High-Performance Liquid Chromatography of Pyridylamino Derivatives of Unsaturated Disaccharides Produced from Chondroitin Sulfate Isomers by Chondroitinases

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A sensitive method was developed for the separation and quantitation of four unsaturated disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S, and ΔDi-diS) by high performance liquid chromatography. The unsaturated disaccharides were coupled with a fluorescent compound, 2-aminopyridine. Complete separation of the resulting pyridylamino derivatives was achieved on a column of μBondapak-C18 with 8 mM KH2PO4-Na2HPO4 (pH 6.0)/methanol (30/1, by volume) as a mobile phase. There was a linear relationship between the fluorescence emission (peak height), and the amount of each authentic disaccharide used for the coupling reaction. This method was applied to analyze commercially available chondroitin sulfates A and C, dermatan sulfate, and urinary glycosaminoglycans obtained from patients with mucopolysaccharidosis after digestion with chondroitinases. The data indicated that the present method is useful for the separation and quantitation of nmol-pmol levels of the unsaturated disaccharides produced from chondroitin sulfate isomers by chondroitinases and can be used for their structural characterization.

Although paper chromatography has been used for many years to estimate unsaturated disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6B, and ΔDi-diS) produced from chondroitin sulfates A and C, and dermatan sulfate by digestion with chondroitinases AC and ABC [EC 4.2.2.4 and EC 4.2.2.5] (1, 2), high-performance liquid chromatography (HPLC) has been shown to be a faster and more sensitive method (3–6).

In our previous study (7), 19 sulfated oligo-
saccharides contained in the deamination products of heparins were coupled with a fluorescent compound, 2-aminopyridine, by a slight modification of the method of Hase et al. (8–10), and successfully analyzed by HPLC. Since the fluorescence-labeling method of Hase et al. (8–10) was also suggested to be applicable for the separation and quantitation of nmol-pmol levels of unsaturated disaccharides produced from chondroitin sulfate isomers by digestion with chondroitinases, we sought to develop in this work a sensitive HPLC method for the separation and quantitation of the pyridylamino derivatives (PA-Ds) of $\Delta$Di-0S, $\Delta$Di-4S, $\Delta$Di-6S, and $\Delta$Di-diS.

MATERIALS AND METHODS

Materials—Unsaturated disaccharides ($\Delta$Di-0S, $\Delta$Di-4S, $\Delta$Di-6S, and $\Delta$Di-diS) and chondroitinases AC II and ABC were kindly donated by Dr. T. Okuyama, Seikagaku Kogyo Co., Tokyo. Chondroitin sulfate A from whale cartilage, chondroitin sulfate C from shark cartilage, and dermatan sulfate from hog skin were obtained from Seikagaku Kogyo Co., Tokyo. A $\mu$Bondapak-C18 column and Sep-pak C18 were purchased from Japan Waters Associates.

Coupling of a Sample with 2-Aminopyridine—Coupling of a sample with 2-aminopyridine was performed by a slight modification of the methods of Hase et al. (11) and Kosakai and Yosizawa (7). A portion (1–5 nmol is suitable, minimum 10 pmol) of an authentic unsaturated disaccharide or an enzymatic digest of chondroitin sulfate isomer was dissolved in 4 $\mu$l of water. To this solution, 40 $\mu$l of a 2-aminopyridine solution (1g in 0.76ml of conc. HCl) and 2 $\mu$l of a sodium cyanoborohydride solution (20 mg in 100 $\mu$l of methanol) were added. The mixture was heated in a sealed tube at 80°C for 20 h. The reaction mixture was diluted with 1 ml of water and then loaded on a column (1.2 x 3 cm) of Dowex 50W x 2 ($H^+$ form), followed by washing of the column twice with the water-washing (1 ml each) of the reaction tube. The products were eluted three times with water (5 ml each). The combined washing and eluate were mixed with 0.25 ml of 1 M sodium acetate and then lyophilized. The residue was dissolved in 3.5 ml of water, and the solution was filtered. An aliquot (5–50 $\mu$l) of the filtrate was used for HPLC analysis of the PA-Ds of $\Delta$Di-diS, $\Delta$Di-6S, and $\Delta$Di-4S. On the other hand, the above column was washed with 10 ml of water and 7 ml of 0.5 M pyridine, in succession. The PA-D of $\Delta$Di-0S was then eluted with 13 ml of 0.5 M pyridine. The eluate was lyophilized. The residue was dissolved in 3.5 ml of water, and 3 ml of this solution was passed through a Sep-pak C18, which was then washed with 3.5 ml of water. The effluent and washing were combined and lyophilized. The residue was dissolved in 1 ml of water and an aliquot (5–30 $\mu$l) of the solution was used for HPLC analysis of the PA-D of $\Delta$Di-0S.

HPLC—HPLC was performed on a $\mu$Bondapak-C18 column (3.9 x 300 mm) equipped with a Waters Associates model 6000A delivery system. After injection of an appropriate amount of an aqueous sample, elution was carried out with 8 mM KH$_2$PO$_4$-Na$_2$HPO$_4$ (pH 6.0)/methanol (30/1, by volume) at a flow rate of 0.4 ml/min for the PA-Ds of $\Delta$Di-diS, $\Delta$Di-6S, and $\Delta$Di-4S and at a flow rate of 0.6 ml/min for the PA-D of $\Delta$Di-0S. The detector used was a Hitachi fluorescence spectrophotometer, Model 650-10LC. The wavelengths of excitation and emission were 310 nm and 375 nm, respectively. The amount of each unsaturated disaccharide was calculated from the peak height of its PA-D on the chromatogram by reference to the standard curve (Fig. 1).

RESULTS AND DISCUSSION

HPLC of the PA-Ds of Authentic Unsaturated Disaccharides—Authentic $\Delta$Di-0S, $\Delta$Di-4S, $\Delta$Di-6S, and $\Delta$Di-diS were separately coupled with 2-aminopyridine, followed by treatments as described in “MATERIALS AND METHODS.” The PA-D of each unsaturated disaccharide was then analyzed by HPLC as described in “MATERIALS AND METHODS.” Among many solvent systems examined, the solvent described in “MATERIALS AND METHODS” gave the best separation of the PA-Ds of these unsaturated disaccharides. The retention times of the PA-Ds of $\Delta$Di-diS, $\Delta$Di-6S, and $\Delta$Di-4S were 10.1, 15.5, and 20.4 min, respectively, while that of $\Delta$Di-0S was 19.5 min. A linear relationship between the amount of a sample used for the coupling reaction and the peak height (fluorescence emission) of its PA-D was observed within the ranges shown in Fig. 1. The
Fig. 1. Relationship between the amounts of $\alpha$-Di-diS, $\alpha$-Di-6S, $\alpha$-Di-4S, and $\alpha$-Di-OS used for the coupling reaction and the peak heights (fluorescence emission) of their PA-Ds on HPLC.

limits of detection of the PA-Ds of these unsaturated disaccharides were 0.1–0.5 pmol with a signal-to-noise ratio of 2 : 1.

A mixture of authentic $\alpha$-Di-diS, $\alpha$-Di-6S, $\alpha$-Di-4S, and $\alpha$-Di-OS was coupled with 2-aminopyridine, and treated as described in "MATERIALS AND METHODS." Figure 2, A and C shows the HPLC of the PA-Ds of these unsaturated disaccharides. Figure 2, B and D, shows the result of blank experiments performed by the same procedures without any sample, corresponding to Fig. 2, A and C, respectively. Although the eluate with 0.5 M pyridine gave several blank peaks (Fig. 2D), these peaks were completely separated from that of the PA-D of $\alpha$-Di-OS (Fig. 2C). Since certain blank peaks overlapped with that of the PA-D of $\alpha$-Di-diS, the eluates with water and 0.5 M pyridine were separately analyzed by HPLC. The data showed complete separation of the PA-Ds of the four unsaturated disaccharides from the mixture.

Analysis of Chondroitin Sulfate Isomers—Chondroitin sulfates A and C, and dermatan sulfate were separately digested with chondroitinases AC and ABC according to the procedures of Fluharty et al. (6) using 10 $\mu$l of 0.2 M Tris-HCl buffer (pH 8.0) instead of 20 $\mu$l. Each digest was lyophilized. The unsaturated disaccharides in each digest were coupled with 2-aminopyridine and the resulting PA-Ds were analyzed by HPLC as described in "MATERIALS AND METHODS." The retention time of the PA-D of $\alpha$-Di-DiSB produced from dermatan sulfate by digestion with chondroitinase ABC was 10.3 min. The results are shown in Table I. The present values differed slightly from those described in the manual, which were obtained by paper chromatography.

Analysis of Urinary Glycosaminoglycans (GAGs) from Mucopolysaccharidosis—GAGs were prepared from urines of patients with Hunter’s, Scheie’s, and Morquio’s diseases by the method
TABLE I. The amounts of unsaturated disaccharides produced from chondroitin sulfate isomers (ChsIs) by digestion with chondroitinases. Each ChsI was separately digested with chondroitinases AC and ABC. The resulting unsaturated disaccharides were coupled with 2-aminopyridine and their PA-Ds were determined by HPLC as described in the text. The values are averages of 4 experiments.

<table>
<thead>
<tr>
<th>ChsI a</th>
<th>Chondroitinase used</th>
<th>ChsI b (nmol)</th>
<th>ADi-diS or (and) ADi-diSB (pmol)</th>
<th>ADi-6S (pmol)</th>
<th>ADi-4S (pmol)</th>
<th>ADi-0S (pmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chs-A</td>
<td>ABC</td>
<td>3.5</td>
<td>16 (0.5) c</td>
<td>594 (17.1) c</td>
<td>2,872 (82.5) c</td>
<td>ND d</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>3.5</td>
<td>9 (0.3)</td>
<td>595 (17.5)</td>
<td>2,802 (82.3)</td>
<td>ND</td>
<td>97.3</td>
</tr>
<tr>
<td>Chs-C</td>
<td>ABC</td>
<td>3.0</td>
<td>174 (6.6) b</td>
<td>2,093 (79.1)</td>
<td>378 (14.3)</td>
<td>ND</td>
<td>88.2</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>3.0</td>
<td>155 (6.1) b</td>
<td>2,015 (79.5)</td>
<td>365 (14.4)</td>
<td>ND</td>
<td>84.5</td>
</tr>
<tr>
<td>DS</td>
<td>ABC</td>
<td>3.6</td>
<td>108 (3.0) b</td>
<td>79 (2.2)</td>
<td>3,410 (94.8)</td>
<td>ND</td>
<td>99.9</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>3.6</td>
<td>ND d</td>
<td>79 (31.0)</td>
<td>176 (69.0)</td>
<td>ND d</td>
<td>7.1</td>
</tr>
</tbody>
</table>

a Chs-A, Chs-C, and DS represent chondroitin sulfate A, chondroitin sulfate C, and dermatan sulfate, respectively.

b The amount of ChsI used for enzymatic digestion.

c Expressed as percent of total unsaturated disaccharides.

Values described in the manual for ADi-4S/ADi-6S of Chs-A and Chs-C were 80/20 and 10/90, respectively.

d Not detected.

TABLE II. The amounts of unsaturated disaccharides produced from urinary GAGs of patients with mucopolysaccharidosis by digestion with chondroitinases. Each GAG was separately digested with chondroitinases AC and ABC. The resulting unsaturated disaccharides were determined as described in Table I.

<table>
<thead>
<tr>
<th>Source of GAG (name, sex, years)</th>
<th>Chondroitinase used</th>
<th>GAG a (nmol)</th>
<th>ADi-diS or (and) ADi-diSB (pmol)</th>
<th>ADi-6S (pmol)</th>
<th>ADi-4S (pmol)</th>
<th>ADi-0S (pmol)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunter's disease (T.H., M c, 8)</td>
<td>ABC</td>
<td>5.67</td>
<td>249 (5.0) b</td>
<td>360 (7.3) b</td>
<td>4,234 (85.7) b</td>
<td>100 (2.0) b</td>
<td>87.2</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>5.67</td>
<td>ND e</td>
<td>350 (35.9)</td>
<td>558 (57.3)</td>
<td>66 (6.8)</td>
<td>17.2</td>
</tr>
<tr>
<td>Scheie's disease (N.O., F, 45)</td>
<td>ABC</td>
<td>4.34</td>
<td>57 (1.9) b</td>
<td>160 (5.4)</td>
<td>2,699 (91.8)</td>
<td>23 (0.8)</td>
<td>67.7</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>4.34</td>
<td>ND d</td>
<td>140 (42.8)</td>
<td>163 (49.8)</td>
<td>24 (7.3)</td>
<td>7.5</td>
</tr>
<tr>
<td>Morquio's disease (K.T., M, 13)</td>
<td>ABC</td>
<td>5.49</td>
<td>54 (1.1) b</td>
<td>2,203 (46.7)</td>
<td>2,117 (44.9)</td>
<td>343 (7.3)</td>
<td>85.9</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>5.49</td>
<td>33 (0.8)</td>
<td>2,233 (51.1)</td>
<td>1,814 (41.5)</td>
<td>290 (6.6)</td>
<td>79.6</td>
</tr>
</tbody>
</table>

a The amount of GAG used for enzymatic digestion.

b Expressed as percent of total unsaturated disaccharides.

c M, male; F, female.

d Not detected.

reported previously (12). These GAG preparations were separately digested with chondroitinases AC and ABC, followed by the same treatments as described above. The resulting PA-Ds were analyzed by HPLC as described above. The results are shown in Table II.

Digestion with chondroitinase ABC of Hunter and Scheie GAGs gave greatly increased quantities of ADi-4S as compared with digestion with chondroitinase AC. In addition, the former yielded ADi-diSB. The data support the concept that there is a catabolic disorder of dermatan sulfate in Hunter's and Scheie's diseases (13). On the other hand, digestion with chondroitinase ABC of Morquio GAG showed slightly increased ADi-4S as compared with digestion with chondroitinase AC. In both cases, large quantities of ADi-6S were found. The present data support the concept that there is a catabolic disorder of chondroitin sulfate C in Morquio's disease (13).
The above observations indicated that the present method is useful for the separation and quantitation of nmol-pmol levels of the unsaturated disaccharides produced from chondroitin sulfate isomers by chondroitinases.

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REFERENCES