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Determination of human urinary hyaluronic acid, chondroitin sulphate and dermatan sulphate as their unsaturated disaccharides by high-performance liquid chromatography

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ABSTRACT

A method for the determination of hyaluronic acid (HA), chondroitin sulphate (CS) and dermatan sulphate (DS) was developed. HA, CS and DS were converted to the corresponding unsaturated disaccharides by digestion with chondroitinase ABC and/or chondroitinase AC-II and determined by high-performance liquid chromatography with fluorimetric detection using 2-cyanoacetamide as a post-column derivatization reagent. The calibration graphs for the unsaturated disaccharides were linear over the range 2 ng – 2 μg for each unsaturated disaccharide. This method was applied to the analysis of normal human urine.

INTRODUCTION

Hyaluronic acid (HA), chondroitin sulphate (CS) and dermatan sulphate (DS) are glycosaminoglycans (GAGs) composed of alternating β1–3-hexuronic acid and β1–4-N-acetylhexosaminidic bonds. The GAGs are converted by enzymatic digestion into oligosaccharides which contain one terminal $\Delta^{4,5}$-unsaturated glucopyranosyluronic acid absorbing at 232 nm. Chondroitinase ABC digests CS, DS and HA to the corresponding unsaturated disaccharides and chondroitinase AC-II functions on CS and HA, but not on DS (Fig. 1). Based on the differences in enzymatic digestion of GAGs, reliable determinations of HA, CS and DS have been performed followed by the separation of unsaturated disaccharides by high-performance liquid chromatography (HPLC) monitoring of the absorption at 232 nm [1–5]. However, UV detection is not suitable for the analysis of biological samples such as urine because it requires time-consuming samples preparation and is not sensitive enough.

2-Cyanoacetamide has been used as a post-column reagent for the determination of reducing carbohydrates to produce strongly fluorescent compounds [6]. We have applied this reagent to the determination of unsaturated disaccharides produced from CS [7,8] and found that the fluorescence intensity was about 400

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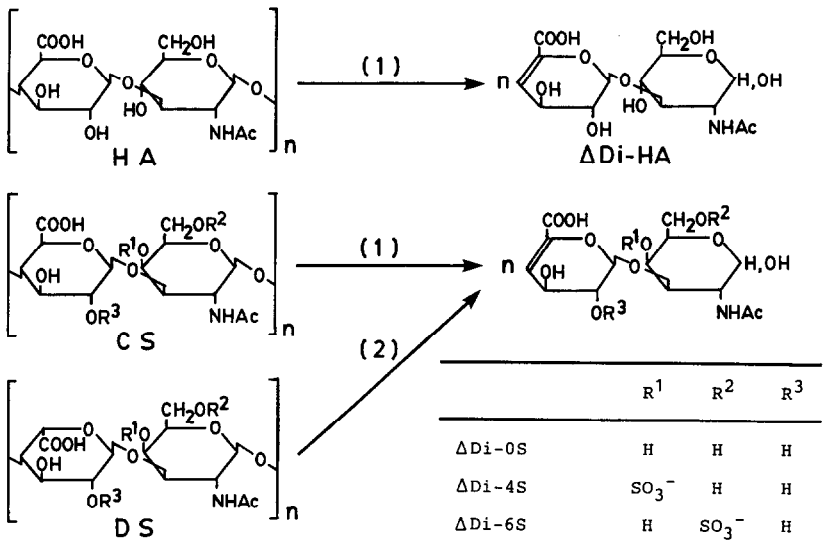

	R^1	R^2	R^3
ΔDi-0S	H	H	H
ΔDi-4S	SO_3^-	H	H
ΔDi-6S	H	SO_3^-	H
$\Delta Di\text{-}diS_B$	SO_3^-	H	SO_3^-
$\Delta Di\text{-}diS_D$	H	SO_3^-	SO_3^-
$\Delta Di\text{-}diS_E$	SO_3^-	SO_3^-	H

(1) Chondroitinase ABC or chondroitinase AC-II

(2) Chondroitinase ABC

Fig. 1. Enzymatic digestion of hyaluronic acid (HA), chondroitin sulphate (CS) and dermatan sulphate (DS).

times stronger than that of neutral sugars [7]. However, in the method, the elution conditions for over-sulphated disaccharides differ from those for non- and mono-sulphated disaccharides, and the separation of ΔDi-HA and ΔDi-0S (for abbreviations, see Experimental) has not been achieved.

This paper reports a method for the simultaneous determination of seven unsaturated disaccharides produced enzymatically from HA, CS and DS, and its application to the analysis of human urinary GAGs.

EXPERIMENTAL

Reagents and chemicals

The standard 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), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-O-sulpho-D-galactose ($\Delta Di\text{-}diS_B$), 2-acetamido-2-deoxy-3-O-(2-O-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-

galactose (ΔDi-diS$_D$) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-O-sulpho-D-galactose (ΔDi-diS$_E$) and chondroitinase ABC (EC 4.2.2.4.) and chondroitinase AC-II (EC 4.2.2.5) were obtained from Seikagaku Kogyo (Tokyo, Japan). 2-Cyanoacetamide was purchased from Kanto Chemicals (Tokyo, Japan). All other chemicals were of analytical-reagent grade. TSKgel NH_2-60 (particle size 5 μm) was purchased from Tosoh (Tokyo, Japan).

Apparatus and chromatographic conditions

The chromatographic equipment included a pump (L-6000; Hitachi Seisakusho, Tokyo, Japan) for the eluting solution, a double-plunger pump (PSU-2.5W; Seishin Pharmaceutical, Tokyo, Japan) for the reagent solution, a sample injector (VMD-350; Shimamura Instruments, Tokyo, Japan), a spectrofluorimeter (RF-530; Shimadzu Seisakusho, Kyoto, Japan), a variable-input recorder (SS-250F; Seconic, Tokyo, Japan), an integrator (Chromatocorder 11; Japan Spectroscopic, Tokyo, Japan) and a dry reaction bath (Type DB-3; Shimamura Instruments).

The HPLC conditions established were as follows: a 10-μl portion of sample solution was loaded via a sample injector with a 20-μl loop. A TSKgel NH_2-60 column (250 mm × 4.0 mm I.D.) was eluted at 30°C with acetonitrile–0.1 *M* Tris–HCl buffer (pH 7.0) containing 0.1 *M* boric acid and 10 m*M* sodium sulphate (64:36, v/v) at a flow-rate of 0.5 ml/min. To the eluate were added 0.3 *M* sodium hydroxide and aqueous 1% 2-cyanoacetamide solution containing 1 m*M* ethylenediaminetetraacetic acid disodium salt at the same flow-rate of 0.2 ml/min with a double-plunger pump. The mixture passed through a PTFE reaction coil (10 m × 0.5 mm I.D.) in a dry reaction bath thermostated at 100°C and a following PTFE cooling coil (2 m × 0.25 mm I.D.). The effluent was monitored fluorimetrically (excitation 346 nm, emission 410 nm).

Urinary creatinine was determined according to the method of Ginman and Colliss [9].

Preparation of human urinary GAGs

Human urinary GAGs were prepared by a modification of Poulsen's method [10] as follows. A 36-ml portion of human urine was adjusted to pH 5 with 2 *M* hydrochloric acid and 2.4 ml of 5% hexadecylpyridinum chloride were added. The sample was kept at 0°C for 4 h. After centrifugation at 2300 *g* for 15 min, the precipitate was washed twice with 6 ml of 0.1% hexadecylpyridinum chloride. The precipitate was dissolved in 4 ml of 2.5 *M* sodium chloride and insoluble materials were removed by centrifugation at 2300 *g* for 15 min. To the supernatant, 44 ml of aqueous 85% (v/v) ethanol were added and GAGs were precipitated by keeping the solution overnight at 0°C. The resulting precipitate was dried, then dissolved in 100 μl of water. Each 20-μl portion was digested with chondroitinase ABC and AC-II, or chondroitinase AC-II.

Enzymatic digestion

A 20-μl portion of human urinary GAGs solution, 10 μl of 0.2 *M* Tris–HCl buffer (pH 8.0) and 10 μl of an aqueous solution containing chondroitinase ABC (0.1 U) and chondroitinase AC-II (0.1 U) were mixed and incubated at 37°C for 3 h. To another 20-μl portion of the sample solution were added 10 μl of 0.2 *M* acetate buffer (pH 6.0) and 10 μl of an aqueous solution of chondroitinase AC-II (0.1 U), then the mixture was incubated at 37°C for 3 h. A 10-μl volume of the reaction mixture was directly subjected to HPLC.

RESULTS AND DISCUSSION

Chromatographic separation and detection of unsaturated disaccharides

In the study of the simultaneous determination of the unsaturated disaccharides from HA, CS and DS, some problems arise. One is how to separate ΔDi-0S and ΔDi-HA, and another is how to elute over-sulphated disaccharides (Δdi-diS_B, ΔDi-diS_D and ΔDi-diS_E), which are adsorbed tightly to the column compared with ΔDi-0S, ΔDi-HA and monosulphated disaccharides (ΔDi-4S and ΔDi-6S). Therefore, it was difficult to carry out the simple and simultaneous determination of these seven unsaturated disaccharides under isocratic conditions.

A variety of eluents for an aminosilica-type column, which is usually used for the separation of unsaturated disaccharides, were examined. As a result, the satisfactory separation of the seven unsaturated disaccharides was achieved with acetonitrile–0.1 *M* Tris–HCl buffer (pH 7.0) containing 0.1 *M* boric acid and 10 m*M* sodium sulphate (64:36, v/v) on a TSKgel NH_2-60 column at 30°C (Fig. 2).

The capacity factors (k') decreased with increasing pH (in the range 6.0–8.0) and decreasing acetonitrile concentration. The column temperature was also important because the resolution of ΔDi-0S and ΔDi-HA decreased with increasing temperature (in the range 30–45°C). To elute over-sulphated disaccharides under

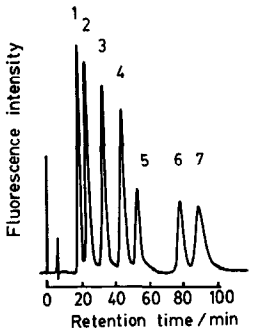

Fig. 2. Typical chromatogram of standard unsaturated disaccharides. Sample size: 10 μl (100 ng of each sugar). Peaks: 1 = ΔDi-HA; 2 = ΔDi-0S; 3 = ΔDi-6S; 4 = ΔDi-4S; 5 = ΔDi-diS_B; 6 = ΔDi-diS_D; 7 = ΔDi-diS_E. Other conditions as described in the text.

isocratic conditions, the addition of sodium sulphate was especially effective compared with other salts tested.

For effective detection, 2-cyanoacetamide was used as a post-column derivatization reagent as described under Experimental.

Calibration graphs for ⊿Di-HA, ⊿Di-0S, ⊿Di-4S, ⊿Di-6S, ⊿Di-diS_B, ⊿Di-diS_D and ⊿Di-diS_E obtained by the peak-area method were linear in the range 2 ng–2 μg. The relative standard deviations at 100 and 10 ng were less than 2% (n = 5) and 4% (n = 5) for each unsaturated disaccharide.

Enzymatic digestion of human urinary GAGs

Human urinary GAGs were prepared according to the procedure described under Experimental. The GAGs were digested by chondroitinase ABC together with chondroitinase AC-II (0.1 U each), chondroitinase ABC (0.1 U) or chondroitinase AC-II (0.1 U). Fig. 3 shows that the amounts of unsaturated disaccharides obtained from urinary GAGs by digestion with chondroitinase ABC are less than those obtained by combined digestion with chondroitinase ABC and chondroitinase AC-II.

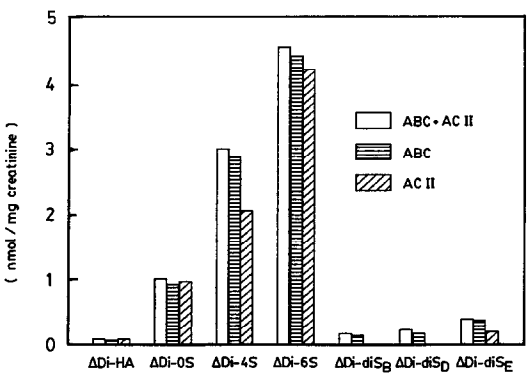

Fig. 3. Comparison of unsaturated disaccharides produced from human urinary glycosaminoglycans after digestion with enzymes. ABC + AC II, digested with chondroitinase ABC together with chondroitinase AC-II; ABC, digested with chondroitinase ABC; AC II, digested with chondroitinase AC-II.

This result may be explained by the fact that the disaccharide unit near to the oligosaccharides linkage (GlcUA–Gal–Gal–Xyl) in CS and DS is not digested with chondroitinase ABC, but is removed by chondroitinase AC-II [11] to generate an unsaturated disaccharide. The molecular sizes of urinary GAGs are relatively small and, consequently, digestion or non-digestion of this disaccharide unit might significantly affect the result in the analysis of urinary CS and DS.

We therefore digested human urinary GAGs with both enzymes simultaneously for complete conversion of HA, CS, and DS into unsaturated disaccharides.

TABLE I

COMPOSITION OF UNSATURATED DISACCHARIDES PRODUCED FROM HUMAN URINARY GLYCOSAMINOGLYCANS

Case No.	Age (years)	Sex[a]	Unsaturated disaccharide (%)									Total amount (nmol/mg of creatinine)
			ΔDi-HA	ΔDi-0S	ΔDi-4S	ΔDi-4S from DS	ΔDi-6S	ΔDi-diS$_B$ from DS	ΔDi-diS$_D$ from DS	ΔDi-diS$_E$	ΔDi-diS$_E$ from DS	
1	22	M	0.2	3.3	29.9	12.2	46.7	1.7	1.9	1.7	2.3	5.7
2	23	M	0.8	5.3	23.9	14.8	46.2	2.0	1.6	1.7	3.7	11.2
3	24	M	0.4	5.4	27.7	10.8	50.1	1.5	1.5	1.2	1.5	8.9
4	24	M	0.4	4.5	27.9	6.6	53.3	1.7	1.2	2.9	1.6	5.2
5	21	F	0.5	7.3	23.3	14.7	44.7	2.5	2.1	1.7	3.2	8.7
6	22	F	0.7	13.7	31.1	8.2	38.4	1.7	1.5	3.0	1.8	8.9

[a] M, male; F, female.

Analysis of human urinary HA, CS and DS

The method was applied to the determination of normal human urinary HA, CS and DS. Fig. 4 shows typical chromatograms of unsaturated disaccharides produced from human urinary HA, CS and DS by combined digestion with chondroitinase ABC and AC-II (Fig. 4A) and by chondroitinase AC-II (Fig. 4B). The differences in the peak heights in the two chromatograms (Fig. 4A and B) correspond to the amounts of DS in human urine.

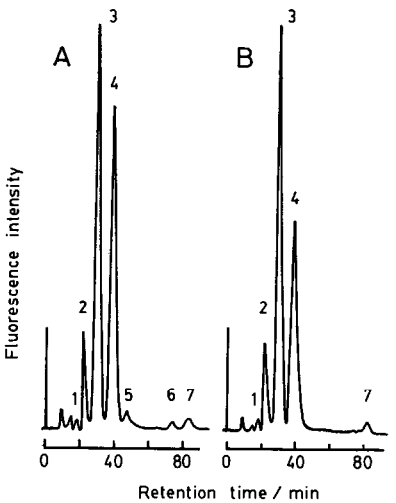

Fig. 4. Chromatograms of unsaturated disaccharides produced from human urinary glycosaminoglycans by digestion with enzymes. (A) Digested with chondroitinase ABC together with chondroitinase AC-II; (B) digested with chondroitinase AC-II. Other conditions as in Fig. 2.

Table I summarizes the results obtained for the compositions of disaccharide units from CS, DS and HA in human urine. It is noteworthy that all amounts of ΔDi-diS_B and ΔDi-diS_D and most of the ΔDi-diS_E are produced from DS. This result may indicate that DS is more easily over-sulphated than CS *in vivo*. Moreover, we have found that part of the GAGs in urine are generated from the kidney and it is very interesting that our results for over-sulphated disaccharides in urine were consistent with those for human kidney GAGs reported by Murata and Yokoyama [12]. On the other hand, the concentration of HA in human urine is very low compared with those of CS and DS (Table I). It may be important to examine low-molecular-mass HA which could not be precipitated by hexadecylpyridinum chloride and were not measured in this work.

The method described here is sensitive and specific for unsaturated disaccharides, and it may be very useful for the simultaneous determination of HA, CS and DS in various biological fluids.

REFERENCES

1 Y. Nomura, H. Tade, K. Takahashi and K. Wada, *Agric. Biol. Chem.*, 53 (1989) 3313.
2 K. Yoshida, S. Miyauchi, H. Kikuchi, A. Tawada and K. Tokuyasu, *Anal. Biochem.*, 177 (1989) 327.
3 T. Gherzghiher, M. C. Koss, R. E. Nordquist and C. P. Wilkinson, *J. Chromatogr.*, 413 (1987) 9.
4 K. Murata and Y. Yokoyama, *J. Chromatogr.*, 415 (1987) 231.
5 M. E. Zebrower, F. J. Kieras and W. T. Brown, *Anal. Biochem.*, 157 (1986) 93.
6 S. Honda, Y. Matsuda, M. Takahashi and K. Kakehi, *Anal. Chem.*, 52 (1980) 1079.
7 H. Toyoda, K. Shinomiya, S. Yamanashi, I. Koshiishi and T. Imanari, *Anal. Sci.*, 4 (1988) 381.
8 H. Toyoda, S. Yamanashi, Y. Hakamada, K. Shinomiya and T. Imanari, *Chem. Pharm. Bull.*, 37 (1989) 1627.
9 R. F. A. Ginman and J. S. Colliss, *Clin. Chem.*, 31 (1985) 331.
10 J. H. Poulsen, *Scand. J. Clin. Lab. Invest.*, 41 (1981) 367.
11 V. C. Hascall, R. L. Riolo, J. J. Hayward and C. C. Reynold, *J. Biol. Chem.*, 247 (1972) 4521.
12 K. Murata and Y. Yokoyama, *J. Chromatogr.*, 423 (1987) 51.