Enzymatic Determination of Urinary Glycosaminoglycans from Orthopedic Patients

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SATAKE, S., OTOTANI, N., ISEMURA, M. and YOSIZAWA, Z. *Enzymatic Determination of Urinary Glycosaminoglycans from Orthopedic Patients*. Tohoku J. exp. Med., 1983, **140** (1), 89-96 — Crude glycosaminoglycan (GAG) fraction was directly precipitated with cetylpyridinium chloride without prior dialysis of urine of orthopedic patients. The crude GAG fraction was then fractionated with trichloroacetic acid (TCA). The TCA-insoluble peptide-bound GAG fraction thus obtained was treated with alkali to eliminate the peptide moiety for enzymatic analysis. The GAG compositions of this fraction and the TCA-soluble fraction were determined by digestion with mucopolysaccharidases (chondroitinase AC, chondroitinase B, chondroitinase C, heparitinase and *Streptomyces* hyaluronidase). When the amount of the crude GAG fraction was small, no significant amount of the TCA-insoluble peptide-bound GAG fraction was obtained. The GAG composition of this case was also determined by the same procedures after direct alkali-treatment of the crude GAG fraction. The data indicated that the proportion of the TCA-insoluble peptide-bound GAG fraction was very small. The alkali-treated TCA-insoluble peptide-bound GAG fraction contained a larger proportion of heparan sulfate than the TCA-soluble GAG fraction. It was clearly demonstrated that the patients with Werner's syndrome and mucopolysaccharidosis I-S (Scheie) excreted large amounts of hyaluronic acid and dermatan sulfate respectively, into urines. It was indicated in most cases that major urinary GAG were chondroitin 4-sulfate, chondroitin 6-sulfate plus chondroitin and heparan sulfate, while minor ones were dermatan sulfate and hyaluronic acid. In addition, the data suggested a wide range of the degree of desulfation of urinary GAG, and the presence of significant amounts of keratan sulfate plus acidic glycopeptides in the urinary GAG fractions. The present data provided more precise information on urinary GAG from orthopedic patients than those reported previously. — glycosaminoglycan; urinary glycosaminoglycans; mucopolysaccharidases; orthopedic patient

Since the presence of mucosubstances (glycosaminoglycans and glycoproteins) in human urine was first recognized by Mörner (1895), numerous investigations have

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Abbreviations: GAG, glycosaminoglycan(s); UA, glycuronic acid; UA-GAG, UA-containing GAG; HexN, hexosamine; Ch-4S, 4-O-sulfated N-acetylgalactosaminyl β (1 $\rightarrow$ 4) glucosiduronyl units (chondroitin 4-sulfate); Ch-6S, 6-O-sulfated N-acetylgalactosaminyl β (1 $\rightarrow$ 4) glucosiduronyl units (chondroitin 6-sulfate); Ch-OS, N-acetylgalactosaminyl β (1 $\rightarrow$ 4) glucosiduronyl units (chondroitin); DS-P, dermatan sulfate-proper (sulfated plus non-sulfated N-acetylgalactosaminyl β (1 $\rightarrow$ 4) L-idosiduronyl units); HS, heparan sulfate; HA, hyaluronic acid; KS, keratan sulfate; A-GP, acidic glycopeptide; CPC, cetylpyridinium chloride; TCA, trichloroacetic acid.

been carried out on these substances in urine under normal and pathological conditions, specifically, concerning mucopolysaccharidosis and other connective tissue diseases (Bourrillon 1972; Dorfman and Matalon 1976; Lundblad 1977). Most of these investigations have been carried out by the procedures employing dialysis of urine and/or treatment with cetylpyridinium chloride (CPC), followed by alkali-treatment or protease-digestion to obtain urinary GAG. Although protease-digestion or alkali-treatment is a useful tool for isolation of GAG, no information is available for the peptide-bound GAG.

In a previous paper (Endo et al. 1979), we observed the presence of small amounts of PCA (perchloric acid)-precipitable GAG fraction and PTA (phosphotungstic acid)-precipitable GAG fraction in the non-dialyzable fraction of normal human urine.

In order to study the nature and the proportion of the peptide-bound GAG in total urinary GAG, crude GAG fraction was directly precipitated with CPC without prior dialysis of urine, followed by fractionation with trichloroacetic acid (TCA). The resulting TCA-insoluble peptide-bound GAG fraction was treated with alkali to eliminate the peptide moiety for enzymatic analysis. The GAG compositions of this fraction and the TCA-soluble GAG fraction were determined by digestion with mucopolysaccharidases. On the other hand, when the amount of the crude GAG fraction was small, no significant amount of the TCA-insoluble peptide-bound GAG fraction was obtained. The GAG composition of this case was also determined by the same procedures after direct alkali-treatment of the crude GAG fraction.

This paper describes the results of the study on the urinary GAG from 20 orthopedic patients.

MATERIALS AND METHODS

Materials. Hyaluronidase from *Streptomyces hyalurolyticus*, chondroitinase AC from *Arthrobacter aureescence*, chondroitin sulfate A from whale cartilage, chondroitin sulfate C from shark cartilage, dermatan sulfate from pig skin, heparan sulfate (HS) from bovine kidney, and hyaluronic acid (HA) from human umbilical cord were supplied by Dr. T. Okuyama. Purified heparitinase, chondroitinase B and chondroitinase C were the preparations reported previously (Ototani and Yosizawa 1979; Ototani et al. 1981). Other materials were commercial products.

Urine. Urine was separately collected from 20 cases of orthopedic patients without any dietary restriction. Collection of 24-hr urine from each patient was carried out as described previously (Tokunaga et al. 1975).

Separation of crude GAG fraction from urine. Urine (200 ml) was diluted with equal volume of water. An aqueous cetylpyridinium chloride (CPC) (5%) was added to the diluted urine to a final concentration of 0.2% with stirring. The mixture was left to stand overnight at 5°C with stirring, and was centrifuged in the cold at 8,000 rpm for 15 min. The sediment was washed twice with 20 ml of 0.2% CPC, and then centrifuged as above. The sediment was dissolved in 30 ml of 3 M sodium acetate at room temperature with stirring. To this solution was added 200 ml of 95% ethanol with stirring. The mixture was then left to stand at 5°C for 2–3 hr. The precipitate was collected by centrifugation at 4,500 rpm for 15 min at room temperature, and washed twice with 95% ethanol saturated with NaCl. The sediment was dried over CaCl₂ in vacuo, yielding crude GAG fraction.

Fractionation of crude GAG fraction with trichloroacetic acid (TCA). The crude GAG fraction was dissolved in 4 ml of 0.2 M NaOH with stirring for 15 min at room temperature, then neutralized with equal volume of 0.2 M HCl. The solution was cooled in an ice-bath. Two ml of chilled 50% TCA were added dropwise to this solution. The mixture was left to stand for 1 hr with stirring, and centrifuged in the cold at 10,000 rpm for 30 min. The sediment was washed twice with 10 ml of 10 % TCA, and centrifuged as above. The sediment was washed twice with 95% ethanol saturated with NaCl, then dried over CaCl_2 in vacuo, yielding TCA-insoluble peptide-bound GAG fraction.

The supernatant of the above 10% TCA-precipitate and the TCA-washings were combined, and 5 volumes of 95% ethanol saturated with NaCl were added with stirring. After standing overnight at 5°C, the precipitate was collected by centrifugation at 4,500 rpm for 15 min. The sediment was washed twice with 95% ethanol saturated with NaCl, then dried over CaCl_2 in vacuo, yielding TCA-soluble GAG fraction (TCA-S-GAG).

Alkali-treatment of TCA-insoluble peptide-bound GAG fraction. The TCA-insoluble peptide-bound GAG fraction was dissolved in 4 ml of 0.2 M NaOH with stirring. The solution was then kept at 37°C for 3 hr with stirring, followed by neutralization with 4 ml of 0.2 M HCl. The neutralized solution was cooled in an ice-bath, then 2 ml of chilled 50% TCA were added to this solution with stirring. The mixture was centrifuged and the supernatant was treated by the same procedures as described above. The GAG fraction obtained from the supernatant was designated as ALK-TCA-IS-GAG (alkali-treated TCA-insoluble peptide-bound GAG fraction).

Direct alkali-treatment of crude GAG fraction. When the amount of crude GAG fraction was small (<80 mg), it was subjected to direct alkali-treatment by the same procedures as described above. The resulting GAG fraction was designated as D-ALK-GAG (GAG fraction obtained by direct alkali-treatment from crude GAG fraction).

Enzymatic quantitation of glycuronic acid-containing GAG (UA-GAG). Glycuronic acid-containing GAG (UA-GAG) were determined by digestion with chondroitinase AC, chondroitinase C, chondroitinase B, heparitinase, and *Streptomyces* hyaluronidase as described in a previous paper (Yosizawa et al. 1983).

Determination of constituents. The contents of glycuronic acid (Kosakai and Yosizawa 1979) and hexosamine (Antonopoulos 1966) were determined by the methods described in the cited references. In case of the former, the value attributable to hexose was subtracted.

RESULTS AND DISCUSSION

The amounts of the urinary crude GAG fractions separated from 20 orthopedic patients are listed in Tables 1 and 2. The crude GAG fractions in Table 1 were then fractionated with TCA, yielding TCA-insoluble peptide-bound GAG fractions and TCA-soluble GAG fractions (TCA-S-GAG). The former was subjected to alkali-treatment to eliminate the peptide moiety for enzymatic analysis. However, when the amount of the crude GAG fraction was small, no significant amount of TCA-insoluble peptide-bound GAG fraction was obtained. Therefore, when the amount of the crude GAG fraction was less than 80 mg (Table 2), it was not fractionated with TCA, but subjected to direct alkali-treatment to obtain total GAG fraction. The yields of TCA-S-GAG, alkali-treated TCA-insoluble peptide-bound GAG fractions (ALK-TCA-IS-GAG), and GAG fractions obtained by direct alkali-treatment of the crude GAG fractions (D-ALK-GAG), expressed as glycuronic acid (UA) and hexosamine (HexN), are shown in Tables 1 and 2. The proportions of ALK-TAC-IS-GAG were very small, which were in accord with the

TABLE 1. *The amounts* of urinary crude GAG fractions separated from 7 orthopedic*

Case	Name	Sex	Age	Disease	Crude GAG Fr.*	Fraction	UA‡	HexN‡
1	H.F.	M	15	Multiple exostosis	98.1	TCA-S-GAG	4.04	5.32
						ALK-TCA- IS-GAG	0.17	0.51
2	M.K.	M	15	Epiphyseal slipping	128.9	TCA-S-GAG	12.77	18.17
						ALK-TCA- IS-GAG	0.79	0.98
3	Y.O.	M	15	Osteosarcoma	93.6	TCA-S-GAG	4.17	8.02
						ALK-TCA- IS-GAG	0.23	0.83
4	K.O.	M	22	Left elbow contracture	86.5	TCA-S-GAG	4.90	6.81
						ALK-TCA- IS-GAG	0.72	1.22
5	K.O.	M	27	Osteosarcoma	102.1	TCA-S-GAG	6.18	6.61
						ALK-TCA- IS-GAG	0.20	0.86
6	F.N.	F	30	Osteosarcoma	92.1	TCA-S-GAG	3.85	6.72
						ALK-TCA- IS-GAG	0.15	0.46
7	S.M.	F	62	Coxarthrosis	85.0	TCA-S-GAG	4.15	5.57
						ALK-TCA- IS-GAG	0.51	1.27

* Expressed as mg per 200 ml of urine. †, ‡ Expressed as mg per day. § Ch-4S and uronyl units and 6-O-sulfated N-acetylgalactosaminyl β (1 $\rightarrow$ 4) glucosiduronyl units plus N-Sulfated plus non-sulfated N-acetylgalactosaminyl β (1 $\rightarrow$ 4) L-idosiduronyl units. ¶ In terms †† HexN $\times$ 2.7- UA $\times$ 2.5, expressed as mg per day. ‡‡ Percent in total UA-GAG. §§ Cal- in total UA-GAG. //// Not detected.

previous findings (Endo et al. 1979). Of 20 cases examined, only 7 cases contained relatively large amounts of crude GAG fractions (Table 1).

The GAG compositions of TCA-S-GAG, ALK-TCA-IS-GAG, and D-ALK-GAG were determined with mucopolysaccharidases (chondroitinase AC, chondroitinase B, chondroitinase C, heparitinase, and *Streptomyces* hyaluronidase) by the procedures as described in a previous paper (Yosizawa et al. 1983). The results are shown in Tables 1 and 2. A large amount of Ch-6S plus Ch-0S and a considerable amount of HS were present in Case 2. Large amounts of HA and DS-P were found in Cases 9 and 15 respectively, which were characteristic of Werner's syndrome (Tokunaga et al. 1975) and mucopolysaccharidosis I-S (Scheie) (Dorfman and Matalon 1976) respectively. The most prominent UA-GAG in Case 19 was Ch-6S plus Ch-0S. Major GAG in other cases were Ch-4S, Ch-6S plus Ch-0S, and HS, while minor ones

patients and the GAG compositions† of their TCA-S-GAG and ALK-TCA-IS-GAG

Ch-4S§	Ch-6S plus Ch-0S§	DS-P''	HS¶	HA**	Total UA-GAG	UA Total UA-GAG	KS plus A-GP††
2.67 (24.8)‡‡	5.29 (49.1)‡‡	0.99 (9.2)‡‡	1.29 (12.0)‡‡	0.54 (5.0)‡‡	10.78	37.5 (70.4)§§	4.26
0.16 (34.8)	0.27 (58.7)	ND''''	0.03 (6.5)	ND''''	0.46	37.0 (77.9)	0.95
4.79 (14.2)	22.68 (67.2)	0.05 (0.1)	4.86 (14.4)	1.38 (4.1)	33.76	37.8 (71.8)	17.13
0.12 (5.6)	1.39 (65.3)	ND	0.62 (29.1)	ND	2.13	37.1 (77.1)	0.67
5.16 (45.3)	3.71 (32.6)	0.26 (2.3)	2.22 (19.5)	0.04 (0.4)	11.39	36.6 (80.9)	11.22
0.24 (38.7)	0.21 (33.9)	ND	0.17 (27.4)	ND	0.62	37.1 (77.1)	1.66
3.79 (28.6)	4.91 (37.1)	0.70 (5.3)	3.50 (26.4)	0.35 (2.6)	13.25	37.0 (77.9)	6.14
0.59 (29.6)	0.18 (9.0)	0.14 (7.0)	1.08 (54.3)	ND	1.99	36.2 (84.0)	1.49
0.51 (3.5)	10.41 (71.6)	0.15 (1.0)	3.40 (23.4)	0.07 (0.5)	14.54	42.5 (35.9)	2.40
0.29 (50.9)	0.10 (17.5)	ND	0.18 (31.6)	ND	0.57	35.1 (92.4)	1.82
1.51 (15.1)	5.83 (58.1)	0.19 (1.9)	2.42 (24.1)	0.08 (0.8)	10.03	38.4 (67.2)	8.51
ND	0.07 (17.1)	0.06 (14.6)	0.28 (68.3)	ND	0.41	36.6 (80.9)	0.86
1.58 (14.4)	6.28 (57.1)	0.06 (0.5)	2.51 (22.8)	0.57 (5.2)	11.00	37.7 (72.5)	4.66
0.60 (41.7)	0.13 (9.0)	ND	0.71 (49.3)	ND	1.44	35.4 (90.1)	2.15

Ch-6S plus Ch-0S were the sum of 4-O-sulfated N-acetylgalactosaminyl $\beta(1\rightarrow4)$ glucosid-acetylgalactosaminyl $\beta(1\rightarrow4)$ glucosiduronyl units $\times 1.2$, respectively, in total urine. '' of bovine kidney heparan sulfate. ** In terms of human umbilical cord hyaluronic acid. †† calculated proportion (%) of sulfated UA-GAG, in terms of the standard chondroitin sulfate A,

were DS-P and HA. The amounts of HA were very small in all cases except for Case 9. Since the yields of ALK-TCA-IS-GAG were small, DS-P and HA were not detected in most cases of these fractions (Table 1). Some differences were observed between the GAG compositions of TCA-S-GAG and those of ALK-TCA-IS-GAG. The latter showed relatively larger proportions of HS than the former except for Case 1. The proportions of their Ch-4S and Ch-6S plus Ch-0S was reversed in Cases 5 and 7.

Since the contents of UA in the representative standard chondroitin sulfate A and hyaluronic acid used in the present study were 34.1 and 47.2% respectively, the values of UA/total UA-GAG provide a basis for the calculation of the proportion of sulfated UA-GAG in total UA-GAG. The calculation was made by using a following equation: $X = (47.2 - Y) / (47.2 - 34.1)$, where X is percent of sulfated

TABLE 2. *The amounts* of urinary crude GAG fractions separated from*

Case	Name	Sex	Age	Disease	Crude GAG Fr*	Fraction	UA†	NexH‡
8	S.T.	M	20	Clavicle fracture	35.1	D-ALK-GAG	2.36	4.22
9	K.S.	M	21	Werner's syndrome	50.3	D-ALK-GAG	2.71	3.75
10	T.K.	M	22	Humerus fracture	43.9	D-ALK-GAG	2.88	6.47
11	H.E.	M	24	Radius fracture	48.0	D-ALK-GAG	1.70	3.26
12	M.S.	M	26	Membranous lipodystrophy	24.5	D-ALK-GAG	2.25	3.74
13	M.U.	M	55	Osteoporosis	44.4	D-ALK-GAG	2.20	4.17
14	K.O.	M	57	Osteoporosis	42.6	D-ALK-GAG	1.81	4.01
15	S.M.	F	8	Mucopolysaccha- ridosis I-S (Sheie)	61.3	D-ALK-GAG	14.57	16.97
16	T.K.	F	27	Giant cell tumor	24.2	D-ALK-GAG	3.24	4.80
17	S.O.	F	27	Coxarthrosis	33.1	D-ALK-GAG	1.68	5.14
18	M.O.	F	31	Coxarthrosis	35.5	D-ALK-GAG	1.23	3.97
19	S.C.	F	62	Coxarthrosis	40.5	D-ALK-GAG	1.36	2.26
20	M.H.	F	67	Multiple myeloma	36.6	D-ALK-GAG	3.08	4.76

*, †, ‡, §, //, ¶, **, ††,

UA-GAG, in terms of the standard chondroitin sulfate A, in total UA-GAG; Y is UA/total UA-GAG. The data thus obtained suggested a wide range of the degree of desulfation of urinary UA-GAG. It is suggested also that the degree of sulfation of UA-GAG in ALK-TCA-IS-GAG was higher than that in the corresponding TCA-S-GAG except for Case 3.

On the other hand, the differences between the values of UA and HexN indicate the amounts of keratan sulfate (KS) plus acidic glycopeptides (A-GP), which were precipitable with CPC, in the urinary GAG fractions. The calculated values ($\text{HexN} \times 2.7 - \text{UA} \times 2.5$) are also listed in Tables 1 and 2. The data suggested that the present urinary GAG fractions contained significant amounts of KS plus A-GP.

Although the GAG compositions of the urinary GAG fractions from orthopedic patients (Nagatsuka et al. 1981; Sato et al. 1982) and normal individuals (Varadi et al. 1967; Endo et al. 1980) have been reported, the present study provided more

13 orthopedic patients and the GAG compositions† of their D-ALK-GAG

Ch-4S§	Ch-6S plus Ch-0S§	DS-P//	HS¶	HA**	Total UA-GAG	UA Total UA-GAG	KS plus A-GP††
3.66 (56.9)††	1.52 (23.6)††	0.09 (1.4)††	1.09 (17.0)††	0.07 (1.1)††	6.43	36.7 (80.2)§§	5.49
0.33 (4.7)	1.11 (15.9)	1.04 (15.1)	0.6 (8.6)	3.89 (55.8)	6.97	38.9 (63.4)	3.35
3.84 (48.9)	1.42 (18.1)	0.62 (7.9)	1.86 (23.7)	0.12 (1.5)	7.86	36.6 (80.9)	10.27
1.56 (34.5)	1.30 (28.8)	0.12 (2.7)	1.45 (32.1)	0.09 (2.0)	4.52	37.6 (73.3)	4.55
1.65 (27.9)	2.04 (34.5)	0.58 (9.8)	1.59 (26.9)	0.06 (1.0)	5.92	38.0 (70.2)	4.47
1.11 (19.6)	2.83 (49.9)	0.03 (0.5)	1.63 (28.7)	0.07 (1.2)	5.67	38.8 (64.1)	5.76
1.94 (39.5)	0.99 (20.2)	0.83 (16.9)	1.06 (21.6)	0.09 (1.8)	4.91	36.9 (78.6)	6.30
3.51 (9.3)	5.23 (13.8)	21.98 (57.9)	6.91 (18.2)	0.31 (0.8)	37.94	38.4 (67.2)	9.39
0.48 (5.9)	4.28 (52.3)	0.06 (0.7)	3.20 (39.1)	0.16 (2.0)	8.18	39.6 (58.0)	4.86
0.49 (11.3)	2.42 (55.8)	0.05 (1.2)	1.19 (27.4)	0.19 (4.4)	4.34	38.7 (64.9)	9.68
1.06 (32.2)	1.71 (52.0)	0.06 (1.8)	0.39 (11.9)	0.07 (2.1)	3.29	37.4 (74.8)	7.64
0.03 (0.9)	2.20 (64.9)	0.34 (10.0)	0.78 (23.0)	0.04 (1.2)	3.39	40.1 (54.2)	2.70
5.7 (67.7)	2.4 (28.5)	0.12 (1.4)	0.15 (1.8)	0.05 (0.6)	8.42	36.6 (80.9)	5.15

††, §§ See Table 1.

precise information on the urinary GAG than those reported previously. Especially, precise information was obtained on the TCA-insoluble peptide-bound GAG in the urinary GAG fraction from orthopedic patients. Also, it is evident that the simple method for the quantitation of UA-GAG with mucopolysaccharidases reported previously (Yosizawa et al. 1983) is useful for diagnosis of mucopolysaccharidosis and other metabolic disorders of UA-GAG, as it was clearly demonstrated in the cases of Werner's syndrome and mucopolysaccharidosis I-S (Scheie).

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