Separation of Unsaturated Chondroitin Sulfate Disaccharides in Thin-Layer Chromatography on Silica Gel and Their Quantitative Determination by Densitometry

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A simple and rapid method has been developed to determine the relative amounts of 4- and 6-sulfate in chondroitin sulfate or proteoglycan preparations. Unsaturated disaccharide products derived from the samples by chondroitinase ABC digestion in 0.01 M Tri-HCl buffer (pH 8.0) were applied to a silica gel-coated thin-layer chromatographic plate and separated in a solvent system of n-propanol-isopropanol-n-butanol-water (55 : 20 : 5 : 20, v/v) containing 0.04 M NaCl and 0.01 M ammonia without a desalting step, which was necessary in paper chromatography or thin-layer chromatography on cellulose. The developing time was as short as 2–2.5 h at room temperature. After staining of the separated products with a carbazole reagent followed by immersing the plate in liquid paraffin, the ratio of monosulfated disaccharides, generally found as major components in chondroitin sulfate chains, was estimated by densitometry.

Keywords — unsaturated chondroitin sulfate disaccharide; chondroitin sulfate; proteoglycan; TLC; densitometry

For the separation of disaccharides with a 4,5-unsaturated uronosyl residue at their nonreducing ends (2-acetamido-2-deoxy-3-O-β-D-glucopyranosyluronic acid)-D-galactose (Di-OS), 2-acetamido-2-deoxy-3-O-β-D-glucopyranosyluronic acid)-4-O-sulfo-D-galactose (Di-4S), and 2-acetamido-2-deoxy-3-O-β-D-glucopyranosyluronic acid)-6-O-sulfo-D-galactose (Di-6S) in the digests of chondroitin sulfates by chondroitinase ABC or AC, thin-layer chromatography (TLC) on cellulose plates has been adapted by many workers1–3) with the same solvents as those used in paper chromatography.4) These chromatographic techniques require a prior developing step for desalting before separation. Wasserman et al.1) found that the total developing time, about 36 h, in the above paper chromatography could be shortened to 12 h in TLC on cellulose. As reported previously,5) we separated hyaluronate oligosaccharides by TLC on a silica gel plate of Kieselgel 60 (Merck & Co.). We attempted to use this plate for the resolution of unsaturated disaccharides derived from chondroitin sulfates and found a suitable solvent system after a series of trials. For the chondroitinase digest of glycosaminoglycans obtained in enriched Tris–HCl buffer,4) a desalting step was desirable for satisfactory resolution even on silica gel plates. No suitable solvent system for one-step development was found. However, the digestion in a low concentration of Tris–HCl buffer alone instead of the enriched buffer permitted a satisfactory separation of disaccharide products without a desalting process. This finding resulted in a considerable reduction of the developing time necessary to characterize these products. The separated disaccharides were visualized as blue spots by carbazole staining and their relative amounts were estimated by densitometric scanning.

In this paper, we report the application of TLC on silica gel for the separation of unsaturated chondroitin sulfate disaccharides and discuss their quantitative analysis by...
Experimental

Materials—Standard unsaturated disaccharides (ADi-0S, ADi-4S, and ADi-6S), chondroitin, chondroitin 4-sulfate (ChS-A), chondroitin 6-sulfate (ChS-C), and chondroitinase ABC (EC 4.2.2.5) were obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Samples of proteoglycan (D1 fraction) were prepared from osteoarthritic human femoral head cartilage by the known method.6 The femoral heads obtained by clinical surgery were kindly supplied by Prof. Y. Kuroki (Fujigaoka Hospital, Showa University). Another unsaturated disaccharide, 2-acetamido-2-deoxy-3-(D-gluco-4-enepyranosyluronic acid)-D-glucose (ADi-HA) was prepared by chondroitinase ABC digestion of hyaluronate tetrasaccharide having unsaturated glucuronic acid at the nonreducing end.5

Digestion of Glycosaminoglycans and Proteoglycans with Chondroitinase ABC—A mixture of chondroitin, ChS-A, and ChS-C (100 µg of each glycosaminoglycan) was digested with 0.2 unit of chondroitinase ABC in 50 µl of enriched Tris buffer (0.05 M Tris, 0.06 M sodium acetate, 0.05 M sodium chloride, and 0.1 mg of bovine serum albumin per ml), pH 8.0,4 for 16 h at 37 ºC. This digest is herein called ET-100. Alternatively, the glycosaminoglycan mixture (20 µg each) was treated in the same manner, except that the digestion was done in 0.01 M Tris-HCl buffer, pH 8.0. This digest is herein called T-20. Judging from the absorbance at 549 nm after application of the periodate-thiobarbituric acid method for unsaturated disaccharides,7 each glycosaminoglycan was quantitatively degraded in 0.01 M Tris alone at the enzyme level mentioned above.

Proteoglycan samples (a-, b-, and c-Dl), each about 30 µg as uronic acid, from three femoral head cartilages were separately digested with chondroitinase ABC in 0.01 M Tris-HCl buffer under the same conditions as described for T-20. Uronic acid was measured by the method of Bitter and Muir,8 using glucuronolactone as a standard.

TLC—Aqueous solutions containing standard disaccharides (ADi-0S, -4S, and -6S) and each digestion mixture (T-20, ET-100, etc.) were applied to a plate (5 x 12 cm) of Kieselgel 60 (plastic plate, 0.2 mm thickness, Merck & Co.) with a microsyringe or glass capillary tubes. Although chromatoplates were usually employed without prior activation, it should be noted that, in the rainy season in Japan, these plates after sample application had to be kept in a desiccator over P2O5 at least overnight to achieve a reproducible separation. After saturation of a closed cylindrical chamber (9 cm diameter) with 25 ml of solvent for 1 h, the plate was placed in the chamber and allowed to develop until the solvent front reached about 10 cm above the origin, then it was air-dried.

Standard disaccharides and degradation products were detected by spraying the plate with 10% H2SO4 in ethanol containing 0.125% carbazole and heating it for a few minutes at 100 ºC; these oligosaccharides appeared as purple spots during the heating at 100 ºC against a pink background and, on cooling to room temperature, they rapidly became dark blue against a pale-blue background. The staining reagent containing carbazole, with which about 0.2 µg (as disaccharide) was detectable, was more sensitive than 10% H2SO4 in ethanol. When silica gel plates are employed in TLC, the carbazole reagent is useful to visualize uronic acid itself or oligosaccharides having it as a major component.

Determination of Unsaturated Disaccharides by Densitometry—A silica gel plate, on which migrating disaccharides had been visualized by carbazole staining, was immersed in liquid paraffin for about 30 min. After removal of excess paraffin on the gel surface, the plate was scanned on a Fuji-Riken FD-AIV densitometer at 600 nm filter and a 0.2 × 3 mm slit. Peak areas corresponding to disaccharide products were measured by weighing. The composition of different disaccharides in a sample determined by densitometry was compared with that found by high-performance liquid chromatography (HPLC).

A UVIDEC-100-II HPLC system (Japan Spectroscopic Co.) equipped with a UV spectrophotometer was employed. Separation was achieved by use of a 250 x 4.6 mm Fine SIL NH4 column (Japan Spectroscopic Co.), which was eluted with 0.01 M Na2SO4 containing 0.005 M sodium acetate, pH 5.0. Peak areas of absorbance at 232 nm were measured by weighing the chart peaks.

Results and Discussion

Developing Solvents

Using standard disaccharides (ADi-0S, -4S, and -6S) as samples, two solvent systems, isopropanol–water (80 : 20, v/v) and n-propanol–water (80 : 20, v/v) each containing NaCl at a final concentration of 0.05 M, were examined. In both systems, the mobilities of ADi-0S, -6S, and -4S increased in this order. The isopropanol system was effective to separate ADi-4S and -6S, while the n-propanol system showed good separation of ADi-0S from ADi-6S. Therefore, the standard mixture was developed with ternary solvent systems in which the total concentration of alcohols was fixed at 80%, by volume. The results are shown in Fig. 1. A small amount of NaCl (0.05 M final concentration) in the solvents was essential to resolve...
these acidic oligosaccharides, as discussed in a previous paper. Appropriate proportions of the alcohols seemed to be in the range of 1 : 3 to 3 : 1 by volume. Addition of small amounts of n-butanol and ammonia was effective to give round spots, though they slightly changed the mobilities of samples. Thus we established a suitable solvent system consisting of n-propanol–isopropanol–n-butanol–water (55 : 20 : 5 : 20, v/v) containing 0.04 M NaCl and 0.01 M ammonia as final concentrations. Figure 2A shows thin-layer chromatograms of individual disaccharides and a mixture of standard disaccharides. Rf values for 4Di-OS, -6S, and -4S were 0.27, 0.20, and 0.13, respectively. ĢDi-HA (Rf= 0.31) moved faster than but they were not separated satisfactorily in this solvent system.

Separation of Digestion Products

Figure 2B illustrates thin-layer chromatograms of the digestion products from a mixture of chondroitin and chondroitin sulfates in two buffer systems (T-20 and ET-100). As can be seen in the figure, 2.3 µl of T-20 permitted the identification of these products but an excess volume (5 µl) of the solution depressed their mobilities. An experiment with ET-100 (Fig. 2B) showed that Rf values of products agreed well with those of the standards only when a very small amount (<0.5 µl) of the digestion mixture was applied to the plate. Rf values gradually decreased with increasing volume above 0.5 µl.

TLC of a mixture of chondroitinase ABC and standard disaccharides indicated that as much as 3 µl of the enzyme solution (0.2 units/50 µl H2O) applied had no influence on the mobilities of standards; the enzyme was found to remain at the origin by staining with Ponceau 3R (0.8% in 6% trichloroacetic acid) followed by washing with 3% acetic acid (data not shown).

These observations suggest that the retardation of products (T-20(5.0) in Fig. 2B) is caused by large amounts of tris(hydroxymethyl)aminomethane applied to the plate.
Quantitative Determination of Unsaturated Disaccharides by Densitometry

Figure 3 illustrates a plot of the quantity of a standard disaccharide applied to a TLC plate against its peak area obtained by densitometry. These areas (expressed as their relative weights to that of 2.0 µg of ΔDi-0S) were proportional to the concentration of each disaccharide type in the range (0.4—2.0 µg) examined, though overlapping between ΔDi-6S and -0S was observed above 1.2 µg. At the same content of each standard, the peak areas (mean ± S.D.) of ΔDi-4S and -6S based on the area of ΔDi-0S were 77 ± 2% and 85 ± 3%, respectively. These ratios (0.77 for ΔDi-4S, 0.85 for ΔDi-6S, and 1.00 for ΔDi-0S) were used to calculate the proportions of different components in chondroitin sulfate chains; peak areas of disaccharide products to be analyzed were divided by the corresponding ratios and expressed as relative values to the sum of them.

To quantify the disaccharide types in glycosaminoglycans (chondroitin, ChS-A, and ChS-C) and proteoglycans (a-, b-, and c-D1), aliquots (2 or 3 µl) of chondroitinase ABC digests of these samples were applied to TLC plates and developed in the solvent system described before. Thin-layer chromatograms (2 µl application) of degradation products derived from commercial glycosaminoglycans are shown in Fig. 4. Their densitograms (not

![Fig. 3. Relations between Amounts of Standard Unsaturated Disaccharides and Their Peak Areas Obtained by Densitometry.](image)

![Fig. 4. Thin-Layer Chromatograms of Digestion Products of Each Commercial Glycosaminoglycan.](image)

Ch, A, and C mean chondroitin, ChS-A, and ChS-C, respectively. Each polysaccharide (100 µg) was digested in 0.01 M Tris-HCl, pH 8.0 (50 µl) with chondroitinase ABC (0.2 units) for 16 h at 37 °C and an aliquot (2 µl) of the reaction mixture was applied to the plate. St means an aqueous solution containing standards (2 mg each/ml): an aliquot of 0.6 µl was applied. Development time was 2.5 h at 24 °C. This photograph was taken after immersing the plate in liquid paraffin for 30 min.
shown) did not show the presence of $\Delta$Di-OS in the chondroitin sulfates. Three D1 preparations behaved like ChS-C. A densitogram of a-D1 (2 $\mu$l application) as a representative pattern among proteoglycan samples is given in Fig. 5C. As can be seen in the figure, $\Delta$Di-0S was not detected. Since the nonsulfated disaccharide was always found by HPLC, the volume to be applied to the TLC plate was increased to 3 $\mu$l. The resulting densitograms of disaccharide products of ChS-A and a-D1 are shown in Figs. 5A and B, respectively. A small and broad peak (or shoulder) of $\Delta$Di-0S was revealed in both samples. This minor component was also found in ChS-C, b-D1, and c-D1. In the application of 3 $\mu$l, the products were not

![Fig. 5. Densitograms of Unsaturated Disaccharides Liberated from ChS-A and a Proteoglycan Preparation (a-D1) by Chondroitinase ABC Digestion](image)

The optical density adjustment of a densitometer was set at 3. Arrows indicate the locations of markers in separate experiments. Scans of plate sites with no sample application are indicated as blank absorbances in A and B by dotted curves, respectively. A, 3 $\mu$l application of the digestion mixture of ChS-A; B and C, 3 and 2 $\mu$l applications of the digestion mixture of a-D1, respectively.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Aliquot applied ((\mu)l)</th>
<th>$\Delta$Di-4S</th>
<th>$\Delta$Di-6S</th>
<th>$\Delta$Di-0S</th>
<th>4S/6S or 6S/4S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin</td>
<td>2</td>
<td>12</td>
<td>4</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>ChS-A</td>
<td>2</td>
<td>82</td>
<td>18</td>
<td>n.d.</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>82</td>
<td>15</td>
<td>3</td>
<td>0.18</td>
</tr>
<tr>
<td>ChS-C</td>
<td>2</td>
<td>20</td>
<td>81</td>
<td>n.d.</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19</td>
<td>78</td>
<td>3</td>
<td>0.24</td>
</tr>
<tr>
<td>a-D1</td>
<td>2</td>
<td>19</td>
<td>81</td>
<td>n.d.</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18</td>
<td>77</td>
<td>5</td>
<td>0.23</td>
</tr>
<tr>
<td>b-D1</td>
<td>2</td>
<td>14</td>
<td>86</td>
<td>n.d.</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15</td>
<td>81</td>
<td>4</td>
<td>0.19</td>
</tr>
<tr>
<td>c-D1</td>
<td>2</td>
<td>16</td>
<td>84</td>
<td>n.d.</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17</td>
<td>79</td>
<td>3</td>
<td>0.22</td>
</tr>
</tbody>
</table>

HPLC data are shown in parentheses. n.d.: not detected.
well resolved, as can be seen in Figs. 5A and B, and so a scan of a plate site with no sample application was necessary for the correction of peak areas. In the application of 2 μl (Fig. 5C), the ΔDi-4S and -6S peaks appeared to be separated to the baseline at the sacrifice of a minor component, ΔDi-0S. In this case, peak areas were determined on the assumption that the baseline was straight in the region of the peaks. Relatively large peaks at the origins of α-D1 (Figs. 5B and C) would be due to chondroitinase ABC itself and materials not degraded by the enzyme.

Table I summarizes the proportion of disaccharide types in glycosaminoglycan and proteoglycan samples. The values obtained by TLC with 3 μl application were comparable with those by HPLC. An applied volume of 2 μl was insufficient to detect a minor component such as ΔDi-0S, whose content was less than about 5%, but sufficient to estimate the ratios of ΔDi-4S and -6S in the chondroitin sulfate isomers examined.

TLC on silica gel for the separation of disaccharide components of chondroitin sulfates is faster than TLC on cellulose and the quantitative analysis by densitometry was convenient to estimate the proportions of ΔDi-4S and -6S. Although the present method is not as sensitive as the recent HPLC methods,9,10 it provides a simple means for preliminary examination of uncharacterized chondroitin sulfates.

References