Profile Analysis of Chondroitin Sulfates in Human Urine and Serum

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A highly sensitive high-performance liquid chromatographic method with fluorometric postcolumn labeling using 2-cyanoacetamide was developed for the profile analysis of chondroitin sulfates (ChS) in normal human urine and serum. Over-sulfated disaccharide units such as di- or trisulfated unsaturated disaccharides in urine were estimated and unsaturated 6-sulfated disaccharide (Δ Di-6S) was found as a major component from ChS in urine, although only small amounts of Δ Di-6S from ChS were present in serum.

Keywords unsaturated disaccharide; chondroitin sulfate; glycosaminoglycan; high-performance liquid chromatography; 2-cyanoacetamide; urine; serum

It has been reported that low-sulfated chondroitin 4-sulfate, mainly in the form of proteoglycan, is predominant in human serum or plasma.¹⁾ On the other hand, many workers have indicated that the principal human urinary glycosaminoglycans (GAGs) are chondroitin (Ch), chondroitin 4-sulfate (Ch4S) and chondroitin 6-sulfate (Ch6S).^{2–4)}

In order to obtain information on the catabolism of chondroitin sulfates (ChS), analytical methods for the determination of urinary and serum ChS have been devised using chromatography and electrophoresis.⁵⁾ However, no sensitive and simple method is yet available, because ChS are heterogeneous in molecular size and chemical composition.

Recently, high-performance liquid chromatographic (HPLC) methods with ultraviolet spectrometric detection were established for the determination of unsaturated disaccharide units produced by chondroitinase ABC digestion.^{6–10)} Although these methods are very useful for accurate and precise determination of ChS in biological materials, a more sensitive and reliable method was required to determine only small amounts of ChS in plasma. Thus, we have developed a highly sensitive HPLC method with fluorometric postcolumn labeling using 2-cyanoacetamide for the determination of unsaturated disaccharides obtained enzymatically and applied it to the analysis of ChS in rabbit plasma.¹¹⁾

In this work, our method was developed for the analysis of normal human serum and urinary ChS, and enabled the determination of minor over-sulfated disaccharide units such as di- or trisulfated unsaturated disaccharides in urine.

Materials and Methods

Materials Unsaturated disaccharides (2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose (Δ Di-OS), 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose (Δ Di-4S), 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose (Δ Di-6S), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo- β -D-glucopyranosyluronic acid)-4-*O*-sulfo-D-galactose (Δ Di-diS_B), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo- β -D-glucopyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose (Δ Di-diS_D) and 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo- β -D-glucopyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose (Δ Di-triS) and chondroitinase ABC (EC 4.2.2.4) were obtained from Seikagaku Kogyo Co., Ltd., Tokyo. 2-Cyanoacetamide was purchased from Kanto Chemicals Co., Ltd., Tokyo, and pronase from Kaken Pharmaceutical Co., Ltd., Tokyo. All other chemicals were of reagent grade. TSK gel NH₂ 60 (Tosoh Co., Ltd., Tokyo) of particle size 5 μ m was packed in a stainless steel tube (3.0 mm i.d. $\times$ 250 mm or 4.0 mm i.d. $\times$ 50 mm).

Peripheral blood was obtained from 6 male and 4 female healthy volunteers and urine from 3 male and 3 female healthy volunteers.

Serum samples were prepared as follows. Fresh blood was incubated at 37°C for 30 min, and then it was centrifuged at 2300 $\times g$ for 15 min after standing overnight at 0°C.

HPLC Conditions The chromatography of unsaturated disaccharides was performed by a modification of the procedure reported previously,¹¹⁾ as shown in Fig. 1.

Preparation of Human Urinary GAGs Human urinary GAGs were separated by a modification of Poulsen's method¹²⁾ as follows. A 9 ml portion of each human urine sample was adjusted to pH 5 with 2 M hydrochloric acid and 600 μ l of 5% hexadecylpyridinium chloride was added. The sample tubes were kept at 0°C for 4 h. After centrifugation at 2300 $\times g$ for 15 min, the precipitate was washed with 1.5 ml of 0.1% hexadecylpyridinium chloride. The precipitate was redissolved in 1 ml of 2.5 M sodium chloride. Insoluble materials were removed by centrifugation at 2300 $\times g$ for 15 min. From the supernatant, GAGs were precipitated overnight at 0°C by adding 11 ml of 85% (v/v) aqueous ethanol. The final precipitate was lyophilized, and then redissolved in 1 ml of water. A 20 μ l portion was used for the determination of Δ Di-OS, Δ Di-4S and Δ Di-6S, 100 μ l for Δ Di-diS_B, Δ Di-diS_D and Δ Di-diS_E, and 400 μ l for Δ Di-triS. Moreover, 250 μ l was used for total uronic acids.

Determination of Uronic Acid and Creatinine A 250 μ l portion of each urinary GAGs solution was diluted with an equal volume water and used for the determination of uronic acids by the carbazole method of Bitter and Muir.¹³⁾ Urinary creatinine was determined by the HPLC method.¹⁴⁾

Enzymatic Digestion of Human Urinary GAGs A 100 μ l (or 400 μ l for determination of Δ Di-triS) portion of each urinary GAGs solution was evaporated and the residue was dissolved in 20 μ l of water. Digestion of urinary GAGs with chondroitinase ABC was carried out according to the method of Kodama *et al.*¹⁵⁾ Human serum GAGs were separated by the method described in the previous paper.¹¹⁾

Results and Discussion

Chromatographic Separation and Detection of Unsaturated Disaccharides A highly sensitive HPLC method was established previously for the determination of Δ Di-OS, Δ Di-4S and Δ Di-6S produced from ChS in rabbit plasma.¹¹⁾ In the present work, we examined the HPLC separation and detection in human urine of over-sulfated disaccharide units, such as Δ Di-diS_B, Δ Di-diS_D, Δ Di-diS_E and Δ Di-triS, by a modification of the previous method.

The concentration of sodium sulfate in the eluent affected the chromatographic behavior of unsaturated disaccharides, and the optimum concentration of the salt was examined under isocratic conditions. For the separation of Δ Di-OS, Δ Di-4S and Δ Di-6S, the optimum concentration of sodium sulfate was 10 mM (Fig. 1, A) for Δ Di-diS_B, Δ Di-diS_D and Δ Di-diS_E 25 mM (Fig. 1, B) and the Δ Di-triS 60 mM (Fig. 1, C).

Calibration curves for Δ Di-OS, Δ Di-4S and Δ Di-6S were linear in the range of 4 pmol–5 nmol; those for Δ Di-

TABLE I. Compositions of Unsaturated Disaccharides Produced from Chondroitin Sulfates in Normal Human Urine

Case No.	Age (years)	Sex	Unsaturated disaccharide proportion (%)							Total amount (nmol/mg creatinine)	Total uronic acid in urinary GAGs ^{a)} (nmol/mg creatinine)
			Δ Di-0S	Δ Di-4S	Δ Di-6S	Δ Di-diS _B	Δ Di-diS _D	Δ Di-diS _E	Δ Di-triS		
1	22	M	15.24	41.32	36.37	2.19	1.38	3.34	0.16	10.24	16.16
2	22	M	26.88	35.90	32.19	1.37	1.37	2.17	0.12	8.91	15.26
3	23	M	16.09	47.93	31.83	1.22	0.85	1.94	0.14	8.69	9.83
4	23	F	13.28	41.10	37.87	2.66	1.62	3.32	0.15	6.00	10.89
5	23	F	9.96	51.12	32.89	1.94	1.09	2.84	0.16	7.74	9.77
6	23	F	13.84	41.02	38.64	2.45	1.24	2.62	0.19	8.66	16.54

M, male; F, female. a) Uronic acid was determined by the carbazole method.

Fig. 1. Chromatograms of Unsaturated Disaccharides Produced from Human Urinary Chondroitin Sulfates

Peaks: 1, Δ Di-0S; 2, Δ Di-6S; 3, Δ Di-4S; 4, Δ Di-diS_B; 5, Δ Di-diS_D; 6, Δ Di-diS_E; 7, Δ Di-triS.

HPLC conditions of A: Column, TSK gel NH₂ 60 (3.0 mm $\times$ 250 mm); eluent, 10 mM ammonium formate buffer (pH 5.0) containing 10 mM sodium sulfate in 4% acetonitrile; flow rate, 0.5 ml/min; sample size, 10 μ l.

HPLC conditions of B: Sodium sulfate concentration in eluent, 25 mM. The other conditions were the same as in A.

HPLC conditions of C: Column size, 4.0 $\times$ 50 mm; sodium sulfate concentration in eluent, 60 mM. The other conditions were the same as in A.

diS_B, Δ Di-diS_D and Δ Di-diS_E were linear in the range of 20 pmol—5 nmol, 10 pmol—5 nmol and 5 pmol—5 nmol, respectively; that for Δ Di-triS was linear in the range of 15 pmol—5 nmol.

Profile Analysis of Urinary ChS The normal human urinary ChS in the fraction of GAGs were analyzed by the proposed HPLC method. Figure 1 shows typical chromatograms of unsaturated disaccharides produced from human urinary ChS. Isomers of disulfated unsaturated disaccharides (Δ Di-diS_B, Δ Di-diS_D and Δ Di-diS_E), which were minor constituents, were separated from each other. A trisulfated disaccharide (Δ Di-triS) was detected from urinary ChS for the first time in the present work. Table I summarizes the compositions of disaccharide units from human urine samples and shows that urinary ChS were diverse in sulfate content and chemical composition.

Analysis of Human Serum ChS Table II gives the analytical results for Δ Di-0S, Δ Di-4S and Δ Di-6S in ten normal human serum samples. The data show that Δ Di-0S and Δ Di-4S were major components and Δ Di-6S is a minor one. Over-sulfated disaccharide units could not be determined in this experiment. The levels of Δ Di-0S and Δ Di-4S were rather similar in young male and female volunteers.

TABLE II. Analysis of Unsaturated Disaccharides Produced from Chondroitin Sulfate in Normal Human Serum after Digestion with Chondroitinase ABC

Case No.	Age	Sex	Unsaturated disaccharide (μ M)			Total amount (μ M)
			Δ Di-0S	Δ Di-4S	Δ Di-6S	
1	23	M	7.63 (29.7)	17.44 (67.8)	0.64 (2.5)	25.71
2	23	M	7.04 (32.5)	14.25 (65.7)	0.39 (1.8)	21.66
3	23	M	10.74 (35.0)	19.42 (63.3)	0.51 (1.7)	30.67
4	24	M	10.02 (29.8)	22.93 (68.3)	0.64 (1.9)	33.59
5	22	M	6.68 (29.6)	15.60 (69.2)	0.26 (1.2)	22.54
6	30	M	6.20 (25.0)	18.65 (74.3)	0.26 (1.0)	25.11
7	21	F	6.92 (33.0)	13.76 (65.5)	0.31 (1.5)	20.99
8	22	F	4.54 (27.7)	11.62 (70.7)	0.26 (1.6)	16.42
9	21	F	8.47 (33.6)	16.36 (68.8)	0.39 (1.6)	25.22
10	24	F	9.06 (37.7)	15.29 (62.2)	0.26 (1.1)	24.61
Mean $\pm$ S.D.			7.73 $\pm$ 1.77	16.53 $\pm$ 3.07	0.39 $\pm$ 0.15	24.65 $\pm$ 4.61

M, male; F, female. The values in parentheses represent the proportions (%) of the disaccharide to the total amount of unsaturated disaccharides.

As described above, our sensitive HPLC method was used for the profile analysis of ChS in normal human urine and serum, and it was found that Δ Di-6S is one of the major components of ChS in urine, while only a small amount of Δ Di-6S is present in serum (Tables I and II). The over-sulfated disaccharide units in urine should be further studied to establish the relationship between degree of sulfation of urinary ChS and disease state.

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