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Insights into the mechanism of separation of heparin and heparan sulfate disaccharides by reverse-phase ion-pair chromatography

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ABSTRACT

Reverse-phase ion-pair high performance liquid chromatography (RPIP-HPLC) and ultra-performance liquid chromatography (RPIP-UPLC) are increasingly popular chromatographic techniques for the separation of organic compounds. However, the fine details of the RPIP separation mechanism are still being debated. Many factors including type and concentration of the ion-pairing reagent, mobile phase pH, organic modifier, ionic strength, and stationary phase all play a role in the overall efficiency and optimization of ion-pairing separations. This study investigates the role that competition between ion-pairing reagents with different steric bulk and hydrophobicity plays in the separation of structural isomers of heparin and heparan sulfate (HS) disaccharides. In addition to providing insights into the mechanism by which RPIP-HPLC can resolve closely related disaccharides, the use of competition between ion-pairing agents could lead to new methods for the separation of larger heparin and HS oligosaccharides. This approach should also be applicable to the analysis of other compound classes, and could lead to a general approach for the chromatographic resolution of mixtures of charged analytes having similar structures.

1. Introduction

Reverse-phase ion-pair high pressure liquid chromatography (RPIP-HPLC) and ultra-performance liquid chromatography (RPIP-UPLC) are promising and increasingly popular methods for the separation of organic and inorganic ionic solutes using lipophilic ions, referred to as ion-pairing reagents (IPR), as mobile phase modifiers to aid in the retention and resolution of these species on a hydrophobic stationary phase [1–3]. The mechanism of retention in RPIP-HPLC is however a matter of some debate. In the classical model of retention in RPIP-HPLC, the hydrophobic IPR and the analyte ion of opposite charge combine in the mobile phase to form a neutral species which then partitions into the hydrophobic stationary phase [4–6]. The dynamic ion-exchange model, however, suggests that the IPR is first adsorbed onto the surface of the stationary phase creating charge sites which then act as ion exchange sites for the oppositely charged analyte [7–9]. The presence of evidence for both theories suggest that RPIP separations occur through a combination of both mechanisms and that the extent to which each contributes to analyte retention may be controlled by experimental conditions [2,10].

To provide further insights into the separation mechanism of RPIP-HPLC, a set of test compounds are needed that provide a diversity of charges as well as subtle structural variations relative to the position of these charges, for example through positional isomers. Such a class of compounds would allow the evaluation of subtle changes in resolution and retention times as a result of varying experimental conditions, e.g., the type and concentration of IPR, mobile phase composition and pH. Through careful observations of the effect of separation conditions on the chromatographic resolution of a class of compounds having regular differences in charge and structure, the mechanistic details of RPIP-HPLC can be better inferred. The heparin and heparan sulfate disaccharides presented in Table 1 are an ideal set of test compounds for this purpose. As a group, they are a family of congeners with a range of molecular charge states as well as several isomeric (as well as anomeric) species that have previously been demonstrated to be resolvable through RPIP-HPLC and UPLC methods [11,12].

Heparin and heparan sulfate (HS) are highly charged anionic polysaccharides that are members of the glycosaminoglycan (GAG) family, which also includes chondroitin sulfate, dermatan sulfate, hyaluronic acid and keratan sulfate. Both heparin and HS are biosynthesized as proteogyclans and can be found stored either in the secretory granules of mast cells, as with heparin, or on the surface of most mammalian cells, like HS [13]. The polysaccharide chains of heparin and HS are highly sulfonated and polydisperse with molecular weights ranging from 5 to 70 kDa. They consist of repeating disaccharide units of uronic acid ($1 \rightarrow 4$) linked to D-glucosamine. The uronic acid can either be α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA), which may be 2-*O*-sulfonated. The D-glucosamine (GlcN) can be *N*-acetylated, *N*-sulfonated or especially in the case of HS, present as unmodified GlcN. In addition,

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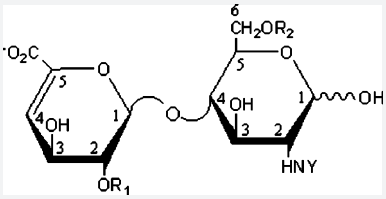
Table 1
Names, structures and charge states at pH 7 of the family of heparin disaccharides studied.



Disaccharide	R_1	R_2	Y
IS	SO_3^-	SO_3^-	SO_3^-
IIS	H	SO_3^-	SO_3^-
IIIS	SO_3^-	H	SO_3^-
IVS	H	H	SO_3^-
IA	SO_3^-	SO_3^-	Ac
IIA	H	$SO3^-$	Ac
IIIA	SO_3^-	H	Ac
IVA	H	H	Ac
IH	SO_3^-	SO_3^-	H
IIH	SO_3^-	H	H
IIIH	H	SO_3^-	H
IVH	H	H	H

O-sulfonation of GlcN is common at the C-6 position, and less so at the C-3 position [13–16].

Heparin is best known for its anticoagulant activity produced by binding through a specific pentasaccharide sequence to antithrombin III, and is commonly prescribed in a low molecular weight form as a treatment for thrombosis [17,18]. Heparin and HS are also exciting pharmaceutical targets due to their involvement in many biological processes including angiogenesis, generation of neural tissue, cell adhesion and differentiation, tumor metastasis, inflammation, and viral/vector-borne infection such as that involved with herpes simplex virus type 1 and malaria [16,19]. This wide range of biological functions is made possible by the great structural diversity of heparin and HS described above. It is this same structural diversity, however, that complicates the characterization of these important GAGs.

Many of the methods used for the structure elucidation of heparin and HS utilize a bottom up approach, whereby the larger oligosaccharide chains are first chemically or enzymatically degraded to smaller oligosaccharides prior to analysis. Most commonly an enzymatic digestion is performed using a cocktail of the enzymes Heparinase I, II, and III, extracted from *Flavobacterium heparinium*, to selectively cleave the glucosamine-(1,4)-uronic acid glycosidic bonds [20]. This enzymatic cleavage produces a double bond at the non-reducing end of each cleaved chain resulting in a UV chromophore that absorbs at 232 nm, greatly facilitating detection without disturbing the sulfonate substituents of the oligosaccharides [21]. Upon exhaustive enzymatic digestion, heparin and HS can be essentially completely reduced to their disaccharide building blocks allowing for full compositional analysis.

Although several studies have shown that direct quantitative analysis of mixtures of heparin and HS disaccharides is possible using mass spectrometric (MS) analysis [22–25], most studies utilize a separation to resolve the individual disaccharide components prior to detection [26]. Capillary electrophoresis (CE) is a useful and increasingly employed method for the separation of many types of carbohydrates [27–29]. It is especially appropriate for the separation of heparin and HS oligosaccharides due to their high negative charge and has been demonstrated for various sized oligosaccharides in both normal and reversed polarity modes [30–33]. This includes a CE method specifically devised for the determination of therapeutically important low molecular weight heparin samples [34]. However, the poor day-to-day reproducibility of migration times limits the regular use of CE due to the need to perform frequent calibrations with disaccharide standards [35].

Alternatively, high performance liquid chromatography (HPLC) can offer a more robust chromatographic separation of heparin and HS oligosaccharides. Strong anion exchange HPLC (SAX-HPLC) is often used for the separation of GAG oligosaccharides (including disaccharides) [32,36–38]. While capable of complete separation of heparin components, including isomers, of di- as well as larger oligosaccharides, SAX-HPLC is difficult to interface with MS detection due to the high ionic strength mobile phases used. Other studies have shown that hydrophilic interaction liquid chromatography (HILIC) can also be useful in the separation of heparin and HS oligosaccharides as well as similar GAGs [39,40]. However, the separations reported thus far are unable to achieve adequate resolution of isomeric disaccharides. The use of graphitized carbon columns for LC separation of heparin disaccharides has also been reported [41], and while isomer separation is possible with this approach, the technique offers poor resolution between the *N*-acetylated and *N*-sulfonated disaccharides hindering quantitative analysis.

As mentioned previously RPIP-HPLC is a promising and increasingly popular method for the separation of heparin and HS oligosaccharides. Studies have shown much success in using this method for the separation of many types of heparin oligosaccharides [42,43], including some of the earliest work in this area which utilized RPIP-HPLC for determining the disaccharide composition of heparin and HS [11]. Several studies have also demonstrated the amenability of RPIP-HPLC to on-line MS detection thus greatly improving the sensitivity and amount of structural information that can be accomplished [43–47].

Smaller sized column packing materials can be used to achieve higher resolution in a shorter amount of time, and the rapid and sensitive analysis of heparin disaccharides can be accomplished using RPIP-HPLC with smaller packing material (e.g., 2 μm) [48]. The introduction and commercial availability of ultra-performance liquid chromatography (UPLC) utilizing 1.7 μm particles and a proprietary mobile phase pump that can withstand pressures up to 10^8 Pa has been demonstrated to provide shorter separation times with improved resolution [49–51]. Recently, it has been shown that full resolution of heparin disaccharides, including isomers, can be achieved through RPIP-UPLC with minimal sample preparation and short equilibration times before and between runs compared to previous studies using HPLC [12]. Because of the potential of RPIP-UPLC for the separation of heparin disaccharides, as well as other classes of closely related charged compounds, this work explores the mechanism of RPIP chromatography through the use of RPIP-UPLC. The ability of this method to resolve heparin disaccharides based on differences in charge and subtle variations in compound structure makes it a useful probe of the mechanism of RPIP separations. This investigation also specifically addresses the interactions between the IPR tributylamine (TrBA) and heparin as well as the potential role that competition between TrBA and other IPRs plays in the resolution of isomeric disaccharides.

2. Experimental

2.1. Materials and reagents

All heparin disaccharide standards were purchased from the Sigma Chemical Company (St. Louis, MO). Table 1 shows the structures, names, and net charge states at pH 7 of the commercially available disaccharides studied. The ion paring reagent TrBA (purity ≥99.5%) was also purchased from Sigma. Butylamine (MBA) (99.5%) and ammonium acetate were purchased from Fisher Scientific (Pittsburgh, PA). Acetonitrile (optima grade) and water (HPLC grade) were purchased from Sigma and Honeywell Burdick & Jackson, respectively.

Table 2
Retention factor, width at half height and peak area for the RPIP-UPLC separation of the 11 commercially available disaccharides with IPR concentration of 2.5 mM TrBA.

Disaccharide	[TrBA]	Retention factor	$W_{1/2}$ (min)	Peak area
IVH	2.5 mM	0.36 ± 0.02	0.076 ± 0.001	1932 ± 33
IVA	2.5 mM	5.85 ± 0.01	0.068 ± 0.002	594 ± 11
IVA anomer	2.5 mM	6.13 ± 0.03	0.078 ± 0.002	N/A
IIH	2.5 mM	5.74 ± 0.03	0.085 ± 0.001	2028 ± 16
IIH anomer	2.5 mM	6.67 ± 0.00	0.091 ± 0.004	988 ± 46
IIIH	2.5 mM	6.66 ± 0.00	0.071 ± 0.002	1407 ± 70
IIIH anomer	2.5 mM	7.52 ± 0.01	0.068 ± 0.001	784 ± 17
IIA	2.5 mM	13.18 ± 0.00	0.120 ± 0.001	4164 ± 20
IIIA	2.5 mM	14.00 ± 0.02	0.117 ± 0.006	3459 ± 14
IH	2.5 mM	14.93 ± 0.00	0.109 ± 0.003	2949 ± 54
IH anomer	2.5 mM	16.08 ± 0.03	0.093 ± 0.002	1764 ± 26
IIS	2.5 mM	17.67 ± 0.00	0.137 ± 0.001	4371 ± 43
IIIS	2.5 mM	18.70 ± 0.02	0.160 ± 0.011	5428 ± 36
IA	2.5 mM	17.98 ± 0.00	0.143 ± 0.001	6071 ± 54
IS	2.5 mM	19.93 ± 0.00	0.253 ± 0.003	10374 ± 36

Table 3
Retention factor, width at half height and peak area for the RPIP-UPLC separation of the 11 commercially available disaccharides with IPR concentration of 5 mM TrBA.

Disaccharide	[TrBA]	Retention factor	$W_{1/2}$ (min)	Peak area
IVH	5 mM	0.37 ± 0.00	0.072 ± 0.002	1666 ± 37
IVA	5 mM	5.05 ± 0.00	0.075 ± 0.003	420 ± 20
IVA anomer	5 mM	5.40 ± 0.03	0.085 ± 0.001	N/A
IIH	5 mM	5.16 ± 0.00	0.088 ± 0.001	1498 ± 24
IIH anomer	5 mM	6.24 ± 0.03	0.093 ± 0.002	669 ± 37
IIIH	5 mM	6.53 ± 0.01	0.080 ± 0.002	999 ± 3
IIIH anomer	5 mM	7.45 ± 0.01	0.075 ± 0.003	624 ± 11
IIA	5 mM	13.00 ± 0.02	0.101 ± 0.001	3066 ± 38
IIIA	5 mM	13.90 ± 0.02	0.102 ± 0.001	2536 ± 19
IH	5 mM	15.06 ± 0.03	0.091 ± 0.001	1921 ± 22
IH anomer	5 mM	16.29 ± 0.02	0.079 ± 0.002	1147 ± 2
IIS	5 mM	17.91 ± 0.01	0.133 ± 0.003	3267 ± 42
IIIS	5 mM	18.94 ± 0.03	0.128 ± 0.001	3783 ± 20
IA	5 mM	18.28 ± 0.00	0.119 ± 0.006	4253 ± 38
IS	5 mM	20.38 ± 0.01	0.201 ± 0.008	7265 ± 143

2.2. UPLC separation

All chromatographic separations were performed on a 2.1 mm × 100 mm Acquity™ UPLC BEH C18 column with 1.7 μm particles (Waters Corporation, MA). A guard column packed with the same 1.7 μm C18 particles was utilized prior to the analytical column. The column temperature was maintained at 40 °C throughout the separation, and a flow rate of 0.5 mL/min was used. A sample volume of 10 μL of a 0.2 mM disaccharide mixture prepared in water was injected for each separation. A binary solvent system was used for gradient elution. Solvent A consisted of 5% acetonitrile in water while solvent B consisted of 80% acetonitrile in water. Both solvents contained the same IPR concentrations and were adjusted using pH meter readings to an apparent pH between pH 6.9 and 7.0 using acetic acid. This yielded an acetate concentration for the 20, 10, 5.0, and 2.5 mM TrBA solutions of 20.0, 9.4, 4.7, and 2.4 mM, respectively. Chromatographic separations using 20 mM TrBA and 2.5 mM ammonium acetate contained an acetate concentration of 22.2 mM.

The pH meter was calibrated using a three-point calibration with pH 4.00 (0.05 M potassium biphthalate buffer), pH 7.00 (0.05 M potassium phosphate monobasic-sodium hydroxide buffer), and pH 12.00 (NaOH, KCl buffer) buffers. The pH 4.00 and pH 7.00 buffers were purchased from Fisher Scientific (Pittsburgh, PA) while the pH 12.00 buffer was purchased from Ricca Chemical Company (Arlington, TX). For separations studying the effects of varying TrBA concentrations, the ion-pair composition of both solvents consisted of 2.5–20 mM TrBA with no other IPR.

Table 4
Retention factor, width at half height and peak area for the RPIP-UPLC separation of the 11 commercially available disaccharides with IPR concentration of 10 mM TrBA.

Disaccharide	[TrBA]	Retention factor	$W_{1/2}$ (min)	Peak area
IVH	10 mM	0.37 ± 0.00	0.069 ± 0.002	532 ± 7
IVA	10 mM	3.88 ± 0.01	0.077 ± 0.004	216 ± 3
IVA anomer	10 mM	4.20 ± 0.01	0.081 ± 0.004	N/A
IIH	10 mM	4.20 ± 0.01	0.074 ± 0.002	444 ± 17
IIH anomer	10 mM	5.26 ± 0.00	0.063 ± 0.005	227 ± 3
IIIH	10 mM	5.77 ± 0.00	0.085 ± 0.004	538 ± 40
IIIH anomer	10 mM	6.61 ± 0.03	0.082 ± 0.004	290 ± 28
IIA	10 mM	12.49 ± 0.02	0.081 ± 0.001	1166 ± 22
IIIA	10 mM	12.78 ± 0.01	0.097 ± 0.002	1203 ± 57
IH	10 mM	13.95 ± 0.01	0.071 ± 0.001	666 ± 8
IH anomer	10 mM	15.13 ± 0.03	0.083 ± 0.002	491 ± 4
IIS	10 mM	17.74 ± 0.00	0.095 ± 0.001	1211 ± 5
IIIS	10 mM	17.87 ± 0.01	0.109 ± 0.004	1298 ± 29
IA	10 mM	18.23 ± 0.00	0.094 ± 0.002	1425 ± 6
IS	10 mM	20.40 ± 0.00	0.123 ± 0.004	1760 ± 25

Table 5
Retention factor, width at half height and peak area for the RPIP-UPLC separation of the 11 commercially available disaccharides with IPR concentration of 20 mM TrBA.

Disaccharide	[TrBA]	Retention factor	$W_{1/2}$ (min)	Peak area
IVH	20 mM	0.44 ± 0.04	0.069 ± 0.002	861 ± 77
IVA	20 mM	3.07 ± 0.00	0.066 ± 0.002	272 ± 14
IVA anomer	20 mM	N/A	N/A	N/A
IIH	20 mM	3.33 ± 0.00	0.068 ± 0.002	945 ± 17
IIH anomer	20 mM	4.15 ± 0.01	0.088 ± 0.003	450 ± 24
IIIH	20 mM	5.20 ± 0.01	0.103 ± 0.007	534 ± 11
IIIH anomer	20 mM	5.96 ± 0.01	0.099 ± 0.005	520 ± 17
IIA	20 mM	11.95 ± 0.01	0.082 ± 0.001	1319 ± 64
IIIA	20 mM	12.40 ± 0.02	0.100 ± 0.008	1436 ± 43
IH	20 mM	13.71 ± 0.01	0.071 ± 0.002	601 ± 22
IH anomer	20 mM	14.75 ± 0.01	0.071 ± 0.002	622 ± 9
IIS	20 mM	17.73 ± 0.01	0.094 ± 0.002	2321 ± 18
IIIS	20 mM	18.00 ± 0.02	0.113 ± 0.004	2665 ± 52
IA	20 mM	18.47 ± 0.02	0.089 ± 0.003	1914 ± 40
IS	20 mM	20.95 ± 0.00	0.117 ± 0.005	3353 ± 42

For the separations studying the effects of varying MBA concentration both solvents contained 10, 15, and 20 mM MBA while the TrBA concentration was maintained at 2.5 mM. The buffers for the MBA experiments contained acetate concentrations of 11.1, 16.7 and 22.2 mM, respectively. Retention factors were calculated for Tables 2–5 by subtracting the retention time of the void from the retention time of the analyte peak and then dividing this value by the retention time of the void.

The gradient profile, shown graphically in Fig. 1b, consisted of a 1 min isocratic step of 100% solvent A after which the fraction of solvent B was increased to 3% over the next 1.5 min. The fraction of solvent B was then increased to 25% over the next 2.5 min and maintained at 25% for 1 min before it was increased to 35% over a 1 min period. The fraction of solvent B was then increased over the next 4 min to 100%. A 5 min equilibration was utilized prior to the next injection. Isocratic experiments to probe the effect of

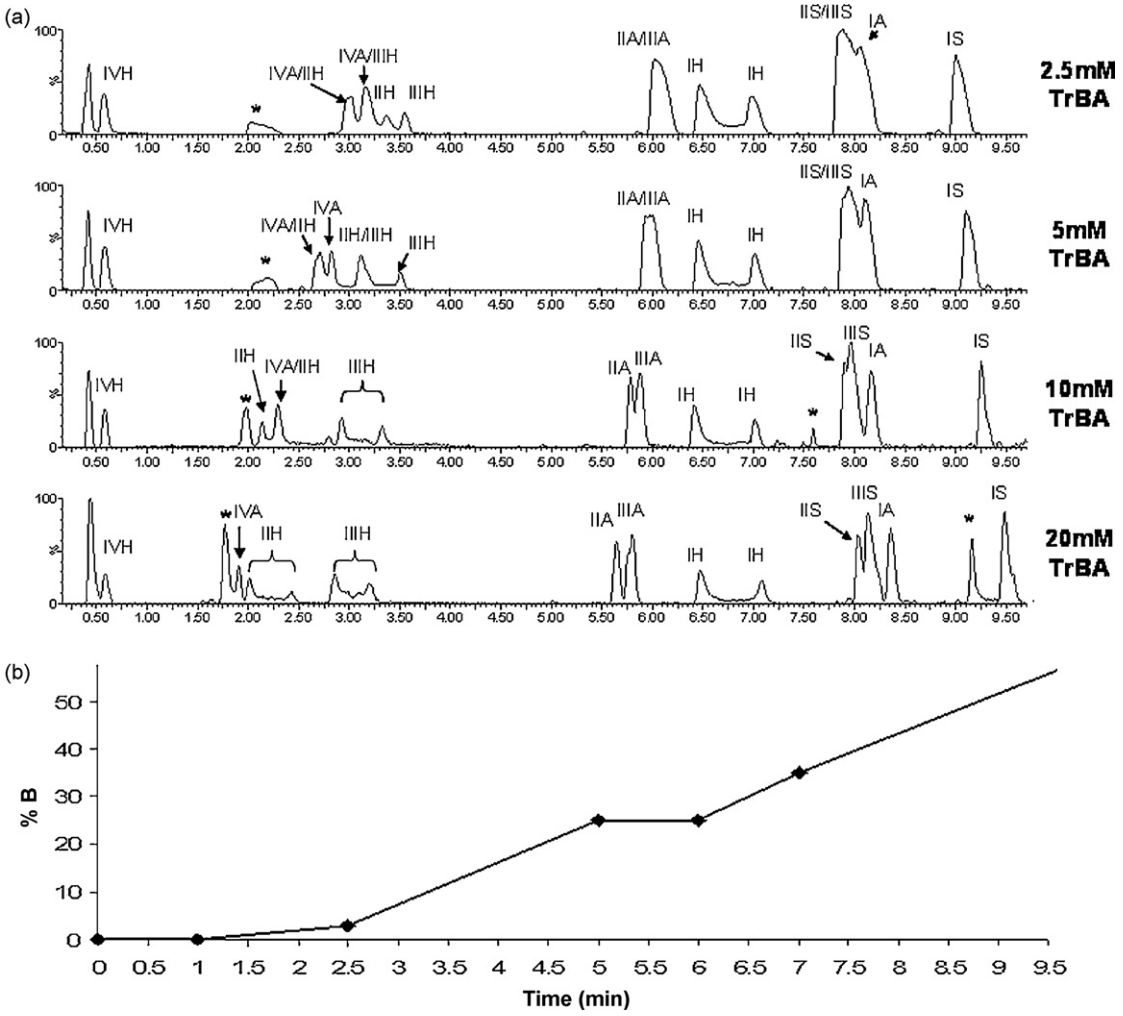


Fig. 1. (a) The effect of increasing TrBA concentration on chromatographic resolution and retention of the commercially available heparin-derived disaccharides. (b) Illustration of the gradient profile showing the change in percentage of mobile phase buffer solution B as a function of time. The peaks marked with an asterisk are impurities.

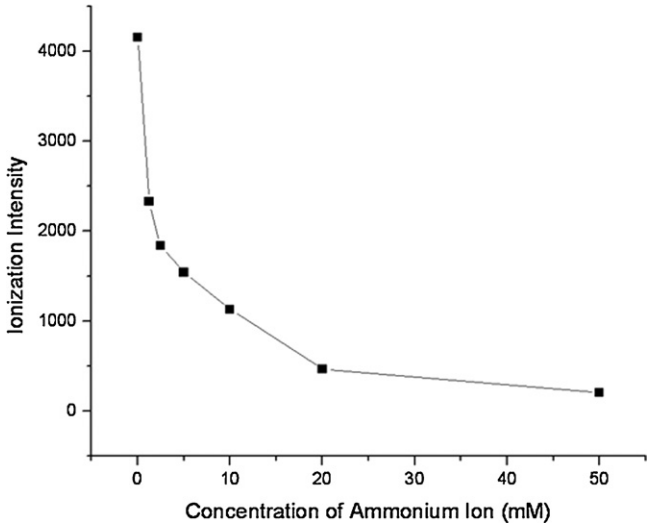


Fig. 2. Graph of ionization intensity of the IS disaccharide as a function of increasing ammonium concentration while maintaining a constant TrBA concentration of 5 mM. Experiment was performed by direct infusion into the MS.

varying TrBA and acetate concentrations were performed with a fixed mobile phase composition of 30% solvent B.

2.3. Mass spectrometry

Total ion chromatograms were obtained using a Waters ESI quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA). Data acquisition was performed using Masslynx 4.1 software. All spectra were obtained in negative mode using the following instrument parameters: capillary voltage, 3 kV; cone voltage, 12 V; source temperature, 120 °C; desolvation temperature, 200 °C; extractor voltage, 1 V; radio frequency lens, 0.5 V; interscan delay, 0.1 s; m/z range, 215–1000.

3. Results

The complete resolution of a mixture of eleven heparin disaccharides was previously demonstrated by Korir et al. [12] by RPIP-UPLC utilizing 50 mM ammonium acetate as a pH buffer and 5 mM TrBA as an IPR. Although this method allowed for the rapid and efficient separation of the mixture components, presumably due to differential ion-pairing interactions of the disaccharides with TrBA, the presence of ammonium ion was also required for complete resolution of isomeric disaccharide pairs IIH/IIIH, IIA/IIIA and IIS/IIIS at the TrBA concentration used. A disadvantage of the use of ammonium as a mobile phase buffer is that it reduces the ESI-MS ionization efficiency, thereby limiting the sensitivity of the analysis. As shown in Fig. 2, the ionization efficiency of heparin disaccharide IS is dramatically reduced by increasing ammonium ion concentration. Motivated by our desire to reduce MS ion suppression while maintaining good chromatographic resolution, experiments were designed to explore the effects of TrBA structure and buffer composition on the chromatographic retention time and resolution of a heparin disaccharide mixture. This study also addresses the possibility that competition between TrBA and other positive IPRs such as ammonium and MBA for ion-pair formation may contribute to the chromatographic resolution of isomeric heparin disaccharides.

The heparin disaccharides shown in Table 1 comprise an interesting class of analytes for exploring the nature of ion-pairing interactions in RPIP chromatography. The disaccharides are all very hydrophilic and are not retained on the reverse phase column unless an ion-pairing reagent is employed. This family is comprised of species with similar structures and charge states, ranging from zero net charge at neutral pH (IVH) to a charge of −4 (IS). In this family, negative charges result from the carboxylate group and from 2-*O* sulfonation of the iduronic acid, and from 6-*O* and *N*-sulfonation of the glucosamine residue. Disaccharides containing an unmodified glucosamine (IH, IIH, IIIH, and IVH) will have a positively charged amino group at the mobile phase pH employed in these experiments. Additionally, the disaccharides comprising charge states −1 (IVA, IIH, and IIIH), −2 (IIA, IIIA, and IH) and −3 (IA, IIS and IIIS) offer structural motifs that differ only in the nature and position of charged functional groups. Although one might expect to be able to resolve components with very different structures but similar charges, for example IIH and IVA, through different ion-pairing interactions, the ion-pairing interactions leading to separation of the isomeric pairs IIH/IIIH, IIA/IIIA and IIS/IIIS are likely to be more complex. Therefore, examining the effects of varying IPR structure and concentration on the resolution of this disaccharide family should yield insights into the finer details of the RPIP separation mechanism.

3.1. Effect of TrBA concentration

Fig. 1a shows the effect of varying mobile phase TrBA concentration on the separation of the heparin disaccharide mixture. The mobile phase used to measure these chromatograms contained no ammonium acetate, so TrBA acetate also served as the pH buffer in these experiments. In cases of peak overlap, compound identity was verified using the mass spectra, and for isomeric species which give rise to identical molecular ions, through injection of spiked samples. Tables 2–5 show calculated retention factors, width at half height and peak area for all 11 commercial disaccharides studied at the varying mobile phase TrBA concentrations. At a TrBA concentration of 2.5 mM, peaks are broad and are poorly resolved. As the TrBA concentration is increased to 20 mM, the peaks sharpen and resolution of all of the disaccharides in this sample is achieved. As expected, ion-pairing with TrBA resolves the disaccharides primarily based on charge. Disaccharide IVH, with a net charge of zero, is barely retained and elutes shortly after the column void while the most highly charged disaccharide, IS, with a net charge of −4 is the most highly retained. Within these extremes, groups of disaccharides having similar charges, elute in the expected order: disaccharides with a −1 net charge (IVA, IIH and IIIH) before those with a −2 net charge (IIA, IIIA, and IH) followed by the disaccharides having a net charge of −3 (IIS, IIIS and IA). For the compounds containing a free glucosamine residue, two peaks are detected for each compound due to resolution of the α and β anomers formed by mutarotation of the reducing-end glucosamine residue [52]. It is interesting that increasing the TrBA concentration does not produce large changes in the retention time for most species, and that the improved resolution achieved at higher concentrations can be attributed at least in part to sharpening of the peaks. It is also important to note that increasing the TrBA concentration of the mobile phase also increases ion suppression during MS detection similar to the behavior observed in Fig. 2 with increasing ammonium concentration. For this reason when using MS detection the IPR mobile phase concentration should be optimized to maintain adequate analyte resolution and retention while minimizing ion suppression. Additionally this change in ion intensity with changing TrBA concentration makes it difficult to extract useful information from the peak areas derived from the total ion chromatograms, for example in Tables 2–5.

3.2. Competition between TrBA and ammonium ion

Except for the incomplete resolution of the isomeric disaccharides IIS and IIIS, the quality of the separation in Fig. 1a obtained

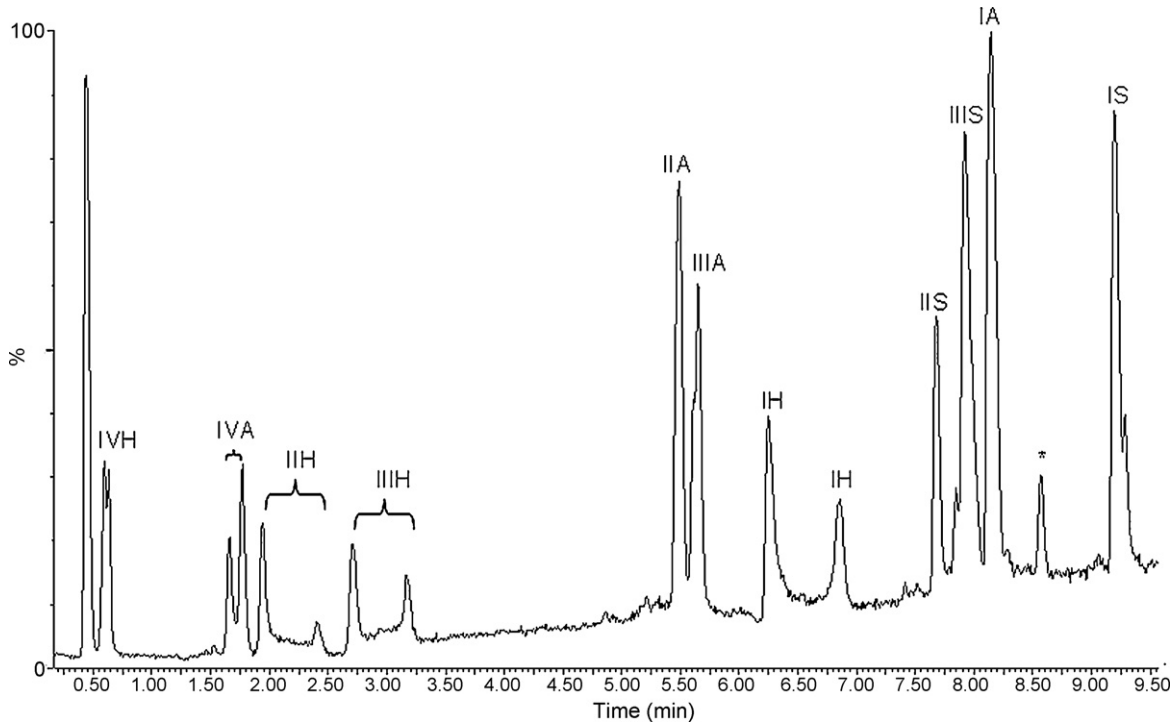


Fig. 3. Optimized ion-pair separation of commercially available heparin-derived disaccharides. Mobile phase IPR concentration: 20 mM TrBA, 2.5 mM ammonium acetate. The peak marked with an asterisk is an impurity.

with 20 mM TrBA is nearly as good as that reported by Korir et al. [12] using 5 mM TrBA and 50 mM ammonium acetate. Fig. 3 shows the effect of adding 2.5 mM ammonium acetate to the mobile phase buffer containing 20 mM TrBA. As can be seen from the chromatogram in Fig. 3, addition of a small amount of ammonium ion results in complete resolution of the disaccharide isomers IIS and IIIS, as well as partial resolution of the α and β anomers of disaccharides IVA and IS. The results achieved in Fig. 3 suggest that competition between TrBA and ammonium for ion-pairing interactions with these disaccharides is at least partially responsible for their separation. Additionally, the overall decrease in retention of all disaccharides observed in Fig. 3 is most likely a result of the lower hydrophobicity of the ammonium ion. Thus, any analyte interactions with ammonium take place in the mobile phase only, causing a decrease in retention time. Fig. 4 also shows the application of the full isomer separation method used for Fig. 3 to a heparin sample that had been exhaustively digested with heparinase I, II and III. The results in Fig. 4 demonstrate the applicability of this separation to a real sample, and confirm the ability of the method to resolve disaccharide IVS, which was not included in Fig. 3.

3.3. Competition between TrBA and MBA

To further investigate the possibility that competition between IPRs occurs and is an important component of the separation mechanism, the effects of varying the MBA concentration while maintaining a constant TrBA concentration of 2.5 mM was studied. MBA was chosen because its single butyl side chain should allow some retention of the MBA-disaccharide ion-pair on the reverse phase column. However, because MBA is less hydrophobic than

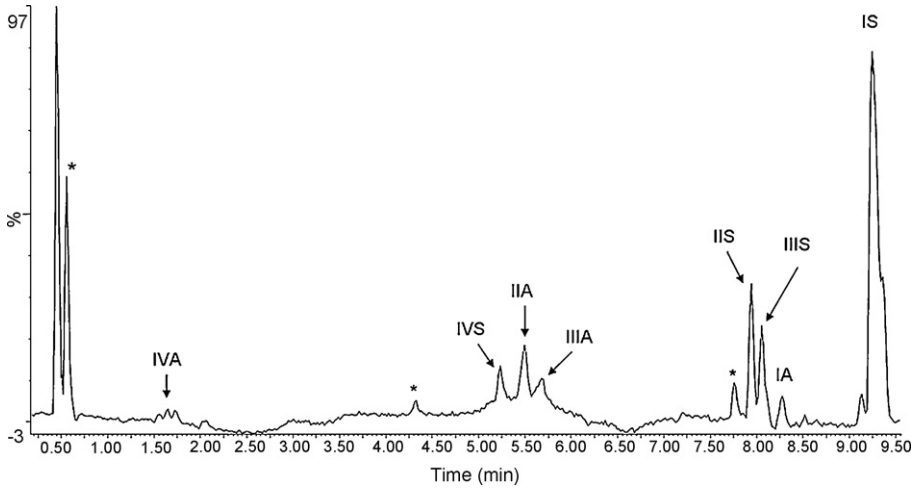


Fig. 4. Separation of a heparin sample exhaustively digested with a cocktail of heparinase enzymes I, II and III. Mobile phase IPR concentration: 20 mM TrBA, 2.5 mM ammonium acetate. The peaks marked with an asterisk are impurities.

TrBA, competition between these ions should be visible through changes in retention time. The results presented in Fig. 5 show the effect of increasing MBA concentration on the separation of the isomeric disaccharides IIA and IIIA, selected because the glucosamine nitrogen of these compounds is *N*-acetylated simplifying the nature of the interactions with the IPR.

As can be seen in Fig. 3, disaccharide IIIA, which elutes after disaccharide IIA, has a greater ion-pairing affinity for TrBA under the separation conditions employed, 20 mM TrBA and 2.5 mM ammonium. In Fig. 5a, when only 20 mM MBA is used as an IPR (no TrBA is present), sharp peaks are obtained for both disaccharides at 0.78 min. With a mobile phase containing 20 mM MBA and 2.5 mM TrBA, shown in Fig. 5b, each disaccharide produces several relatively sharp chromatographic peaks between 1.00 and 2.00 min. Close examination reveals that IIIA has greater peak dispersion and retention of the later eluting components. We attribute these peaks to the partial chromatographic resolution of species in which the disaccharides are bound to MBA and TrBA over the course of the separation, and are in slow exchange on the time scale of the chromatographic separation. Even though TrBA is more hydrophobic and should have a greater effect on retention, MBA is present at a much higher concentration and the poor retention suggests that MBA interactions dominate. At an intermediate concentration of 15 mM MBA, shown in Fig. 5c, a transition point is reached in the retention of both the IIA and the IIIA isomers at which two types of ion-pair species are both present and resolved in the chromatogram. As expected from the separation in Fig. 3, disaccharide IIIA appears to have a greater ion-pairing interaction with TrBA than disaccharide IIA in Fig. 5c. The broad peaks detected in this separation further suggest that exchange between MBA and TrBA ion-pairs is occurring on the chromatographic time scale. It is also interesting that there appear to be two different types of ion-pair species formed, and with different populations for disaccharides IIA and IIIA. The broad peaks observed between 2 and 3 min suggest involvement of both MBA and TrBA in ion-pairing. The sharper and more highly retained peaks at around 4.5 min likely results from stable TrBA ion-pairs. Fig. 5d shows that when the MBA concentration is decreased to 10 mM, a single chromatographic peak is now detected for each disaccharide. The longer retention time observed in Fig. 5d likely reflects the larger relative population of TrBA–disaccharide ion-pairs. The sharpness of these peaks suggests either that competition between the TrBA and MBA is fast on the time scale of the separation, or more likely that TrBA dominates the ion-pairing interactions. MS analysis of the mass spectra for the chromatographic peaks did not yield further insights into this matter as no MBA or TrBA adducts to the heparin disaccharides could be detected in either negative or positive mode ESI. In fact, no disaccharide ions were detected in positive mode. It is also important to note that the overall lower retention of the disaccharides in Fig. 5b–d as compared to Fig. 1a where only 2.5 mM TrBA is present is most likely due to the presence of the less hydrophobic MBA thus reducing the surface potential of the stationary phase.

4. Discussion

In RPIP separations, the retention of analytes is determined by several factors. These include the hydrophobicity of the stationary phase, organic concentration of the mobile phase, charge of analytes at the experimental pH, as well as charge, concentration and hydrophobicity of the IPR [2,10]. In the electrostatic model of retention through ion-pairing, the hydrophobic IPRs are first absorbed onto the surface of the hydrophobic stationary phase where, in the case of this study, the disaccharides interact electrostatically with the positive charge of the alkyl ammonium IPR through the negative surface potential that is generated by their carboxylate and

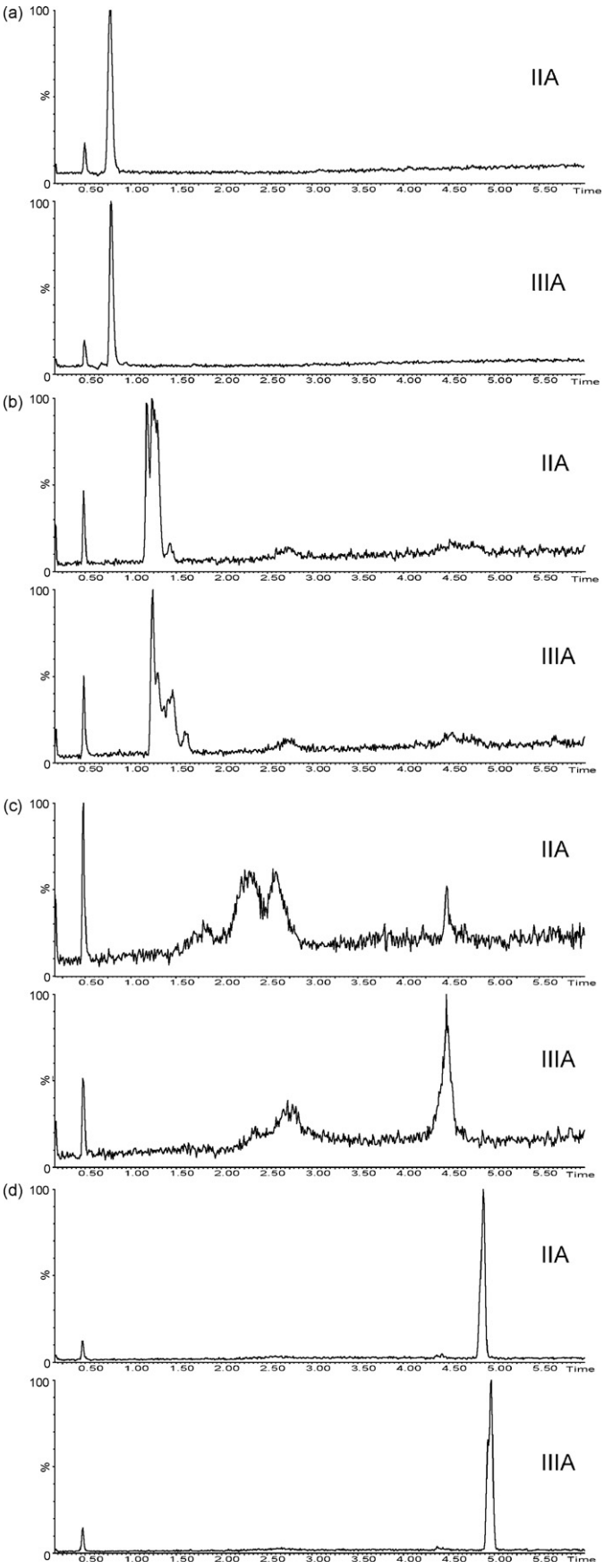


Fig. 5. The effect of competition between the less hydrophobic IPRs MBA and TrBA on the *N*-acetylated disaccharides IIA and IIIA. Mobile phase IPR concentration: (a) 20 mM MBA, (b) 20 mM MBA, 2.5 mM TrBA, (c) 15 mM MBA, 2.5 mM TrBA and (d) 10 mM MBA, 2.5 mM TrBA.

Table 6
Retention factors for the 11 commercially available disaccharides using 30% mobile phase buffer B at 2.5, 5, 10 and 20 mM TrBA concentration.

Disaccharide	[TrBA]	Retention factor	[TrBA]	Retention factor	[TrBA]	Retention factor	[TrBA]	Retention factor
IVH	2.5 mM	0.11 ± 0.00	5 mM	0.13 ± 0.00	10 mM	0.12 ± 0.01	20 mM	0.11 ± 0.00
IVA	2.5 mM	0.34 ± 0.00	5 mM	0.37 ± 0.00	10 mM	0.35 ± 0.02	20 mM	0.34 ± 0.00
IIH	2.5 mM	0.30 ± 0.00	5 mM	0.33 ± 0.00	10 mM	0.30 ± 0.02	20 mM	0.27 ± 0.01
IIIH	2.5 mM	0.30 ± 0.00	5 mM	0.33 ± 0.00	10 mM	0.33 ± 0.00	20 mM	0.27 ± 0.00
IIA	2.5 mM	0.62 ± 0.00	5 mM	0.65 ± 0.00	10 mM	0.64 ± 0.02	20 mM	0.59 ± 0.01
IIIA	2.5 mM	0.65 ± 0.02	5 mM	0.70 ± 0.00	10 mM	0.67 ± 0.03	20 mM	0.58 ± 0.01
IH	2.5 mM	0.69 ± 0.00	5 mM	0.65 ± 0.00	10 mM	0.66 ± 0.02	20 mM	0.61 ± 0.00
IIS	2.5 mM	0.89 ± 0.00	5 mM	1.07 ± 0.00	10 mM	1.08 ± 0.05	20 mM	1.09 ± 0.02
IIIS	2.5 mM	0.99 ± 0.02	5 mM	1.17 ± 0.00	10 mM	1.14 ± 0.03	20 mM	1.08 ± 0.02
IA	2.5 mM	0.96 ± 0.02	5 mM	1.17 ± 0.00	10 mM	1.16 ± 0.03	20 mM	1.21 ± 0.00
IS	2.5 mM	1.30 ± 0.00	5 mM	1.83 ± 0.00	10 mM	1.89 ± 0.04	20 mM	2.21 ± 0.00

sulfonate groups. Electrostatic ion-pair formation can also occur in the mobile phase prior to interaction with the stationary phase with retention of the ion-pair occurring at the stationary phase surface.

From these models, certain retention trends for this series of disaccharides can be hypothesized. First, as discussed previously, as the concentration of the IPR is increased the retention of the disaccharides should also increase [45]. However, as seen in Fig. 1a, this is only the case for the latter eluting disaccharides IIS, IIIS, IA, and IS. The retention times of disaccharides IH and IVH are unaffected by the increase in TrBA concentration, while disaccharides IIA, IIIA, IVA, IIH, and IIIH show a decrease in retention time as the concentration of TrBA is increased. This phenomenon could be explained in part by the mechanism of stationary phase volume exclusion resulting from coating of the stationary phase with TrBA, similar to the "cation exclusion" mechanism described by Loeser and Drumm [53]. In this mechanism, instead of the surface charge of the stationary phase causing volume exclusion, it is the size of the TrBA molecules coating the surface of the stationary phase that blocks the disaccharides from accessing the stationary phase pore volume. Thus as the TrBA concentration is increased, more of the pore volume will be blocked resulting in less retention of the disaccharides by the stationary phase. However, at higher organic mobile phase concentrations, the TrBA coating is washed away and normal retention trends are expected, as observed in Fig. 1a for disaccharides IIS, IIIS, IA, and IS which elute at higher organic concentrations produced by the gradient profile shown in Fig. 1b.

An alternative explanation for these results is that as the TrBA concentration is increased the concentration of the counterion, in this experiment acetate, is also increased relative to the constant concentration of heparin disaccharides. Because acetate is negatively charged it can compete with heparin for interaction with TrBA and thus lower the overall surface potential of the stationary phase causing a decrease in retention of the disaccharides. Additionally, it is possible that the disaccharides with a −1 and −2 net charge have a higher propensity to ion-pair in the mobile phase which would remove these analyte ions from interacting with the stationary phase decreasing their retention factor [1]. With increasing TrBA concentration there would be an increased percentage of TrBA in the mobile phase making this effect more pronounced and producing decreased retention of these disaccharides with increasing TrBA concentration.

To test which theory best explains the observed retention behavior, a series of isocratic separations were performed with 30% buffer B (the approximate percentage at which disaccharide IS elutes) while increasing the TrBA concentration. Table 6 summarizes the changes in retention factor for each disaccharide as a function of TrBA concentration. As can be seen from this table, the poorly retained disaccharides IVH, IVA, IIH, and IIIH show an initial small increase in retention factor when the TrBA concentration is increased from 2.5 to 5.0 mM followed by a small decrease in retention factor when the concentration of TrBA is increased further to 10 and 20 mM. A similar but more pronounced trend is observed for disaccharides IIA and IIIA. The retention factor for disaccharide IH decreases with increasing TrBA concentration while disaccharides IIS, IIIS, IA and IS show an increase in retention factor with increasing TrBA concentration. It is interesting that while disaccharide IIIS has a higher retention factor for 20 mM TrBA than for 2.5 mM there is an initial spike in its retention factor at 5.0 mM TrBA similar to that observed for the −1 and −2 charge state disaccharides. This is in contrast to the trend seen for IIS, for which the retention factor increases with increasing TrBA concentration. For purposes of comparison, an experiment with isocratic elution was also performed using a buffer containing 0 mM TrBA (data not shown), however, the disaccharides were not retained and all eluted in the column void. The results summarized in Table 6 show similar retention trends to those seen in Fig. 1 but with a higher organic concentration. This suggests that the reason the −1 and −2 charge state disaccharides show a decrease in retention with increasing TrBA concentration may be due to the increasing concentration of acetate, which can also ion pair with TrBA and that increasing TrBA increases interactions with the disaccharides in mobile phase. However, because the −1 and −2 charge state disaccharides are so poorly retained in this isocratic experiment it is hard to judge whether the observed trends are sufficiently significant to completely eliminate a role of pore exclusion effects on the disaccharide retention factors.

The separation of groups of disaccharides according to their net negative charge, as is observed in Figs. 1 and 3, can be explained simply by an increase in the average number of ion-pairs with increasing disaccharide charge. However, this simple mechanism fails to fully explain the chromatographic resolution of the structurally similar isomeric pairs IIH/IIIH, IIA/IIIA, and IIS/IIIS, for which the only difference is sulfonation at either the C6 position of the glucosamine residue or the C2 position of the uronic acid residue. To understand how these compounds might be resolved in RPIP separations, the sites of electrostatic interaction with the IPR must be considered as well as the role that competition between different IPRs may play in the resolution of the individual isomers.

As shown in Fig. 1a, all of the disaccharide isomers except IIS and IIIS can be resolved simply by using 20 mM TrBA as the IPR. Further examination of the structures of the IIH/IIIH and IIA/IIIA isomers suggests that a mechanism based on the sterics of the ion-pairing interaction may be involved in this separation. In disaccharides IIH and IIA, the sulfonate at the glucosamine C6 is in close proximity to the negatively charged carboxylate moiety of the uronic acid residue in comparison to length of the TrBA butyl arms. Interaction of a TrBA ion with either the glucosamine C6 sulfonate or the carboxylate group would sterically hinder ion-pairing with a second TrBA ion at the other site. However, in the case of disaccharides IIIH and IIIA, which are more highly retained than their isomeric analogs, the uronic acid C2 sulfonate and the carboxylate moiety are oriented away from each other in space allowing ion-paring with TrBA at both sites simultaneously, leading to greater inter-

actions with the stationary phase. For disaccharides IIS and IIIS, *N*-sulfonation of the glucosamine adds another point of interaction with the IPR, increasing their retention relative to IIH/IIIH or IIA/IIIA. The glucosamine *N*-sulfonate and uronic acid C2 sulfonate are sufficiently close in disaccharide IIIS that ion-pairing with one TrBA sterically hinders interaction of second TrBA ion with the other site. Therefore, the net ion-pairing interactions of IIS and IIIS are similar, and they have similar retention times as shown in Fig. 1a.

Addition of 2.5 mM ammonium ion improves the resolution of disaccharides IIS and IIIS, as shown in Fig. 3, suggesting that competition between the TrBA and ammonium ions is responsible for the separation of these isomers. A separation mechanism involving competition of the smaller ammonium ion with the much larger TrBA should be reflected in changes in the retention time of the isomer with the greatest degree of steric crowding at TrBA interaction sites. Comparison of Figs. 1a and 3 indicate that the retention time of IIIS is essentially unchanged in the presence of 2.5 mM ammonium ion, while the retention time of IIS decreases from 7.93 min in Fig. 1a to 7.67 min in Fig. 3. As was observed for disaccharides IIH and IIA, this result suggests that steric effects have a more significant impact on ion-pairing interactions with TrBA and disaccharide IIS.

The results shown in Fig. 5 further probe the nature of competition between IPRs in this separation. Fig. 5c shows that at an intermediate concentration of 15 mM MBA a transition point is reached in the retention of the isomeric pair IIA/IIIA where two ion-pair species are resolved in the chromatogram shown in Fig. 5c. In addition to the sharper peak at 4.48 min that we attribute to the TrBA ion-pair, two broad peaks are also detected between 2 and 3 min for each disaccharide. Because these peaks have a retention time that is intermediate between that of MBA and TrBA alone, they might be attributed to ion-pairs with both MBA and TrBA. Even at concentrations of 20 mM MBA and 2.5 mM TrBA (Fig. 5b), slow exchange between multiple ion-pair species leads to partial resolution and broadening compared to the peak widths obtained when only 20 mM MBA is present. Overall lower retention of the disaccharides is observed in Fig. 5b–d compared with Fig. 1a where only 2.5 mM TrBA is present. As mentioned previously, this is most likely due to a reduction in the surface potential of the stationary phase induced by MBA, a less hydrophobic IPR. Although this study examined only the mass spectrometry-compatible IPRs: TrBA, MBA, and ammonium, other IPRs used for ion-pairing with heparin such as tetrabutylammonium (TBA), should have similar separation mechanisms as previous heparin disaccharide separations using these alternate IPRs show identical elution orders as those presented in this work [11,48]. One would expect similar separation mechanisms to apply to other classes of closely related hydrophilic anions, but the extent of competition effects should be highly dependent on the strength of the ion-pairing interactions themselves. If the IPR interacts tightly with the analyte it would be more difficult for another IPR to compete for that interaction. In this regime steric interactions would be expected to dominate the separation mechanism.

Beyond the factors already discussed, peak width also affects the chromatographic resolution of the heparin disaccharide family. As observed in Fig. 1a, increasing the TrBA concentration decreases the peak width. It is this decrease in peak broadening at high TrBA concentrations, more so than changes in relative retention times, that is responsible for the partial resolution of the IIS and IIIS isomers. With such a complex system, peak broadening effects must be interpreted with some caution, considering that multiple equilibria are involved. However, the results in Fig. 1a suggest that when TrBA is at high concentration, stable ion-pairs are formed, decreasing the peak broadening that would result from partial resolution of ion-pairs with different configurations (e.g., those having either one or two associated TrBA ions). This hypothesis is reinforced by the results presented in Fig. 5 showing clear chromatographic resolution of different types of disaccharide ion-pairs.

Finally, while the previous mechanisms serve well to explain isomer separation, the phenomena of anomeric resolution require an alternative explanation. As seen in Figs. 1 and 3, chromatographic resolution of the α and β anomers is most significant for disaccharides with an unsubstituted glucosamine residue, suggesting that the partial positive charge state of this amine plays a role in the chromatographic resolution of these anomers. This phenomenon has also been observed in CE separations of heparin disaccharides and glucosamine [30,52]. We attribute the resolution of the α and β anomers of the disaccharides IH, IIH and IIIH in these separations to differences in the pK_a values of the glucosamine primary amine group. For example, Blaskó et al. [54] report that the β-anomer ammonium group of glucosamine is more acidic (pK_a = 7.87) compared to the α anomer (pK_a = 8.12). This suggests that under our separation conditions, the α-anomers of disaccharides IH, IIH and IIIH have a higher average population of species with a positively charged primary amine, and thus an overall lower net negative charge. This lower negative electrostatic charge would reduce the overall interaction of the α anomers with the IPRs causing them to elute prior to the corresponding β anomers. However, unless the specific goal of the experiment required resolution of the α and β anomers, better chromatographic resolution and quantitation of heparin disaccharides could be achieved by adjusting the mobile phase pH away from the glucosamine pK_as, providing that effective resolution of the entire disaccharide family could still be achieved.

5. Conclusions

Because of the potential versatility and effectiveness of RPIP-HPLC and UPLC in the separation and study of a wide range of charged hydrophilic analytes, including heparin and HS disaccharides, it is important to improve our mechanistic understanding of separations based on ion-pairing interactions. An improved understanding of the separation mechanism assists in the development and refinement of new separation methods for complex mixtures of structurally similar analytes. This study focused on the effects of competition between TrBA and two other positive but less hydrophobic IPRs, MBA and ammonium, on the UPLC resolution of structural isomers of heparin disaccharides having subtle differences in charge and structure. Although the results of this work focused only on the separation mechanism of heparin disaccharides using TrBA as an IPR, it should be noted that we expect other bulky cationic IPRs such as TBA to operate by a similar separation mechanism. Future work to explore the interactions of anionic IPRs with a closely related family of cationic analytes could provide additional insights into the nature of interactions between the analyte, the IPRs and the stationary phase. The knowledge gained from this study could provide significant insights leading to the development of RPIP-UPLC methods for the separation of larger heparin oligosaccharides, as well as other complex mixtures of structurally similar ionic compounds.

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