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Short communication

Separation of hydroxyl protected heparin derived disaccharides using reversed-phase high-performance liquid chromatography

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Abstract

A simple and efficient method for the separation of hydrophobic derivatives of glycosaminoglycan-derived disaccharides is described. Hydroxyl-protected derivatives of a trisulfated disaccharide, prepared from heparin using heparin lyase, were separated by reversed-phase high-performance liquid chromatography. These disaccharide derivatives differed by the number, position, and stereochemistry of acetyl and pivaloyl groups. Separation was achieved on a C_{18} column using a reversed gradient of ammonium sulfate in water. This method has application in the purification of disaccharide derivatives being used as chiral synthons in the preparation of higher oligosaccharides.

1. Introduction

High-performance liquid chromatography (HPLC) is commonly used in the analysis of oligosaccharides that are chemically and enzymatically derived from glycosaminoglycans (GAGs); including heparin, heparan sulfate, chondroitin sulfates, and dermatan sulfate. Amino columns [1–5], reversed-phase (RP) columns [6–11], strong anion-exchange (SAX) columns [12–15], and others [16–19] have been used for separating these highly polar acidic oligosaccharides. While a number of RP-HPLC methods have been developed for the analysis of GAG-derived oligosaccharides, pre-column derivatization and/or ion-pairing reagents are usually required for separation of these highly polar compounds. Pre-column derivatization has included lipophilic dansylhydrazone [16] and pyridylamino [10] groups attached through the sugars reducing-end. These derivatives can be detected by ultraviolet absorbance, fluorescence, and chemiluminescence. Ion-pairing reversed-phase (IPRP)-HPLC has utilized quaternary ammonium groups (tetrapropylammonium, tetrabutylammonium) to cap the negatively charged groups of GAG-derived oligosaccharides, providing hydrophobic interaction sites [6–9,11]. Most derivatization reactions are irreversible and the complete removal of the ion-pairing agents is often difficult. Thus, after a separation has been accomplished there is no practical way to recover the starting acidic oligosaccharides. This has limited RP- and RPIP-HPLC to the analysis of acidic oligosaccharides, precluding its use for preparative chromatography.

SAX-HPLC has been widely used for both the analysis and preparation of oligosaccharides en-

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zymatically-derived from GAGs [12]. This is the optimal method for the fractionation of underivatized oligosaccharides containing unsaturated uronic acid residues allowing detection based on UV absorbance. Pre-column derivatization, with agents, such as 7-amino-1,3-naphthalene disulfonic acid (AGA), facilitates sensitive fluorescence detection [20]. These fluorescent tags are often charged to minimize hydrophobic interactions that are deleterious to fractionation on SAX-HPLC.

Enzymatic depolymerization of heparin with heparin lyases (as well as heparan sulfate, chondroitin sulfates, dermatan sulfate, and hyaluronic acid with their corresponding polysaccharide lyases) provides a reproducible distribution of unsaturated uronic acid containing oligosaccharide products [21]. The trisufated disaccharide 1 (Fig. 1) is the most abundant disaccharide obtained from heparin. In our laboratory, we have recently synthesized a number of hydroxyl-protected derivatives (2–8) of disaccharide 1 (Fig. 1) [22]. Purification of reaction mixtures utilizing SAX-HPLC afforded poor resolution. The pivaloyl derivatives (7 and 8) underwent hydrophobic collapse, precipitating on the column, while the various acetylated derivatives were poorly resolved, possibly the result of the minimal effect of acetate groups on their elution position. These results suggested that RP-HPLC might be useful for the separation of these highly charged disaccharide derivatives, which differ only in position, number, and stereochemistry of relatively small hydrophobic groups. The results presented describe a simple RP-HPLC method for the separation of these disaccharide derivatives. Derivatization and the use of ion-pairing reagents are not required, making this method potentially useful for preparative applications.

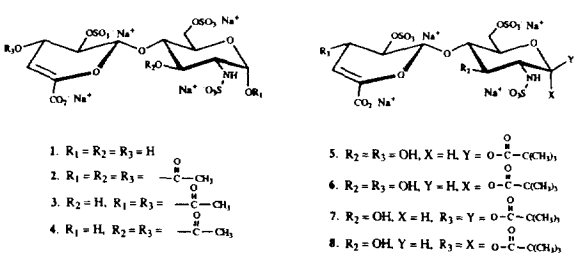

Fig. 1. Structures of heparin disaccharide derivatives.

2. Experimental

Trisulfated disaccharide (1) and derivatized disaccharides (2, 3 and 5–8) were prepared and characterized in our laboratory as previously reported [22]. Compound 4 was prepared from 2 by selective removal of the anomeric acetate using standard conditions [23]. All high-resolution NMR and FAB-MS data were consistent with structure. Composition of mixtures and purity of the disaccharide derivatives were determined by capillary electrophoresis as previously reported [22].

RP-HPLC used dual, face programmable, Shimadzu (Kyoto, Japan) LC-7A titanium-based pumps. The system was equipped with a Rheodyne (Cotati, CA, USA) No. 7125 titanium injector and a Pharmacia LKB (Piscataway, NJ, USA) 2141 variable-wavelength UV detector with both Shimadzu Chromatopac C-R2A integrating recorder and Rainin (Woburn, MA, USA) analog converter connected to a Macintosh system utilizing Dynamax chromatography software. Analytical separations were performed using a reversed-phase C_{18} column (25 cm × 4.6 mm I.D.), 5 μm particle size from Vydac (Hesperia, CA, USA). UV detection of these compounds was at 232 nm based on the chromophore of the unsaturated uronic acid residues [21].

All chemicals were of analytical or reagent grade. Ammonium sulfate (200 m*M*) was adjusted to pH 6 using 5% ammonium hydroxide. De-ionized, distilled water was adjusted to pH 6.0 with NaOH. All mobile phase solutions were filtered through a 0.45-μm membrane filter and degassed prior to use. Specific conditions for all separations are given below.

3. Results and discussion

The aim of this investigation was to develop a rapid and efficient method for the separation of

highly charged, structurally complex, heparin-derived disaccharide derivatives containing hydrophobic hydroxyl protecting groups. Both SAX-HPLC and RPIP-HPLC, used to separate enzymatically prepared disaccharide 1 [11,12], require conditions that create problems with the fractionation of derivatives 2–8.

SAX-HPLC was first used to purify these disaccharide derivatives on a semi-preparative scale [22]. This separation method has limited utility for purification of compounds 2–8 due to low resolution and long separation times. The acetate derivatives (2–4) are poorly resolved by SAX-HPLC, but this separation was satisfactory for the preparation of small quantities of these compounds. The more hydrophobic monopivaloylated derivatives, 5 and 6, are not resolvable by SAX-HPLC. Strong hydrophobic interactions between the dipivoylated derivatives 7 and 8 and the stationary phase of the SAX column occur at the high salt concentrations (>2 *M*) required for their elution. These hydrophobic interactions combined with the strong ionic interactions from the disaccharide's four acidic groups, give long retention times and broad peaks. The addition of up to 10% methanol to the aqueous salt eluent provided little improvement in resolution.

RP-HPLC separation of acidic oligosaccharides generally requires the use of quaternary ammonium ion-pairing reagents in the mobile phase [6–9,11]. Removal of the ion-pairing reagent following separation poses a serious purification problem. Additionally, when a fully hydroxyl-protected disaccharide derivative, such as 2, is converted to a tetrabutylammonium salt (prepared by neutralizing the acid form, obtained by passing 2 through H^+ Dowex, with tetrabutylammonium hydroxide) water solubility is lost and the regeneration of the sodium salt form becomes very difficult.

Pyridylamino derivatization of dermatan sulfate and chondroitin sulfate disaccharides through reductive amination has been reported [10]. This derivatization facilitates RP-HPLC separation in sodium phosphate buffer mobile phase containing methanol without the use of an ion-pair [10]. This observation suggested that compounds 2–8, also containing hydrophobic functionality, might be separated effectively using RP-HPLC without further derivatization and in the absence of ion-pairing reagents.

Compounds 1, 2, 7, and 8 were first examined using a C_{18} column with water (at pH 6.0) as the mobile phase. While 1 and 2 eluted with the void volume, 7 and 8 were slightly retained, giving broad overlapping peaks (chromatograms not shown). These preliminary results suggested that loading the samples in salt, followed by elution at decreasing salt concentrations might afford an improved separation. Ammonium sulfate was chosen to be added to the mobile phase since it had been used similarly in the analysis of phosphorylated peptides [24]. Ammonium sulfate can be easily removed after fractionation using a desalting column or by evaporating to dryness and recovering and disaccharide derivative from salt by dissolving it in methanol. Initially, this separation was performed isocratically with 200 m*M* ammonium sulfate. Trisulfated disaccharide 1 again eluted with the void, but disaccharide derivatives 2 and 7 were retained until the column was washed with water.

The optimum system for the separation of all of the positional and stereochemical isomers of the seven derivatized disaccharides (2–8) consisted of loading the sample in pH 6, 200 m*M* ammonium sulfate followed by a linearly decreasing ammonium sulfate gradient (200 m*M* to 0 m*M*) over 10 min. Analytical RP-HPLC chromatograms of disaccharride 1 (Fig. 2a) and acetylated disaccharides 2–4 (Figure 2b,c,e) show all components are well resolved. Resolution is based on the number of acetylated hydroxyl groups as well as the position of these groups (i.e., 3 and 4 are positional isomers). Although compound 4 was analyzed as >95 percent pure by capillary electrophoresis, a double peak was observed in Fig. 2e. A previous report [25] suggests that, under certain RP-HPLC conditions, the α- and β-anomers of a free anomeric center can result in a double peak.

Analytical RP-HPLC chromatography of the pivaloylated derivatives (5–8) are shown in Fig. 3. Differing degrees of pivaloylation results in dramatic differences in elution position. However, the α- and β-isomers of the monopivaloylated derivatives 5 and 6 (Fig. 3b) and the

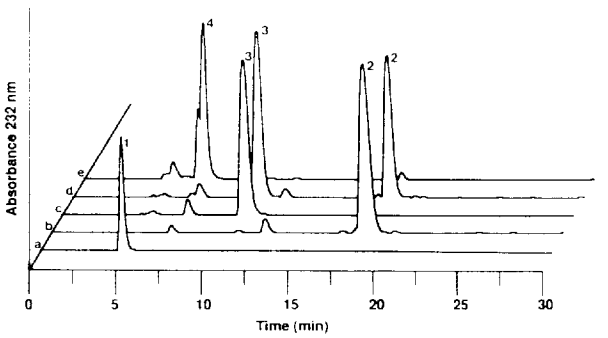

Fig. 2. Analytical RP-HPLC chromatograms of disaccharides 1–4 (peak labels correspond to the structures in Fig. 1). The stationary phase is a Vydac RP C-18 (5 μm), 250 × 4.6 mm I.D. column. The mobile phase is composed of solution A (200 m*M* ammonium sulfate, pH 6.0) and solution B (water, pH 6.0). A linear gradient elution of $t = 0.0$ min [solution A (0.6 ml/min), solution B (0.0 ml/min)] to $t = 10$ min [solution A (0.0 ml/min), solution B (0.6 ml/min)] and $t = 10$–50 min [solution B, 0.6 ml/min] was used. The injection volume was 30 μl and 40–60 μg of sample was analyzed per injection. Detection relied on UV absorbance at 232 nm.

dipivaloylated derivatives 7 and 8 (Fig. 3e) also gave well-resolved peaks. Compounds 5 and 6 had previously coeluted when analyzed by SAX-HPLC.

In conclusion, these data show that RP-HPLC provides a rapid and efficient separation of highly acidic heparin-derived disaccharide derivatives and that this separation is based on degree, position, and stereochemistry of both acetyl and pivaloyl hydroxyl protecting groups. The mobile phase used contains only water and ammonium sulfate making this method generally applicable for preparative separations and avoids the problems associated with using ion-pairing reagents and mixed solvent systems. These methods should facilitate the purification of GAG-derived disaccharides containing limited hydrophobicity or differing slightly in the hydrophobic moieties that are present. Current efforts include applying these separation techniques in the preparation of protected chiral synthons useful for the chemical synthesis of larger, biologically important GAG-like oligosaccharides.

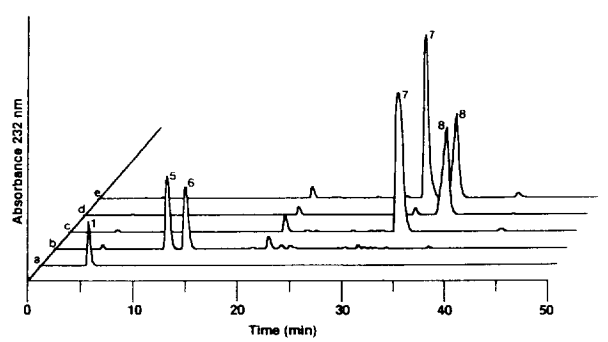

Fig. 3. Analytical RP-HPLC chromatograms of disaccharides 1, 5–8 (peak labels correspond to the structures in Fig. 1). Analysis was conducted as described in the legend of Fig. 2.

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