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Separation of a complex mixture of heparin-derived oligosaccharides using reversed-phase high-performance liquid chromatography

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Abstract

A reversed-phase ion-pairing high-performance liquid chromatography (RP-HPIPC) method for the separation of a complex mixture of heparin-derived oligosacchrides has been developed by a stepwise optimization of the mobile phase, in which the concentration of ion-pairing reagent, mobile phase pH, and acetonitrile concentration were varied. The resolution of more than 30 oligosaccharide components was obtained, under optimized conditions, in an analysis time of less than 30 min. This represents the first RP-HPLC method that can separate a complex mixture of both small and large sulfated oligosaccharides in a single chromatographic step. The heparin-derived oligosaccharides, in this mixture, can also be separated under a second set of RP-HPIPC conditions using a volatile ion-pairing reagent, tributylammonium acetate, to aid in the recovery of individual sulfated oligosaccharides. Moreover, it was possible to replace sodium chloride gradient, required for eluting highly sulfated oligosaccharides, with a fixed, low concentration of a volatile salt, ammonium acetate, by utilizing an acetonitrile gradient. This solvent system might make it possible to directly interface this RP-HPIPC separation with mass spectral analysis.

Keywords: Ion-pairing reagents; Gradient elution; Oligosaccharides

1. Introduction

Glycosaminoglycans (GAGs) are a family of highly sulfated, linear polysaccharides that display a wide array of biological activities [1–3]. GAGs can be selectively depolymerized by site-specific enzymes [4–8] to obtain oligosaccharide mixtures [9,10]. Heparin, the most prominent GAG, exhibits an array of important biological activities through its interaction with coagulation proteins, growth factors, and chemokines [3,11]. A deeper understanding of the biological functions of GAGs requires new analytical methods for the elucidation of their fine structures.

A number of chromatography and electrophoresis techniques have been explored for the separation of GAG-derived oligosaccharides. These include polyacrylamide gel electrophoresis (PAGE) [10,12–14], capillary electrophoresis (CE) [15–18], affinity chromatography, low-pressure size-exclusion chromatography (SEC) [19], and high-performance liquid chromatography (HPLC) [20–25]. No single method affords high-purity homogeneous oligosaccharides from complex mixtures. A major limitation in under-

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standing the biochemistry of highly sulfated GAGs, such as heparin, is the lack of high resolution analytical techniques that are efficient in fractionating oligosaccharide mixtures ranging in charge from −4 to −32 and degree of polymerization (DP) from 2 to 16.

Amino [26,27], reversed-phase ion-pairing (RP-IPC) [23,28], strong anion-exchange (SAX) [19,22,23] HPLC-based methods have been applied to the analysis of the GAG-derived oligosaccharides. These chromatographic separations represent a critical step in determining oligosaccharide structure, metabolism and function [19,25]. These oligosaccharides are often available in only small quantities further complicating their analysis.

SAX-HPLC has been the preferred technique for the separation and analysis of heparin-derived oligosaccharides [19,22,25]. However this method is limited by long separation times (>90 min), works poorly or not at all for chemically modified oligosaccharides containing hydrophobic protecting groups, and requires extensive desalting of purified oligosaccharide compounds. RP-HPIPC, while offering an interesting alternative to SAX-HPLC has only been employed for the analysis of relatively small (DP≤6) oligosaccharides [28–30]. Furthermore, most widely used ion-pairing reagents, i.e., tetrabutylammonium salts, are difficult to remove from purified oligosaccharides, limiting the recovery of pure oligosaccharides suitable for biological evaluation.

In the current study, RP-HPIPC on an octadecyl (C_{18}) silica column was optimized for separation of heparin-derived oligosaccharides, ranging in size from disaccharide to large oligosaccharides, obtained from the controlled depolymerization of heparin with heparinase (EC 4.2.2.7). Volatile ion-pairing reagent, tributylammonium acetate (TrBA), and volatile salt, ammonium acetate, were also examined to provide an eluent compatible with HPLC–MS analysis.

2. Materials and methods

2.1. Chemicals

Bovine lung heparin, sodium salt (≥140 USP units per mg, 500 000 units) was from Sigma (St. Louis, MO, USA). Heparin lyase I (EC 4.2.2.7) was from IBEX (Montreal, Canada). Acetonitrile, HPLC grade, and all other chemicals, of the purest grade available, were obtained from Aldrich (Milwaukee, WI, USA). Ultra-pure water was obtained using a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Preparation of heparin oligosaccharide mixtures

The heparin oligosaccharide mixture was prepared from bovine lung heparin by controlled enzymatic depolymerization with heparin lyase I as previously described [19]. Briefly, 5 g of heparin was prepared in 80 ml of 50 m*M* sodium phosphate buffer (pH 7.0) containing 2 mg/ml bovine serum albumin and sterilized with a filter (0.22 μm). To this solution 0.5 IU of heparin lyase I was added at time 0 and again at 24 h, and the mixture was incubated at 30 °C until the digestion was 30% complete. The mixture was adjusted to pH 3 with concentrated hydrochloric acid. The precipitated protein was removed by centrifugation, and the solution was passed through a sulfopropyl-Sephadex column to remove the remaining protein. The oligosaccharide mixture was adjusted to pH 7.0, freeze–dried, redissolved in water, and separated by pressure filtration with a 5000 molecular mass cut-off (MWCO) membrane into low-molecular-mass (M_r<5000) and high-molecular-mass (M_r>5000) oligosaccharides. The resulting low-molecular-mass oligosaccharide fraction, corresponding to the mixtures of disaccharide through tetradecasaccharide, was desalted by a Bio-Gel P-2 column and freeze–dried.

2.3. HPLC separations

SAX-HPLC separation of heparin oligosaccharide mixture was performed on a 5 μm Waters Spherisorb column (250×4.5 mm) from Waters (Milford, MA, USA). The mobile phase was water (solution A) and 2 *M* sodium chloride (solution B), with linear gradient elution 0.2–2 *M* sodium chloride and a flow-rate of 1.0 ml/min. Both solutions A and B were adjusted the pH 3.5 by hydrochloric acid.

RP-HPIPC separations of the same oligosaccharide mixture were performed on a 5 μm Discovery C_{18} column (250×4.6 mm) (250×20 mm for semi-preparative column) from Supelco (Bellefonte, PA, USA). For conditions relying on non-volatile eluent,

the mobile phases were acetonitrile–water containing tetrabutylammonium hydrogen sulfate in both solutions A and B, and solution B contained 2 *M* sodium chloride. The mobile phase pH was adjusted by acetic acid. A linear gradient elution of 0.2–2 *M* sodium chloride over 120 min at a flow-rate of 1.0 ml/min was used. For conditions relying on volatile eluent, the mobile phase was acetonitrile–water containing tributylamine and ammonium acetate in both solutions A and B. The mobile phase pH was adjusted by acetic acid. A linear gradient elution from 20% to 65% acetonitrile in 120 min and a flow-rate of 1.0 ml/min were used.

The liquid chromatography system consisted of two Shimadzu LC-10Ai pumps, and a Shimadzu UV–visible spectrophotometric detector (Model SPD-10A). The elution profiles in the HPLC separations were monitored by absorbance at 232 nm at 0.02 AUFS. All HPLC experiments were repeated, and all separations were performed at room temperature unless otherwise indicated.

2.4. Recovery of purified oligosaccharides from the volatile ion-pairing reagent

A sized uniform oligosacchride fraction, corresponding to tetrasaccharides (5 mg), was purified at the semi-preparative scale using the mobile phase conditions relying on the volatile eluent. The main peak corresponding to the fully sulfated tetrasaccharide was separately collected, freeze–dried to remove the volatile ion-pairing reagent, and desalted on a Bio-Gel P-2 column (45×1.5 cm I.D.) eluted with water at the flow-rate of 0.3 ml/min. The eluent was collected and freeze–dried. The pure oligosaccharide sample was dissolved in $^{2}H_{2}O$ (99.9%), filtered through a 0.45-μm syringe filter and freeze–dried to remove exchangeable protons. After exchanging the sample three times, the sample was re-dissolved in $^{2}H_{2}O$ (99.9%). ^{1}H Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker DRX-600-MHz instrument.

3. Results and discussion

RP-HPIPC has several advantages compared to SAX-HPLC in the separation of charged analytes. Among these advantages are enhanced sensitivity, increased separation efficiency and improved column stability [28–30]. The objective of this study was to develop an RP-HPIPC method to separate heparin-derived oligosaccharides with resolution greater than the conventional SAX-HPLC separation [19,22,25], involving shorter analytical times and utilizing eluents that might be compatible with MS detection.

3.1. Preparation of analyte

Bovine lung heparin was subjected to controlled partial enzymatic depolymerization using heparinase. Pressure filtration using a MWCO 5000 membrane yielded a mixture of oligosaccharides ranging in size from disaccharide (DP 2) to tetradecasaccharide (DP 14, M_r 4655). The composition of this mixture was confirmed by low-pressure SEC and gradient PAGE [19] (data not shown). The oligosaccharide mixture prepared from bovine lung heparin is known to contain oligosaccharides having only an even number of sugar residues (i.e., DP 2, 4, 6, 8, 10, 12, and 14). Furthermore, one oligosaccharide sequence predominates in each size class (Fig. 1) and disaccharides (DP 2) through tetradecasaccharides (DP 14) have been purified from this mixture and the structure of each was completely characterized by multi-dimensional NMR spectroscopy [19].

3.2. Preparation of mobile phase

Acetonitrile–water (15:85, v/v) was initially selected as the mobile phase containing ion-pairing reagent and a linear gradient of salt was employed to elute the oligosaccharide samples. The development of the separation was carried out through systematically altering various experimental parameters.

3.3. Selection of an ion-pairing reagent

Because of the presence of multiple negative charges associated with N- and O-sulfo groups, heparin oligosaccharides are not expected to be retained on a reversed-phase column. Initial studies set out to select an appropriate ion-pairing reagent to partition heparin oligosaccharides onto the solid phase. Several tetraalkyl ammonium salts were investigated as ion-pairing reagents, including tetraethyl-, tetrabutyl-, and tetrahexylammonium salts. Tetrabutylammonium (TBA) salts, capable of retain-

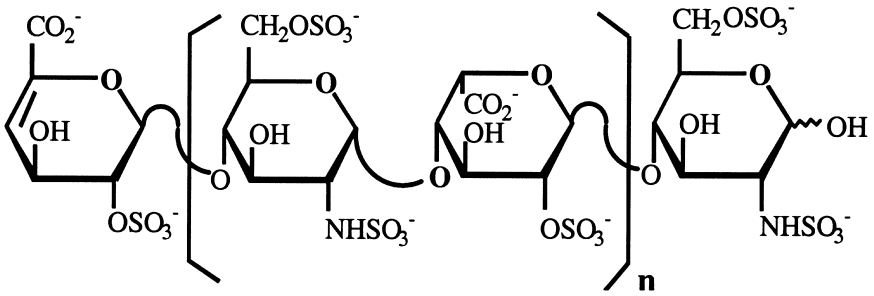

Fig. 1. Structures of major heparin oligosaccharides derived from heparinase treatment of bovine lung heparin. The net charges and molecular masses for the disaccharide (DP 2, $n=0$) are −4 and 665 (as the sodium salt) and for the tetradecasaccharide (DP 14, $n=6$) are −28 and 4655.

ing both small (DP 2) and large (DP 14) oligosaccharides on a C_{18} column, were selected for evaluation in further optimization studies. Ion-pairing reagents with larger alkyl groups gave very long retention times, while ion-pairing reagent with smaller alkyl groups did not show satisfactory retention of larger oligosaccharides.

3.4. Effect of ion-pairing reagent concentration on retention time

The mobile phase composition and pH were first fixed (15% CH_3CN, pH 3.5) to investigate the influence of TBA ion-pairing reagent concentration on the separation. The concentration of TBA was varied from 1 to 10 m*M*. The chromatogram showed baseline separation of many of the small (DP 2) and large (DP >10) oligosaccharides in mobile phase containing 10 m*M* TBA (Fig. 2a). It should be noted that the major peaks in each size class, labeled by DP, coeluted with authentic standards [19] and corresponded to the structures shown in Fig. 1. Minor peaks between DP 2 and DP 4, for example, correspond to undersulfated (<3 sulfo groups/disaccharide repeating unit) tetrasaccharides, ones between DP 4 and DP 6, correspond to undersulfated

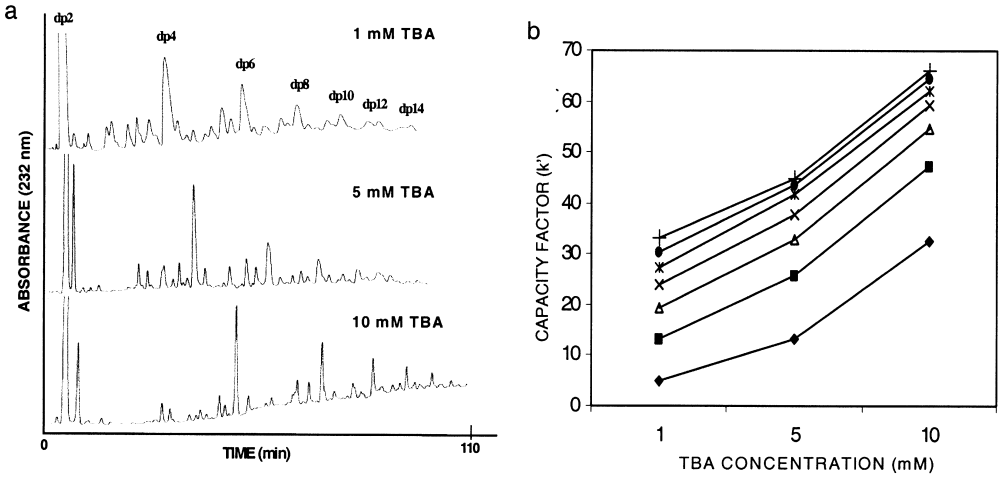

Fig. 2. The effect of TBA on (a) separation resolution (b) the capacity factor (k') of heparin-derived oligosaccharides. An elution gradient of 0.2–2 *M* NaCl in 120 min was formed utilizing mobile phases: A=15% CH_3CN, pH 3.5 and B=15% CH_3CN, 2 *M* NaCl, pH 3.5. At 10 m*M* TBA, the major disaccharide (DP 2) and tetradecasaccharide (DP 14) components eluted at 53.6 and 107.2 min, respectively. The curves in panel (b) correspond to (♦) disaccharide, (■) tetrasaccharide, (△) hexasaccharide, (×) octasaccharide, (*) decasaccharide, (•) dodecasaccharide, and (+) tetradecasaccharide.

hexasaccharides, and so on. Peak shape was also affected by concentration of ion-pairing reagent, peaks were broad and tailing, especially for large oligosaccharides, in mobile phase containing 1 m*M* TBA. The effect of TBA concentration on the capacity factors (k') of each heparin oligosaccharide is shown in Fig. 2. An increase in TBA concentration resulted in increasing k' values for each oligosaccharide studied as well as an increase in analysis time.

The basis for RP-HPIPC separation is still controversial. Two different processes are possible. In process 1, the retention of the oligosaccharides can be explained by the formation of ''ion-pairs'' between the positively charged ion-pairing reagent and negatively charged oligosaccharides. These ion-pairs mask the charges on the oligosaccharides, which in turn facilitates their partitioning on the hydrophobic column. In process 2, the adsorption of the positively charged ion-pairing reagent onto the C_{18} column, renders it into an ion-exchange column. In both retention processes, an increase in the concentration of the ion-pairing reagent in the mobile phase should lead to an increase in analyte retention.

Increasing the TBA concentration above 10 m*M* resulted in peak doubling, presumably through the resolution of the α and β anomeric forms of the individual oligosaccharides further the complicating the chromatogram. These doublet peaks coalesce into single peaks when the column temperature was increased to 50 °C (data not shown). High temperature is known to accelerate the rate of interconversion between α and β anomers of oligosaccharides. Since increasing temperature did not improve the resolution nor decrease retention time, the remaining optimization experiments were carried out using 10 m*M* TBA at room temperature.

3.5. Effect of mobile phase pH on retention time

Different eluent pH values, from 3.5 to 7.0, were examined for the separation of oligosaccharides. Higher pH values enhanced resolution (Fig. 3a) with an increase in mobile phase pH leading to decreasing k' values for each oligosaccharide. While little decrease in k' was observed for disaccharide (DP 2) though hexasaccharide (DP 6) from pH 5.5 to 7.0, significant changes were observed for larger (DP 8 to 14) oligosaccharides. The pK_a of O- and N-sulfo groups and carboxyl groups in heparin are <1, 1–1.5 and 3–4, respectively [3]. Because of the polyelectrolyte effect, the pK_a of carboxyl groups should increase as oligosaccharide size (and net charge) increases. Thus, while complete ionization of smaller

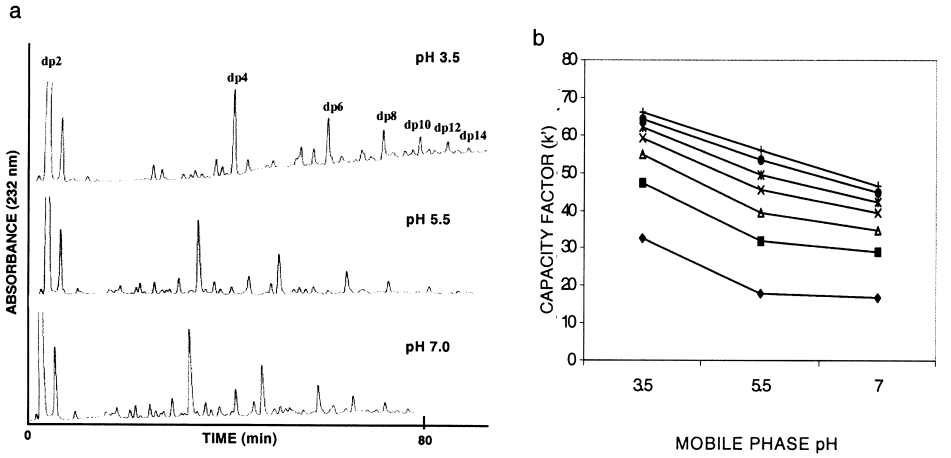

Fig. 3. The effect of mobile phase pH on (a) separation resolution (b) the capacity factor (k') of heparin-derived oligosaccharides. An elution gradient of 0.2–2 *M* NaCl in 120 min was formed utilizing mobile phases: A=15% CH_3CN, 10 m*M* TBA and B=15% CH_3CN, 2 *M* NaCl, 10 m*M* TBA. At pH 7.0, the major disaccharide (DP 2) and tetradecasaccharide (DP 14) components eluted at 28.3 and 76.3 min, respectively. The curves in panel (b) correspond to (♦) disaccharide, (■) tetrasaccharide, (△) hexasaccharide, (×) octasaccharide, (*) decasaccharide, (●) dodecasaccharide, and (+) tetradecasaccharide.

oligosaccharides might take place at pH 5.5, higher pH might be required to fully ionize the large oligosaccharides. At pH 7.0, both sulfo and carboxyl groups in all the oligosaccharides present in the mixture are expected to be completely ionized, permitting their full interaction with tetrabutylammonium cations and enhancing resolution. Based on these experiments, pH 7.0 was used to further optimize mobile phase composition.

3.6. Effect of mobile phase composition on retention time

While the chromatogram using 15% CH_3CN containing 10 m*M* TBA at pH 7.0 and a 0.2–2 *M* linear sodium chloride gradient, gave excellent resolution of oligosaccharides from DP 2 to DP 14, the analysis time was very long (>70 min for the elution of the tetradecasaccharide). The content of organic modifier in the mobile phase was next optimized in an attempt to reduce analysis time. Increasing the concentration of the CH_3CN organic modifier in the mobile phase at a constant concentration of ion-pairing reagent led to a decrease in analyte retention. This was expected, as the organic solvent both lowers either ion-pair adsorption to the stationary phase (separation process 1) and lowers the amounts of absorbed ion-pairing reagents to the stationary phase (separation process 2).

The concentration of CH_3CN was increased from 15 to 25% (Fig. 4). A dramatic decrease in k' is seen when the amount of CH_3CN increased, with the change in k' for each incremental increase in the concentration of CH_3CN becoming more pronounced as the size of the oligosaccharide increased (Fig. 4b). The chromatographic resolution was only slightly affected by the concentration of CH_3CN between 15% and 25% (Fig. 4a). Based on these experiments, a mobile phase of 25% CH_3CN containing 10 m*M* TBA at pH 7.0 was selected as optimal for the separation of heparin-derived oligosaccharides. This mobile phase composition gave baseline separation for both small and large heparin oligosaccharides with an analysis time of under 30 min.

3.7. Comparison of SAX-HPLC and RP-HPIPC methods on the separation of heparin-derived oligosacchrides

The chromatograms shown in Fig. 5 compare the optimized separation of heparin-derived oligosaccharides by RP-HPIPC to SAX-HPLC. RP-HPIPC shows markedly enhanced resolution in the separation of both small and large heparin oligosac-

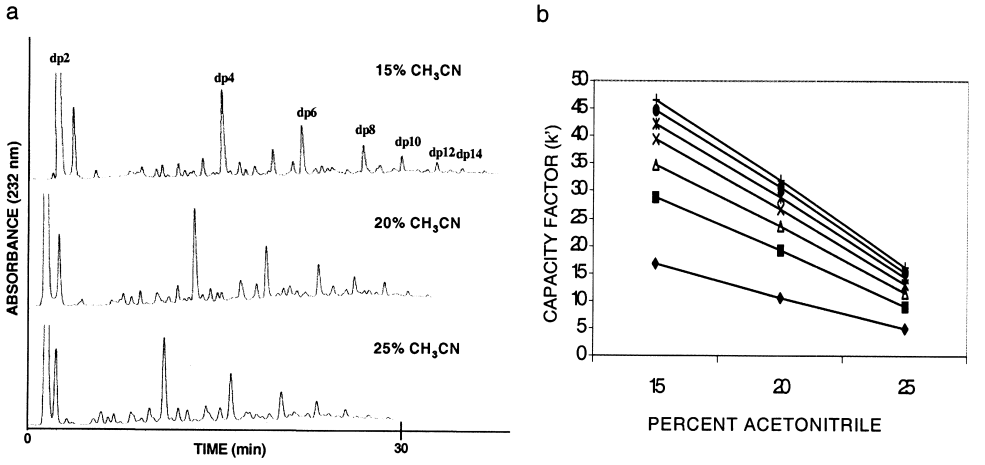

Fig. 4. The effect of acetonitrile concentration on (a) separation resolution (b) the capacity factor (k') of heparin-derived oligosaccharides. An elution gradient of 0.2–2 *M* NaCl in 120 min was formed utilizing mobile phases: A=CH_3CN–water, 10 m*M* TBA, at pH 7.0 and B=CH_3CN–water, 2 *M* NaCl, 10 m*M* TBA at pH 7.0. At 25% CH_3CN the major disaccharide (DP 2) and tetradecasaccharide (DP 14) components eluted at 9.3 and 27.5 min, respectively. The curves in panel (b) correspond to (♦) disaccharide, (■) tetrasaccharide, (△) hexasaccharide, (×) octasaccharide, (*) decasaccharide, (•) dodecasaccharide, and (+) tetradecasaccharide.

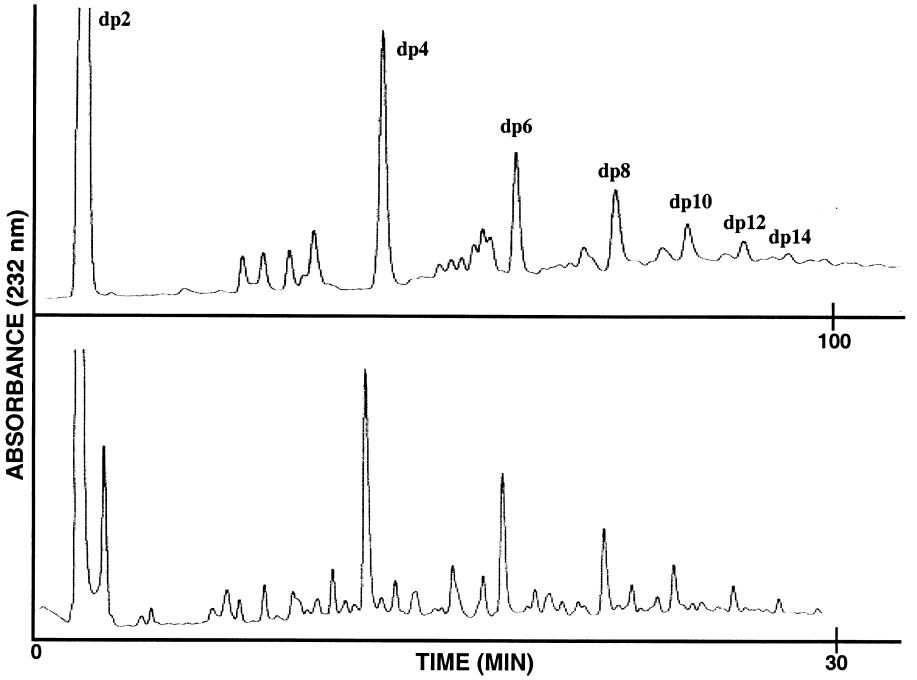

Fig. 5. Comparison of chromatograms obtained from SAX-HPLC (upper panel) and from RP-HPIPC (lower panel) on the separation of heparin-derived oligosaccharides. An elution gradient of 0.2–2 *M* NaCl in 120 min was obtained in SAX-HPLC using mobile phases: A=water, pH 3.5 and B=2 *M* sodium chloride solution, pH 3.5. The major disaccharide (DP 2) and tetradecasaccharide (DP 14) eluted at 42.5 and 93.2 min, respectively. For chromatographic conditions and eluting times for RP-HPIPC as in Fig. 4 using 25% CH_3CN.

charides. In addition to major peaks (labeled according to DP), corresponding to the fully sulfated oligosaccharides (Fig. 1), numerous addition minor peaks, corresponding to undersulfated oligosaccharides, are observed in RP-HPIPC. Furthermore, the analysis time required for RP-HPIPC was 27.5 min to elute tetradecasaccharide compared to 93.2 min for SAX-HPLC.

3.8. Replacement of TBA with the volatile ion-pairing reagent

SAX-HPLC has been used successfully in a semi-preparative scale to recover pure heparin oligosaccharides for NMR characterization [19]. Oligosaccharides are collected and desalted on an SEC column or through dialysis. Unfortunately, removal of ion-pairing reagents, introduced during RP-HPIPC, using dialysis is problematic. We have had some preliminary success using organic solvent [80% (v/v) methanol after bringing the sample to 16% (w/w) sodium chloride] to precipitate oligosaccharides without precipitating ion-pairing reagents. Unfortunately, this recovery method is limited to larger oligosaccharides. Thus, we sought to examine the use of a volatile ion-pairing reagent to facilitate its removal. The volatile ion-pairing reagent, tributylammonium acetate (TrBA) was used in place of TBA. The resolution was maintained (Fig. 6a) and the recovery of individual oligosaccharides could be easily accomplished by simple evaporation of TrBA followed by desalting using an SEC column or dialysis to remove sodium chloride. The complete removal of residual ion-pairing reagent from the recovered oligosaccharide components was confirmed by the absence of peaks from 0.8 to 1.8 ppm in the ^{1}H-NMR spectrum.

3.9. Replacement of sodium chloride with the ammonium acetate

Sodium chloride poses a number of problems in

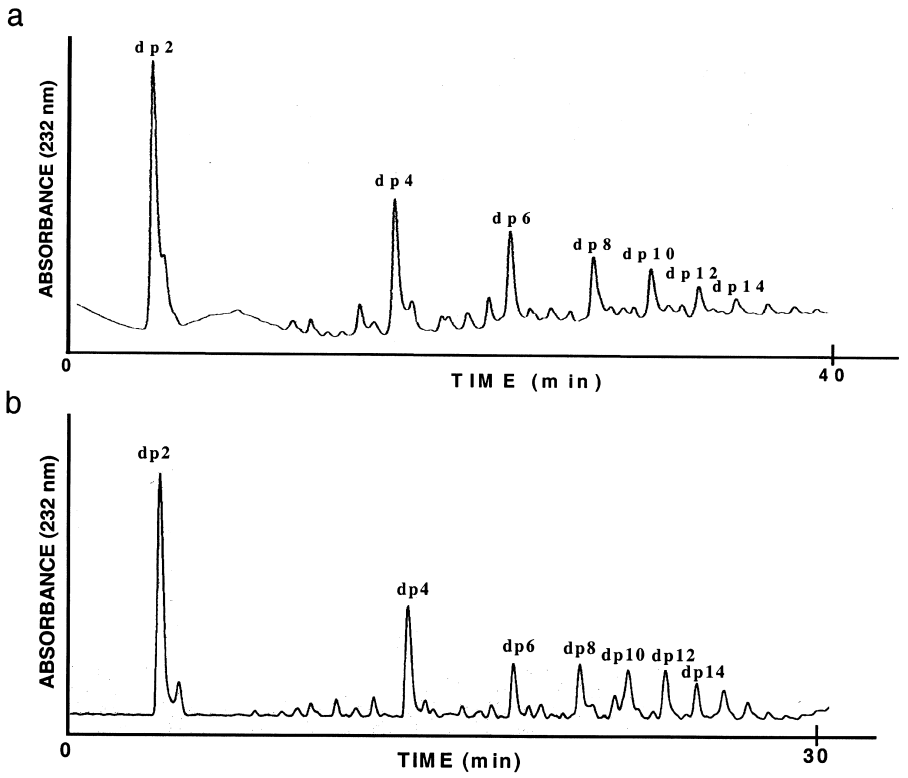

Fig. 6. The RP-HPIPC chromatogram by using TrBA as a volatile ion-pairing reagent on the separation of heparin-derived oligosaccharides. (a) An elution gradient of 0.2–2 *M* NaCl in 120 min was formed utilizing mobile phases: A=25% CH_3CN, 15 m*M* TrBA, adjusted pH to 7.0 with CH_3COOH and B=25% CH_3CN, 2 *M* NaCl, 15 m*M* TrBA, adjusted pH to 7.0 with CH_3COOH. The major disaccharide (DP 2) and tetradecasaccharide (DP 14) components eluted at 10.6 and 34.4 min, respectively. (b) An elution gradient of 20–65% CH_3CN in 120 min was formed utilizing mobile phases: A=20% CH_3CN, 50 m*M* ammonium acetate, 15 m*M* TrBA, adjusted pH to 7.0 with CH_3COOH and B=65% CH_3CN, 50 m*M* ammonium acetate, 15 m*M* TrBA, adjusted pH to 7.0 with CH_3COOH. The major disaccharide (DP 2) and tetradecasaccharide (DP 14) components eluted at 10.3 and 22.1 min, respectively.

HPLC separations. First, chloride is corrosive to stainless steel used in most pumps, requiring the use of titanium, polyether ether ketone (PEEK), or ceramic pump heads. Second, as previously mentioned, sodium chloride must be removed by desalting column or dialysis in preparative applications. Third, MS detection is severely compromised by the presence of sodium chloride. Ammonium acetate is a non-corrosive, volatile salt and hence, is more favorable for preparative applications and MS detection. At first, an ammonium acetate salt gradient was optimized to keep the salt concentration as low as possible and still provide adequate resolution, and short analysis times. However, lower salt concentration did not provide satisfactory resolution for large oligosaccharides (>DP 8) and an unstable baseline was observed because of the volatility of ammonium acetate (data not shown). As an alternative, an acetonitrile gradient was applied to facilitate the elution of large, highly sulfated oligosaccharides. We found that without the presence of ammonium acetate in the mobile phase, an acetonitrile gradient alone did not afford separation of oligosaccharide mixture. After some trials, an optimal gradient profile of acetonitrile with a fixed low concentration of ammonium acetate afforded separation of all oligosaccharide components. A mobile phase of water–acetonitrile, containing 50 m*M* ammonium acetate and 15 m*M* TrBA adjusted the pH to 7.0, with the gradient 20 to 65% of acetonitrile over

120 min gave baseline separation for both small and large heparin oligosaccharides in the mixture (Fig. 6b).

4. Conclusions

We have developed the simple, rapid, and sensitive conditions permitting analysis heparin-derived oligosaccharides, from disaccharide (DP 2) to tetradecasaccharide (DP 14), by RP-HPIPC. This method was successfully applied on a semi-preparative scale using a volatile ion-pairing reagent. Finally, replacement of sodium chloride with ammonium acetate and the use of acetonitrile gradient afforded a separation system amenable to on-line electrospray ionization MS detection.

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