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Note

High-performance liquid chromatographic separation of unsaturated disaccharides derived from heparan sulfate and heparin

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An enzymatic method has been developed for the elucidation of the structures of heparan sulfate and heparin[1–4] which exhibit a more complex composition than most other glycosaminoglycans[5–7]. Heparinase digests heparin and "heparin-like" portions of heparan sulfate whereas heparitinase (heparanase) degrades the non-sulfate and low-sulfate portions of heparan sulfate. Both enzymes produce mainly disaccharides containing an α,β-unsaturated uronic acid on the nonreducing end. Five disaccharides have been isolated and characterized by Hovingh and Linker[8], which probably represent the disaccharide repeating units of heparan sulfate. The quantitation of the yield of disaccharides from different preparations of heparan sulfate and heparin was accomplished by small-scale enzymatic digestion of the polymers followed by paper chromatographic determination of the products. A similar paper chromatographic technique was used by Silva *et al.*[9] for the analysis of the disaccharide products formed from heparan sulfate.

We have developed a high-performance liquid chromatographic (HPLC) analytical method that is more rapid and sensitive than paper chromatographic procedure for quantifying these unsaturated disaccharides and is well suited to the investigation of the structures of heparan sulfate and heparin.

EXPERIMENTAL

Materials

Four unsaturated disaccharides from heparan sulfate and heparin were obtained as a much appreciated gift from Dr. Alfred Linker, University of Utah (Salt Lake City, UT, U.S.A.). These disaccharides are ΔDi–HS_b–I, a non-sulfated disaccharide from heparan sulfate, ΔDi–HS_b–II, a disaccharide from heparan sulfate containing one sulfate group on the acetylglucosamine unit, ΔDi–HS_b–III, a disaccharide from heparan sulfate containing N-sulfate glucosamine, and, ΔDi–H_a–I, a trisulfated disaccharide from heparin. The structures and preparation of these disaccharides have been reported previously[8,10]. The unsaturated disaccharides from chondroitin sulfates (ΔDi–OS, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi–6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulfo-D-galactose; and ΔDi–4S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose) were purchased from

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Miles Labs. (Elkhart, IN, U.S.A.). The disulfated disaccharide, ΔDi–diS$_B$ (2-acetamido-2-deoxy-3-O-(2-O-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4-O-sulfo-D-galactose) was prepared and purified chromatographically from the chondroitinase ABC-digest of dermatan sulfate from pigskin (Miles Labs).

Methanol and acetonitrile (HPLC grade) and ammonium acetate were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). PIC-A reagent (tetrabutylammonium phosphate) for ion-pair chromatography was purchased from Waters Assoc. (Milford, MS, U.S.A.).

Apparatus

A modular HPLC system was used for the chromatographic studies which consisted of two Model 6000A solvent delivery systems, a Model 660 solvent programmer, a U6K universal injector, and a Model 440 two-channel absorbance detector (Waters Assoc.). The recorder used was a Houston Instrument (Austin, TX, U.S.A.) OmniScribe A5211-5 dual-pen recorder. Peak heights, peak areas and retention times were measured by an on-line Model Supergrator-1 integrator (Columbia Scientific Industries, Austin, TX, U.S.A.).

Prepacked HPLC columns, Partisil 10 PAC and Partisil 10 ODS, 10 μm, 25 cm $\times$ 4.6 mm I.D. (Whatman, Clifton, NJ, U.S.A.) were employed.

Procedure

In the first HPLC procedure, a column packed with a bonded cyano-amino-type polar material (Whatman Partisil 10 PAC) was used. The mobile phase was a ternary solvent system of acetonitrile, methanol and 0.5 *M* ammonium acetate (pH 6.5). This procedure was used for isocratic elution of non-sulfated, monosulfated and disulfated unsaturated disaccharides. The elution of the trisulfated disaccharide which is a more strongly retained compound in this system was achieved by increasing the aqueous ammonium acetate content in the mobile phase. However, more satisfactory results were achieved by employing a second column.

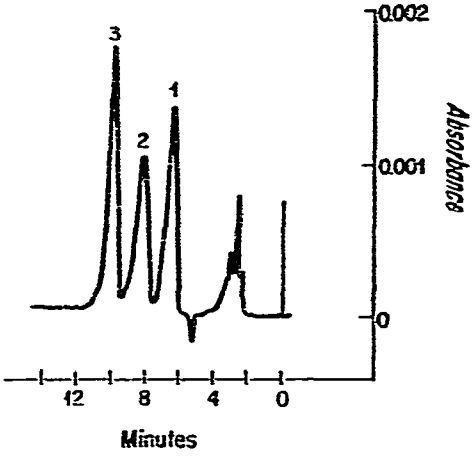
The second HPLC procedure was an ion-pair reversed-phase method which was designed specifically for the rapid separation and sensitive quantitation of disulfated and trisulfated disaccharides. A bonded C_{18} reversed-phase column was employed. The mobile phase consisted of 0.005 *M* PIC-A reagent mixed with methanol. The PIC-A reagent was prepared by mixing 15 ml (one bottle) of the pre-packaged reagent with 1 l of glass distilled water. The pH of the solution was 7.0. A solution of 10% methanol and 90% of this PIC-A reagent gave satisfactory results.

The flow-rate used for both procedures was 1.0 ml/min. All separations were performed at ambient temperatures. Details are given separately with each chromatogram.

RESULTS AND DISCUSSION

The results of HPLC separation and quantitation of the unsaturated disaccharides derived from chondroitin sulfates and dermatan sulfate have been reported previously[11–14]. An attempt was made to apply the same technique for the effective determination of the unsaturated disaccharides derived from enzymatic

degradation of heparan sulfate and heparin because of the similar structures of the two classes of disaccharides. Fig. 1 represents the isocratic separation of the three disaccharides, produced by digestion of heparan sulfate with heparitinase (ΔDi–HS$_b$–I, ΔDi–HS$_b$–II and ΔDi–HS$_b$–III), achieved by the Partisil 10 PAC column. The time required for this separation was 12 min. The composition of the mobile phase was systematically varied in order to select the optimal conditions for separation. The capacity ratio (k') of the disaccharides was determined as a function of the acetonitrile and methanol content of the mobile phase, keeping the content of aqueous ammonium acetate constant at 20% as shown in Fig. 2. The retention behavior of ΔDi–HS$_b$–I, ΔDi–HS$_b$–II and ΔDi–HS$_b$–III are essentially similar to that of ΔDi–OS, ΔDi–6S and ΔDi–4S, respectively. The k' values for the sulfated disaccharides first decrease with increasing acetonitrile content, pass through a minimum at about equal content of acetonitrile and methanol and then increase sharply. The k' values of the nonsulfated disaccharides increase slightly in the region in which k' values of sulfated disaccharides decrease and then increase sharply as did the sulfated disaccharides. The acetonitrile/methanol ratio in the mobile phase can be used for adjusting the relative retention of the three disaccharides. At a constant



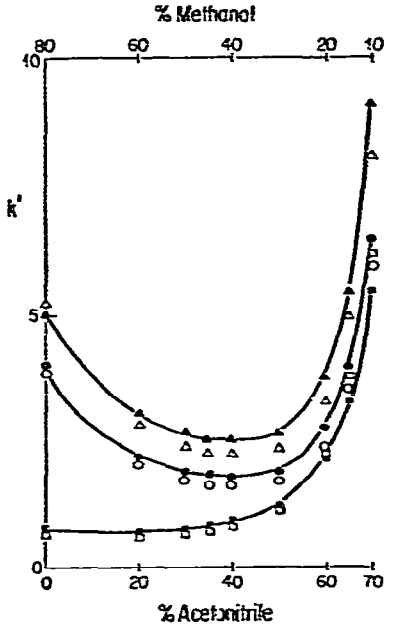


Fig. 1. HPLC of the three unsaturated disaccharides obtained from heparan sulfate: 1 = ΔDi–HS$_b$–I; 2 = ΔDi–HS$_b$–II; 3 = ΔDi–HS$_b$–III. Column, Whatman Partisil 10 PAC; mobile phase, acetonitrile–methanol–0.5 *M* ammonium acetate, pH 6.5 (60:20:20); flow-rate, 1.0 ml/min; pressure, 600 p.s.i.; UV detection at 254 nm, 0.005 a.u.f.s.

Fig. 2. Capacity ratio (k') of the unsaturated disaccharides as a function of the acetonitrile and methanol content of the mobile phase. ■ = ΔDi–HS$_b$–I; ● = ΔDi–HS$_b$–II; ▲ = ΔDi–HS$_b$–III; □ = ΔDi–OS; ○ = ΔDi–6S and △ = ΔDi–4S. Column, Whatman Partisil 10 PAC; mobile phase, acetonitrile–methanol–0.5 *M* ammonium acetate, pH 6.5 (X:Y:20), X, % acetonitrile, and Y, % methanol, X + Y = 80%; flow-rate, 1.0 ml/min.

ratio of the two organic solvents, an increase in the amount of aqueous ammonium acetate buffer resulted in the decrease of retention time of each disaccharide, which is probably due to the increase of the ionic strength and the increased solvation of the solutes as the solvent becomes more polar. When the pH of ammonium acetate was below 6.0, two peaks were observed for each disaccharide, especially for the nonsulfated one which suggests that anomeric forms were separated.

It was possible to separate the disulfated and trisulfated disaccharides derived from degradation of heparan sulfate and heparin with heparinase from non-sulfated and monosulfated disaccharides in one chromatogram by a concave gradient elution (curve No. 10, solvent programmer) with increasing ammonium acetate content from 20% to 45% within a 20-min period. The total chromatographic time was 30 min. However, a specific ion-pair reversed-phase HPLC method appears to be more rapid and sensitive for the determination of these disulfated and trisulfated disaccharides. Fig. 3 shows a separation of ΔDi–He_a–I from other disaccharides. The ΔDi–diS_B appeared at 5.5 min and was completely separated from ΔDi–He_a–I under these conditions. This peak probably locates the positions of disulfated disaccharides expected from heparan sulfate and heparin. The k' value of ΔDi–He_a–I decreases with increasing methanol content in the mobile phase.

Linear relationships between the amount of disaccharides injected (100 ng to 5 μg) and the peak area of disaccharides were observed in both HPLC procedures as shown in Fig. 4. Seven injections of a fixed amount of ΔDi–HS_b–III employing the

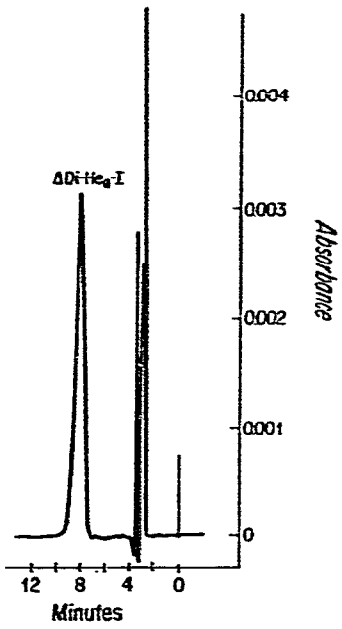


Fig. 3. Ion-pair chromatographic elution of the trisulfated disaccharide from a reversed-phase column. The peaks at about 3 min contain ΔDi–HS_b–I, ΔDi–HS_b–II, ΔDi–HS_b–III and solvent front. Column, Whatman Partisil 10 ODS; mobile phase, methanol–water (10:90) with PIC-A reagent; flow-rate: 1 ml/min; pressure, 800 p.s.i. The amount of ΔDi–He_a–I injected was 2.25 μg. UV detection at 254 nm, 0.005 a.u.f.s.

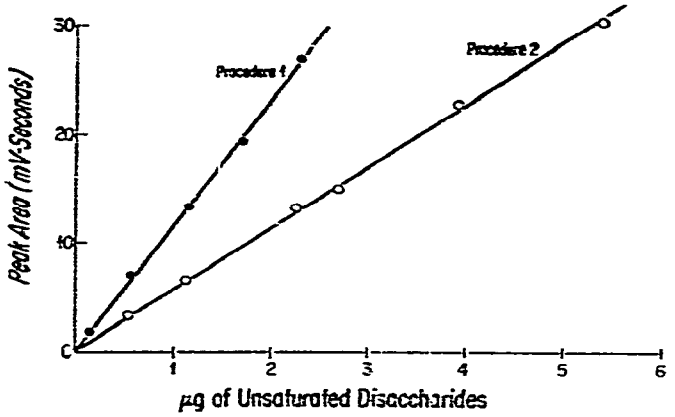


Fig. 4. Linearity of response plots for HPLC determination. ● = ΔDi–HS_b–III in Partisil 10 PAC column procedure; ○ = ΔDi–He_a–I in ion-pair reversed-phase (Partisil 10 ODS column) procedure.

Partisil 10 PAC column procedure and of ΔDi–He_a–I in the ion-pair chromatographic procedure gave relative standard deviations of 1.04% and 1.43% respectively. The detection limits were 20 ng (ΔDi–HS_b–III) employing the Partisil 10 PAC column and 40 ng (ΔDi–He_a–I) with the Partisil 10 ODS column with a signal-to-noise ratio of 3:1. These results demonstrate that the two HPLC procedures described here together provide a useful means for the quantification of unsaturated disaccharides which are produced from enzymatic degradation of heparan sulfate and heparin.

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