# Compositional Analysis of Hyaluronan, Chondroitin Sulfate and Dermatan Sulfate: HPLC of Disaccharides Produced from the Glycosaminoglycans by Solvolysis

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**An ion-exchange high-performance liquid chromatography procedure was developed for analysis of mixtures of *N*-acetyldermosine, *N*-acetylchondrosine and *N*-acetylhyalobiuronic acid produced quantitatively by heating dermatan sulfate, chondroitin sulfate and hyaluronan in dimethyl sulfoxide (DMSO) containing 0.1% (v/v) $H_2O$ at 80 °C for 48 h. These disaccharides were eluted from a TSK gel SAX column using 0.1 M acetic acid containing KCl, and detected fluorometrically by post column derivatization. The eluate was mixed with 2-cyanoacetamide solution and alkaline solution, and heated at 110 °C for 4 min. The resultant compounds were detected fluorometrically (Ex. 335 nm and Em. 390 nm). The usefulness and practicality of the present method were verified by applications to the determination of glycosaminoglycans in tissues.**

**Key words** solvolysis; chondroitin sulfate; dermatan sulfate; hyaluronan; saturated disaccharide

The major glycosaminoglycans (GAGs) constituting the extracellular matrix (ECM) of connective tissues are chondroitin sulfate, dermatan sulfate and hyaluronan.[1–3] The contents of these GAGs and/or proteoglycans (PGs) in ECM fluctuate dynamically in association with repair and regeneration of tissues.[4–10]

So far, the most reliable methods for quantitating GAGs involve determining the unsaturated disaccharides produced by digestion with various chondroitinases and hyaluronidases.[11–14] However, these methods can not readily discriminate between dermatan sulfate and chondroitin sulfate. Dermatan sulfate, chondroitin sulfate and hyaluronan consist of characteristic disaccharide units (uronic acid–hexosamine).[15] In this work, we have developed a chromatographic determination method of these disaccharides, which reflect the structural characteristics of the GAGs.

Pyridinium complexes of GAGs were suspended in dimethyl sulfoxide (DMSO) containing a small amount of $H_2O$, then were quantitatively degraded to *N*-acetyldermosine, *N*-acetylchondrosine or *N*-acetylhyalobiuronic acid by solvolysis. The disaccharides produced were determined simultaneously by HPLC equipped with a fluorometric detector using 2-cyanoacetamide as a post column reagent.[16]

## Materials and Methods

**Materials** Unsaturated disaccharides [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (ΔDi-HA), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S)], dermatan sulfate (porcine skin), chondroitin sulfate (type A, shark cartilage), hyaluronan (human umbilical cord), and heparan sulfate (bovine kidney) were purchased from Seikagaku Kogyo Co. (Japan). TSK gel SAX was obtained from Tosoh Co. (Japan). 2-Cyanoacetamide (α-cyanoacetamide) was purchased from Wako Pure Chemicals (Japan). Actinase E was purchased from Kaken Pharmaceutical Co. (Japan). All other chemicals were of analytical grade.

**Isolation of GAGs from Tissues** GAGs were isolated from tissues as follows[17]: tissues were minced, and then homogenized in four volumes of acetone under ice-cooling. After centrifugation at 1000 × *g* for 15 min, the precipitate was washed with ether and dried *in vacuo*. The defatted dry sample (0.1 g) was suspended in 3 ml of 0.5 M NaOH and kept overnight at 4 °C. After having been neutralized with 3 M HCl, the solution was boiled for 5 min, and then centrifuged at 3000 × *g* for 30 min. The supernatant was mixed with 1 ml of Actinase E solution (1% in 0.01 M Tris–acetate buffer, pH 8.0), and the reaction mixture was heated at 45 °C for 24 h. The solution was mixed with 1 ml of 50% trichloroacetic acid, and the mixture was allowed to stand at 4 °C for 1 h. After centrifugation at 2300 × *g* for 15 min, the supernatant was dialyzed against distilled water, and then lyophilized.

**Preparation of Standard Disaccharides** Standard disaccharides, *N*-acetyldermosine, *N*-acetylchondrosine and *N*-acetylhyalobiuronic acid were prepared from dermatan sulfate, chondroitin sulfate and hyaluronan individually as follows; 10 ml of 1 mg/ml GAG solution was passed through Dowex 50W-X8 ($H^+$-form, 2 cm i.d. × 10 cm), and the column was washed with 40 ml of $H_2O$. The GAG fraction was neutralized with pyridine, and lyophilized. The GAG–pyridinium complex (5 mg) was suspended in 5 ml of DMSO containing 0.1% (v/v) $H_2O$, and heated at 80 °C for 2 d. The disaccharides were isolated from the reaction mixture using HPLC (column, TSK gel SAX (7.6 mm i.d. × 500 mm); eluent, 0.1 M acetic acid). The fractions containing disaccharides were lyophilized, and the disaccharides were dissolved in water. The disaccharide solutions were standardized by the orcinol method.[18]

## Results and Discussion

**Solvolysis of Dermatan Sulfate, Chondroitin Sulfate and Hyaluronan** *N*-Acetylhexosaminide bonds in dermatan sulfate, chondroitin sulfate and hyaluronan are unstable compared with hexuronide bonds.[19] When the GAG–pyridinium complex was heated in DMSO containing $H_2O$, these *N*-acetylhexosaminide bonds were cleaved. This procedure was generally used to hydrolyze the sulfate ester.[19] By regulating the solvolysis conditions, dermatan sulfate, chondroitin sulfate and hyaluronan were degraded into disaccharides, *N*-acetyldermosine (IdoA–GlcNAc), *N*-acetylchondrosine (GlcA–GalNAc) and *N*-acetylhyalobiuronic acid (GlcA–GlcNAc), respectively. The structures of these disaccharides are shown in Fig. 1. In order to find suitable solvolysis conditions, an HPLC method for the determination of disaccharides was established. The disaccharides were separated from the other oligosaccharides on an Asahipak GS-320 gel-filtration column. Disaccharides were eluted at the same retention time on this column. The most important factor in solvolysis was the reaction temperature. At 70 °C, the cleavage of *N*-acetylhexosaminide bonds was incomplete, whereas heating at 90 °C degraded GAGs to monosac-

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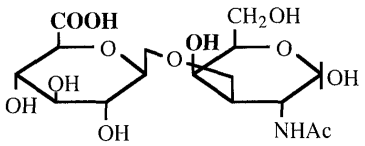
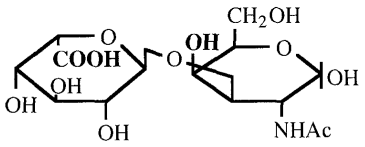
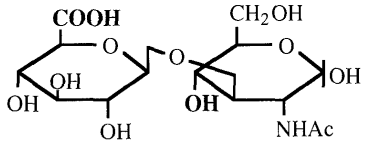
charides. The solvolysis was therefore conducted at 80 °C, and the effects of the content of $H_2O$ and reaction time are shown in Fig. 2. Finally the solvolysis conditions selected for the simultaneous determination of dermatan sulfate, chondroitin sulfate and hyaluronan are as follows; heating the GAGs in DMSO containing 0.1% (v/v) $H_2O$ at 80 °C for 48 h. Under these conditions, the GAGs were solvolyzed into disaccharides in more than 90% yield.




**N-Acetylchondrosine**




**N-Acetyldermosine**




**N-Acetylhyalobiuronic acid**

Fig. 1. Structure of Disaccharide Units Produced from Connective Tissue GAGs by Solvolysis

**Chromatographic Determination of Disaccharides** In order to determine these disaccharides selectively and sensitively, we employed HPLC with a fluorometric detector using 2-cyanoacetamide as a post column reagent (Fig. 3). The reaction temperature and reaction time for post column reaction were established as 110 °C

Table 1. Comparison of Fluorescence Intensities of Products Derived from Various Carbohydrates

| Carbohydrate | Relative intensity | Carbohydrate | Relative intensity |
|---|---|---|---|
| IdoA–GalNAc | 100 | Fructose | 94 |
| GlcA–GalNAc | 192 | Lactose | 139 |
| GlcA–GlcNAc | 113 | Fucose | 121 |
| GlcA–GalN | 185 | Xylose | 113 |
| ΔDi–HA | 915 | GlcA | 83 |
| ΔDi–0S | 559 | Mannose | 90 |
| Galactose | 96 | GlcN | 91 |
| Glucose | 118 | GalN | 92 |

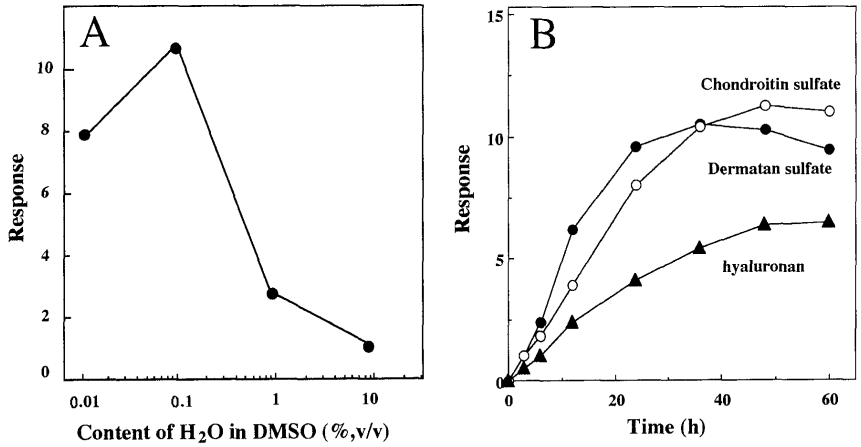



Fig. 2. Solvolysis of Chondroitin Sulfate, Dermatan Sulfate and Hyaluronan to Disaccharides

Dermatan sulfate–pyridinium complex (100 μg) was suspended in 1 ml of DMSO containing $H_2O$, and heated at 80 °C for 48 h (A). Each GAG–pyridinium complex (100 μg) was suspended in 1 ml of DMSO containing 0.1% (v/v) $H_2O$, and heated at 80 °C (B). The amount of disaccharides was determined by HPLC using an Asahipak GS-320 column.

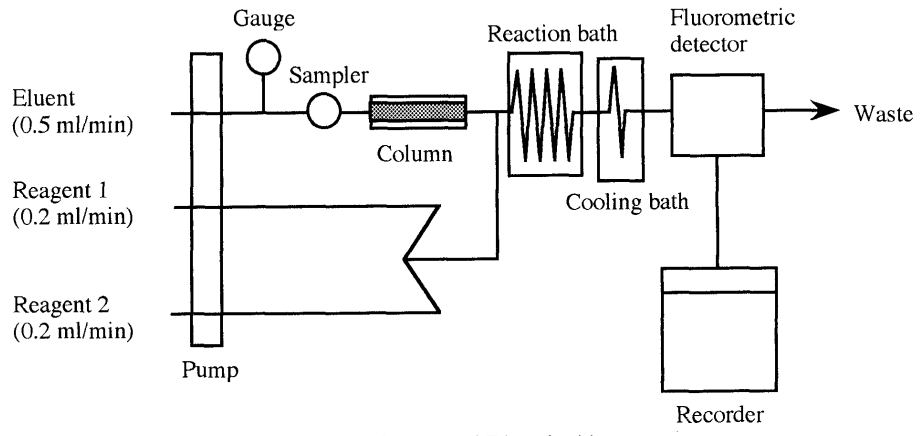



Fig. 3. Schematic Diagram of HPLC for the Determination of Saturated Disaccharides

Column, TSK gel SAX (4.6 mm i.d. × 250 mm); eluent, 0.1 M acetic acid containing 6 mM KCl; column temperature, 80 °C; flow rate, 0.5 ml/min, reagent 1, 2 M NaOH solution; reagent 2, 1% 2-cyanoacetamide solution; reaction temperature, 110 °C; reaction coil, 0.5 mm i.d. × 20 m; cooling coil, 0.25 mm i.d. × 2 m; sample volume, 10—20 μl; detection, Ex. 335 nm, Em. 390 nm.

for 4 min. Table 1 shows relative fluorescence intensities of these disaccharides and related saccharides at the same molarity. These values were calculated from the peak areas. The relative fluorescence intensity of *N*-acetylchondrosine was almost twice those of *N*-acetyldermosine and *N*-acetylhyalobiuronic acid, and these disaccharides were detectable with similar sensitivity as reducing monosaccharides.

*N*-Acetyldermosine, *N*-acetylchondrosine and *N*-acetylhyalobiuronic acid are isomers of each other, but complete separation was achieved by chromatography using an anion-exchange resin (TSK gel SAX, 4.6 mm i.d. × 250 mm) column, and acidic solution as an eluent.

Commercial dermatan sulfate (porcine skin), chondroitin sulfate (shark cartilage), hyaluronan (human umbilical cord) and heparan sulfate (bovine kidney) were submitted

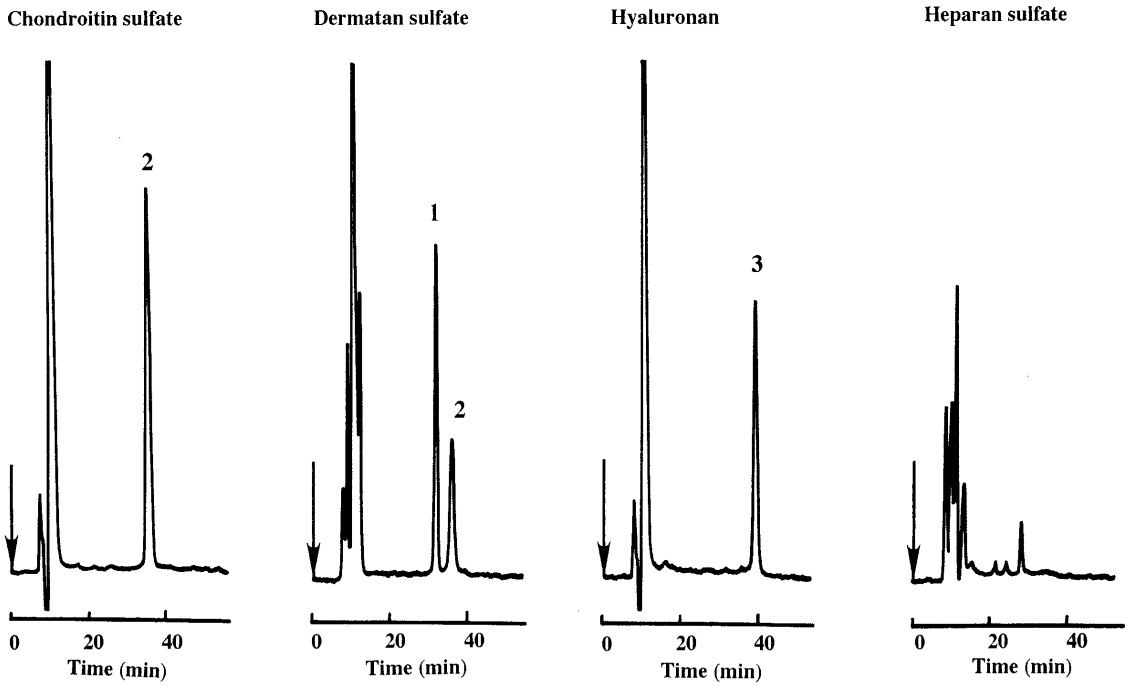



Fig. 4. Chromatograms of Saturated Disaccharides Produced from Commercially Available GAGs by Solvolysis

Pyridinium complexes (100 μg) of chondroitin sulfate (shark cartilage), dermatan sulfate (porcine skin), hyaluronan (human umbilical cord) and heparan sulfate (bovine kidney) were suspended in 1 ml of DMSO containing 0.1% (v/v) $H_2O$ and heated at 80 °C for 48 h. Peaks; 1, *N*-acetyldermosine; 2, *N*-acetylchondrosine; 3, *N*-acetylhyalobiuronic acid.

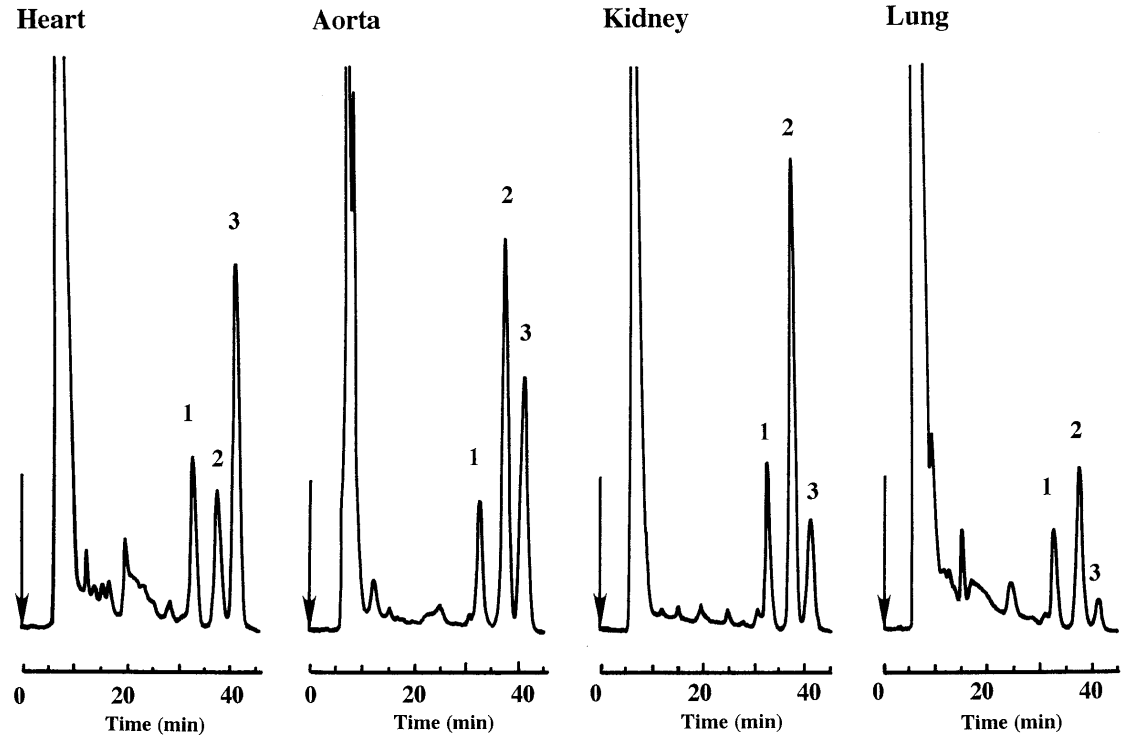



Fig. 5. Chromatograms of Saturated Disaccharides Produced from GAGs Isolated from Rabbit Tissues

GAGs isolated from defatted dry powder (0.1 g) of rabbit tissues were submitted to the present method. Peaks; 1, *N*-acetyldermosine; 2, *N*-acetylchondrosine; 3, *N*-acetylhyalobiuronic acid.

to the present method, and chromatograms of the resultant disaccharides are shown in Fig. 4. Though the major component of uronic acid of porcine skin dermatan sulfate is iduronic acid, which is produced from glucuronic acid by C-5-uronosyl epimerase, a part of the glucuronic acid residues remains unepimerized. The ratio of *N*-acetyldermosine and *N*-acetylchondrosine obtained from porcine skin dermatan sulfate was 85:15 (mol/mol). Heparan sulfate was hardly degraded to disaccharides under this solvolysis condition, so heparan sulfate did not interfere with the simultaneous determination of dermatan sulfate, chondroitin sulfate and hyaluronan. Amino groups of glucosamines in heparan sulfate chains were sulfated, as well as acetylated. This sulfate ester was labile and was hydrolyzed in preference to cleavage of the hexosaminide bonds during solvolysis. The resultant *N*-non-substituted glucosaminide bonds were more stable than *N*-acetylhexosaminide bonds.

**Applications** GAGs were extracted and isolated from rabbit heart, aorta, kidney and lung, and examined by the present method. The chromatograms of the resultant disaccharides are shown in Fig. 5. The component of hyaluronan, chondroitin sulfate and dermatan sulfate are characteristic in each tissue.[19] As described above, the present method can be used for accurate determination of disaccharide units in hyaluronan, chondroitin sulfate and dermatan sulfate.

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