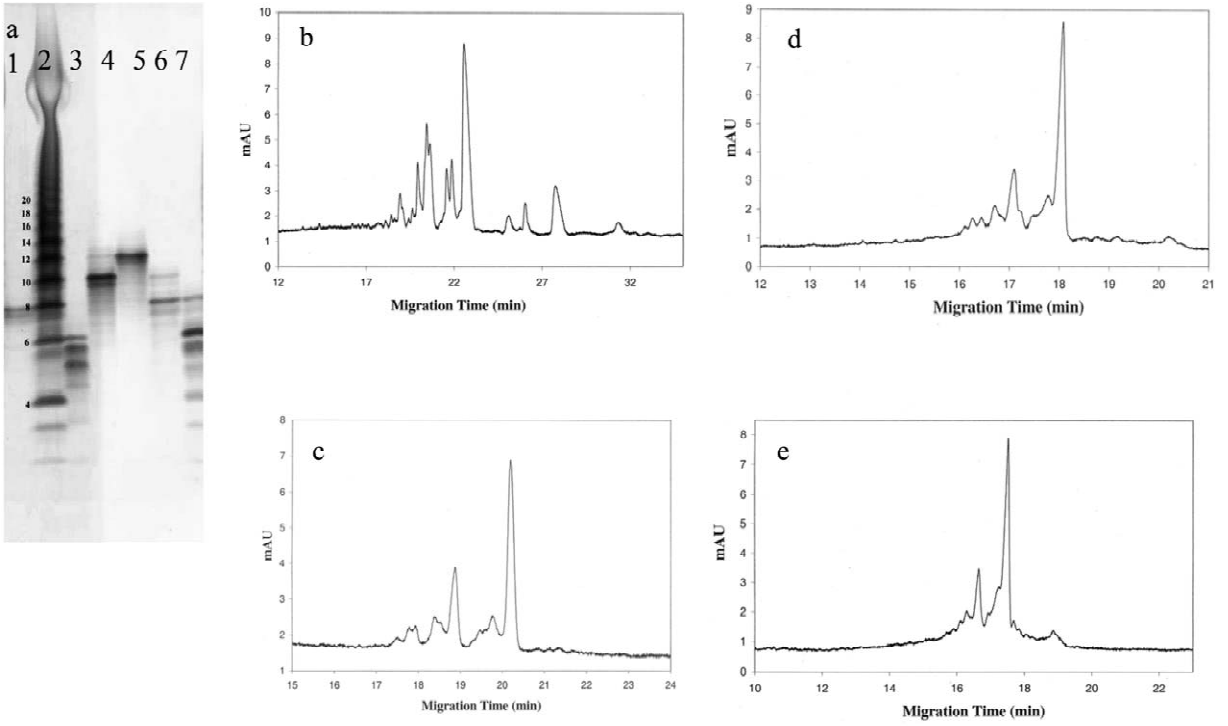

Fig. 5. PAGE analysis and partial capillary electropherograms of BL heparin-derived oligosaccharide mixtures in normal polarity mode. (a) PAGE gel: (lane 1) fully sulfated octasaccharide standard, (lane 2) oligosaccharide mixture with DP of fully sulfated oligosaccharides (DP 4–20) labeled, (lane 3) PI hexasaccharide mixture, (lane 4) BL decasaccharide mixture, (lane 5) BL dodecasaccharide mixture, (lane 6) BL octasaccharide mixture, and (lane 7) BL hexasaccharide mixture. CE electropherograms of BL oligosaccharide mixtures, applied voltage: 22 kV, 30 m*M* ammonium hydrogencarbonate, 10 m*M* triethylamine (b) hexasaccharide mixture, (c) octasaccharide mixture, (d) decasaccharide mixture, (e) dodecasaccharide mixture.

run migration times and day-to-day peak area precision (RSD<2.7%) with limits of detection of 0.9 to 6.9 μ*M* were obtained. On-line CE–MS and CE–MS–MS using these running electrolytes should provide composition and structural information of complex heparin-derived oligosaccharide structures.

Table 1
Run-to-run, day-to-day precision of migration times, areas, and limits of detection for CE performed in 30 m*M* ammonium hydrogencarbonate, 10 m*M* TEA, pH 9.10

	Run-to-run precision		Day-to-day precision		Detection limit	
	RSD t_M[a] (%)	RSD area (%)	RSD t_M[a] (%)	RSD area (%)	μg ml^{-1}	μ*M*
FS tetrasaccharide	2.41	1.93	2.54	11.81	1.2	0.9
FS hexasaccharide	2.03	2.32	2.11	10.47	6.0	3.0
FS octasaccharide	1.83	1.93	1.89	11.17	12.00	4.5
FS decasaccharide	1.75	2.62	1.76	12.42	23.00	6.9

[a] Retention times (t_M) vary from capillary to capillary. Average retention times observed on the capillary used in these experiments were: fully sulfated (FS) tetrasaccharide, 28 min; FS hexasaccharide, 23 min; FS octasaccharides, 20.5 min; and FS decasaccharide, 18 min.

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