Characterization and Determination of Human Urinary Keratan Sulfate

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Keratan sulfate was isolated from normal human urine and was characterized by sugar compositional analysis and ^{1}H-NMR spectroscopy. It was found that KS from human urine is classified as skeletal type (KS-II type chain) with an *O*-glycosidic linkage between galactosamine and serine (or threonine). ^{1}H-NMR studies revealed that urinary KS is not a proteoglycan but a polysaccharide (molecular weight is about 5 kDa). The quantitation of human urinary KS by HPLC showed that urinary KS is excreted at constant levels (0.07 ± 0.015 μg/mg creatinine).

Key words human urine; keratan sulfate; keratanase; HPLC; ^{1}H-NMR

Keratan sulfate (KS) is a glycosaminoglycan (GAG) that was initially isolated from the bovine cornea,[1,2] and later detected in human and bovine skeletal cartilage. KS species have been classified depending on their linkages to protein as KS-I, for the *N*-linked chains derived from cornea (or cartilage fibromodulin) and KS-II, for the *O*-linked chains from skeletal tissues such as cartilage.[3–6] KS isolated from brain tissue possesses an *O*-linked bond between mannose and threonine and may represent a third type.[7] The isolation and quantitation of KS from human urine have been a difficult problem of long-standing date back to the early studies on Morquio's disease.[8] Unlike the other GAGs, it contains no uronic acid and the sugar composition is similar to that of various sugar chains from glycoprotein found in large quantities in urine. Thus, the characteristics of the structure of KS excreted in human urine are still not completely explained. We have investigated the methodology for the determination of hyaluronan, dermatan sulfate and chondroitin sulfate in normal human urine with highly sensitive HPLC methods using fluorescence[9,10] and chemiluminescence[11] detection. In this work, we established HPLC methods for analysis of KS using highly sensitive fluorometric detection. KS from human urine was isolated and subsequently characterized by sugar compositional analysis and ^{1}H-NMR spectroscopy.

Experimental

Materials The following materials were purchased from the companies indicated: keratan sulfate from bovine cornea, keratanase (from *Pseudomonas* sp.) (EC 3.2.1.103), and chondroitinase ABC protease free (from *Proteus vulgaris*) (EC 4.2.2.4) from Seikagaku Kogyo (Tokyo, Japan); heparin lyase I (EC 4.2.2.7), heparin lyase II and III (EC 4.2.2.8) from Sigma Chemical (U.S.A.). DEAE-cellulose (DE-52) was purchased from Whatman (U.S.A.). Sephadex G-100 and Phenyl Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). The HPLC column packings of TSKgel SCX and TSKgel Sugar AXI were obtained from Tosoh (Tokyo). Spectra/Por 7 Molecularporous dialysis membrane (molecular weight cut off, 3500) was obtained from Spectrum (U.S.A.). All other chemicals and reagents used were of analytical grade. Unit definitions followed the commercial catalogs.

Instruments The HPLC equipment consisted of a high-pressure pump (L-6000, Hitachi, Tokyo), a sample injector (Model 7125, Rheodyne, CA, U.S.A.), a column thermocontroller (Mini-80, Taitec, Tokyo), a double plunger pump for the reagent solutions (PSU-2.5W, Shimamura, Tokyo), a dry reaction bath (DB-3, Shimamura, Tokyo), a fluorescence spectrophotometer (F-1050, Hitachi, Tokyo), a UV-VIS spectrophotometric detector (SPD-6AV, Shimadzu, Kyoto, Japan), a conductivity monitor (CM8, Tosoh, Tokyo) and a chromato-integrator (D-2500, Hitachi, Tokyo).

Preparation of Human Urine for the Determination of KS Human urinary GAGs were prepared by a modification of Poulsen's method.[12] To a 9 ml portion of human urine sample, 600 μl of 5% hexadecylpyridinum chloride was added. The sample tubes were kept at 0 °C for 4 h. After centrifugation at $2300 \times g$ for 15 min at room temperature, the precipitate was washed twice with 1.5 ml of 0.1% hexadecylpyridinum chloride. The precipitate was dissolved in 1 ml of 2.5 M NaCl and insoluble materials were removed by centrifugation at $2300 \times g$ for 15 min at room temperature. To the supernatant, 11 ml of aqueous 85% (v/v) ethanol was added and GAGs were precipitated by keeping the solution overnight at 0 °C. The recovery of 2 μg of KS ranged from 68 to 81% with an average of about 75%. Urinary creatinine was determined by HPLC method.[13]

Electrophoresis Electrophoresis of the GAGs was carried out in 1 M pyridine–acetate buffer (pH 3.5) at 0.5 mA/cm on a cellulose acetate membrane for 20 min.[14] The position of the GAGs on electropherograms was then visualized by immersion in an Alcian blue solution (0.5% Alcian blue in 0.3% acetic acid) for 20 min and washed with 1% acetic acid. This method was also employed for purification of urinary KS at the final step.

Compositional Analysis of KS The neutral and amino sugars from KS were recovered by hydrolysis in 6 N HCl for 60 min at 80 °C. Determination of neutral sugars such as galactose and mannose was performed by HPLC as follows: Briefly, the complete separation of mannose, fucose, galactose, xylose and glucose was achieved by TSKgel Sugar AXI column (4.6 mm i.d. × 50 mm) eluted with 0.35 M boric acid–NaOH buffer (pH 8.2) (0.4 ml/min, 60 °C) and high sensitivity was achieved by post-column reaction with 1% (v/v) 2-cyanoacetamide[15] and 0.1 M NaOH at 110 °C, monitoring on wavelength at 280 nm. Aminosugars derived from KS by hydrolysis were separated on a TSKgel SCX column (4.6 mm i.d. × 150 mm), eluted with 0.35 M boric acid–NaOH buffer (pH 7.6) at 60 °C, and detected by the same post-column derivatization system for the neutral sugars. The amounts of sulfate group[16] and neuraminic acid (NueAc)[17] were determined by HPLC methods as described in the references cited.

^{1}H-NMR Study For ^{1}H-NMR spectroscopy, about 100 μg of purified human urinary KS and 2 mg of bovine corneal KS were treated repeatedly with 0.5 ml portions of ^2H_2O, followed by desiccation over P_2O_5 *in vacuo* to exchange the labile protons with deuteron. Then, thoroughly dried sample was redissolved in 0.5 ml of ^2H_2O, and transferred to an NMR tube (5.0 mm o.d. × 18 cm, PP-528; Wilmad Glass Co., Buena, NJ). All spectra were obtained by a JNM-A500 spectrometer of JEOL equipped with a DEC VAX station 3200 computer system, a process controller and an array processor. The operation conditions for one-dimensional (1D) spectra were as follows: frequency, 500 MHz; sweep width, 10 kHz; flip angle, 90° (11.5 μs); sampling points, 32 k; accumulation, 2000 pulses; temperature, 333K. Chemical shifts were indicated by parts/million from the signal of 3-trimethylsilyl[$^2H_4$] propionic acid as an internal standard. Double quantum filtered (DQF)-[18] and triple quantum filtered (TQF)-[19] COSY spectra, and homonuclear Hartman–Hahn (HOHAHA)[20] spectra were recorded in the phase-sensitive mode. All two-dimensional (2D) spectra were recorded with 512 × 1024 data points and spectral width of

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3200 Hz. HOHAHA spectra were recorded with a mixing time of 90 ms. The water resonance was suppressed by selective irradiation during the relaxation delay. A total of 128—256 scans were accumulated for each t_1, with a relaxation delay of 1.4 s. The digital resolution was 3.2 Hz/point in both dimensions with zero-filling in the t_1 dimension. A phase-shifted sinebell function was applied for both t_1 dimensions in the case of DQF-COSY, and a Lorentz–Gauss function was applied in other cases.

Results

Measurement of KS by Post-Column HPLC with Fluorometric Detection A 20 μl portion of KS sample, 10 μl of 0.01 M Tris–HCl buffer solution (pH 7.4) and 10 μl of an aqueous solution containing 0.01 U of keratanase were mixed and incubated at 37 °C for 24 h. A 10 μl volume of the reaction mixture was directly subjected to HPLC. The major product by keratanase digestion, disaccharide monosulfate (Fig. 1), was determined using an anion-exchange column. A TSKgel Sugar AXI column (4.6 mm i.d. × 50 mm) was eluted with 20 mM ammonium acetate buffer (pH 7.5) containing 0.1 M NaCl at a flow rate of 0.5 ml/min (column temperature, 60 °C). To the eluate were added 0.3 M NaOH and aqueous 1% (v/v) 2-cyanoacetamide solution at the same flow rate of 0.25 ml/min with a double plunger pump. The mixture was passed through a reaction coil (0.5 mm i.d. × 10 m) in a dry reaction bath thermostated at 110 °C and a following cooling coil (0.25 mm i.d. × 2 m). The effluent was monitored fluorometrically (excitation 346 nm, emission 410 nm). A TSKgel sugar AXI column sufficiently resolved the disaccharide monosulfate from interfering materials such as *N*-acetylneuraminic acid (NeuAc) (Fig. 2). Other oligosaccharides in the keratanase digest were adsorbed on the column under the isocratic elution condition employed. A calibration graph was linear in the range of 20 ng—20 μg for the injected sample as KS. The relative standard deviation at 50 ng was less than 3% ($n=5$).

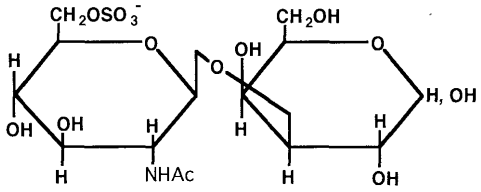

Fig. 1. Structure of Disaccharide Monosulfate Produced from KS by Keratanase Digestion

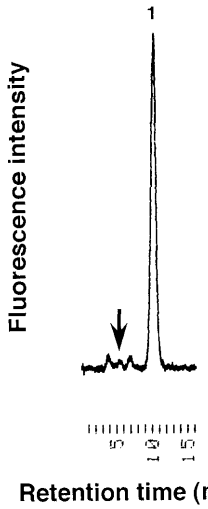

Fig. 2. Separation of Disaccharide Monosulfate on a TSKgel Sugar AXI Column

A 20 μl portion of bovine corneal KS sample (50 ppm), 10 μl of 0.01 M Tris–HCl buffer solution (pH 7.4) and 10 μl of an aqueous solution containing 0.01 U of keratanase were mixed and incubated at 37 °C for 24 h. A 10 μl volume of the reaction mixture was directly subjected to HPLC. An arrow indicates the elution position for NeuAc. Peak 1, disaccharide monosulfate.

Isolation of KS from Human Urine The crude GAG sample (540 mg) containing hyaluronic acid, dermatan sulfate, chondroitin sulfate, heparan sulfate and KS from pooled human urine (20 l) was prepared without any pronase digestion by the method described by Poulsen.[12] The crude GAG extract was enriched in the KS by anion exchange chromatography on a column of DE-52 (Fig. 3A). By this procedure most of the low-sulfated chondroitin 4-sulfate was removed at around 0.3 M NaCl, whereas KS was eluted with 0.4—0.5 M NaCl. The fraction was digested with chondroitinase ABC, and produced disaccharides were separated from KS by a Sephadex G-100 column (Fig. 3B). KS fractions contaminated with heparan sulfates were separated by hydrophobic chromatography[21] on a column of Phenyl Sepharose CL-4B (Fig. 3C). After dialysis against water, the KS was recovered by lyophilization and then treated with the mixture of heparin lyases I (10 U), II (2 U) and III (2 U) followed by gel-filtration HPLC on an Asahipak GS-320 (7.6 mm i.d. × 500 mm) column, eluted with 10 mM ammonium hydrogen carbonate (0.15 ml/min). Finally, the KS pool was subjected to purification by electrophoresis on cellulose acetate membrane. The KS purified by electrophoresis was as pure as possible for a human urinary KS and it was used in further studies (approximately 100 μg of KS was isolated). KS in each fraction was measured by the HPLC method using 2-cyanoacetamide as a post-column reagent (described above). Chondroitin sulfates[9] and uronic acids[22] were determined by the methods cited.

Compositional Analysis of Human Urinary KS As shown in Table 1, KS from human urine contains about equimolar amounts of *N*-acetylglucosamine and galactose, and it is slightly oversulfated (sulfate/GlcNAc = 1.6). GalNAc residue could also be detected. This suggests that human urinary KS can be classified as a skeletal type (type II), whereas bovine corneal KS (classified as type I) shows the presence of mannose, which is a characteristic component of *N*-linked carbohydrate chain.[3-6] Generally, it is very difficult to measure the molecular weights of GAGs because of their structural heterogeneity. In this report, we speculated that the molecular weight of urinary KS is about 5 kDa, based on the presumption that the amount of galactosamine is one mol/mol KS. The content of sulfate groups in urinary KS is larger than that of bovine corneal KS.

^{1}H-NMR The one dimensional 500 MHz ^{1}H-NMR spectra of human urinary and bovine corneal KS (Fig. 4), and the two dimensional spectra of bovine corneal KS were obtained. Especially, the TQF-COSY spectrum of KS, which may be the first application case for KS, is known to be very useful to assign H-6 protons bound to sulfated and nonsulfated C-6 of galactose and that of *N*-acetylglucosamine, respectively (Fig. 5). The assignments of all the signals are summarized in Table 2.

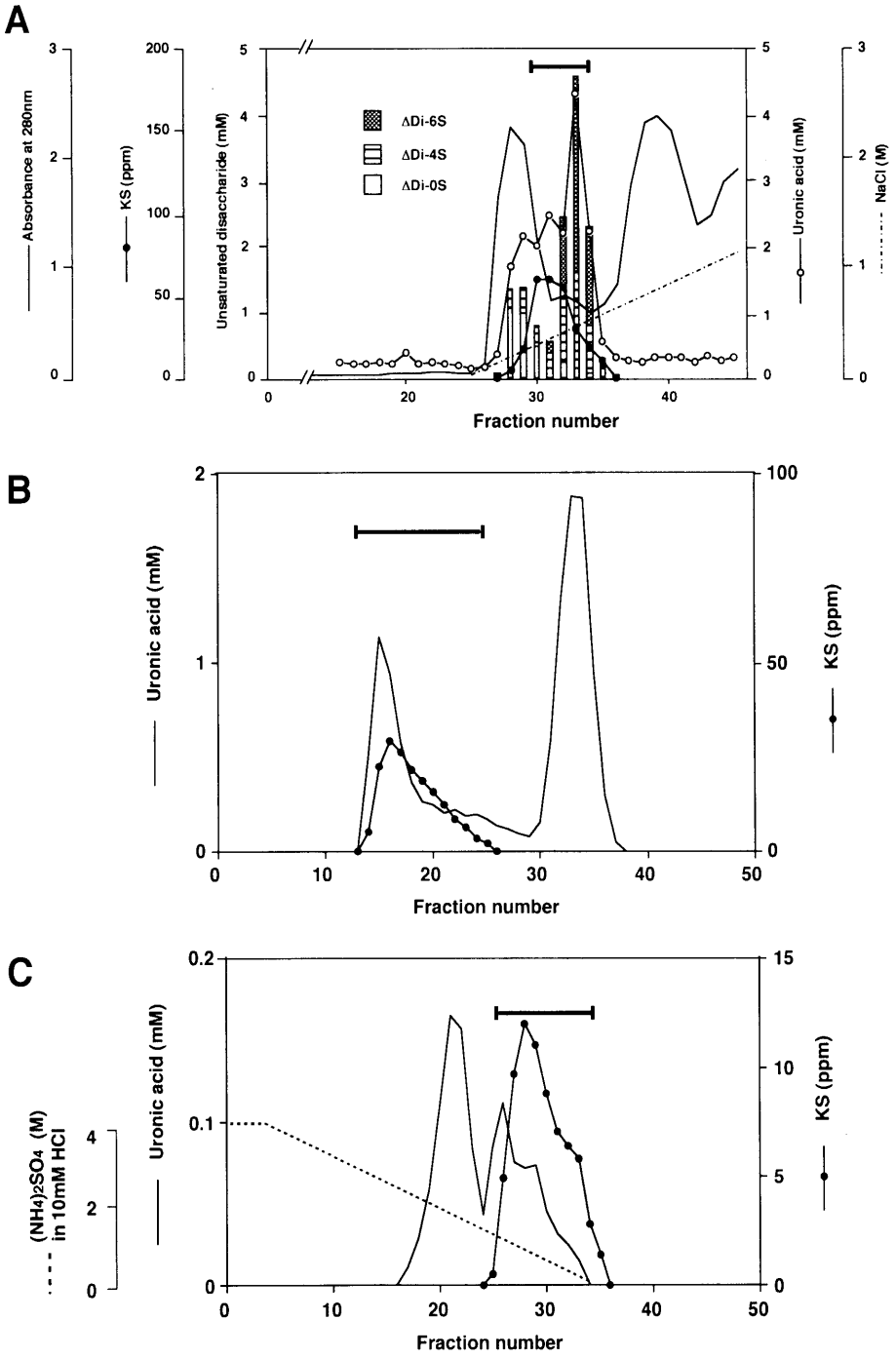

Fig. 3. Isolation of KS from Human Urine

A, DE-52 anion exchange column (22 mm i.d. × 310 mm) was eluted with a linear gradient of 0—2.0 M NaCl/50 mM Tris–HCl buffer (pH 7.0) at a flow rate of 20 ml/h. Fractions (10 ml) were assayed for chondroitin sulfates, uronic acids and KS. B, fractions (tubes No. 29—34 in Fig. 3A) were pooled, and digested with chondroitin ABC for 24 h. The digest was chromatographed on a Sephadex G-100 column (22 mm i.d. × 900 mm) eluted with 0.2 M NaCl at the flow rate of 15 ml/h. C, Fractions (tubes No. 13—25 in Fig. 3B, 10 ml/tube) were pooled, and chromatographed on a Phenyl Sepharose CL-4B column (10 mm i.d. × 150 mm) eluted with a linear gradient of 4.0—0 M ammonium sulfate/10 mM HCl at a flow rate of 25 ml/h. Abbreviations: ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose.

Unfortunately, because of the irradiation at water resonance at 4.43 ppm for long-term measurement and overlapped resonances, we were unable to quantify the ratios of the sulfation at *N*-acetylglucosamine and/or galactose C-6 positions by the integration of the signals. The unambiguous assignment of the ^{1}H-NMR spectra from fully sulfated blocks of KS will facilitate the quantitative and qualitative analysis of human urinary KS. On the other hand, we could not observe signals corresponding to any peptide or protein bound to KS. The results obtained by both ^{1}H-NMR spectroscopy may suggest that the human urinary KS is excreted as a GAG derived from proteo-keratan sulfate by the action of some endoglycosidases.

Determination of Normal Human Urinary KS by HPLC The amount of urinary KS was estimated by the HPLC method (purified KS from human urine was used as a standard, Table 3). Crude GAG from healthy volun-

Table 1. Compositional Analysis of Human Urinary and Bovine Corneal KS

Compound	Molar ratio					
	GlcNAc	Gal	GalINAc	Man	NeuAc	Sulfate
Urinary KS	8.3	7.3	1.0[a]	N.D.	1.0	13.2
Corneal KS	37.5	33.0	N.D.	3.0[b]	0.9	48.0

a) Amount of GalNac is presumed to be 1.0 mol per 1 mol of human urinary KS. *b*) Amount of Man is presumed to be 3.0 mol per 1 mol of bovine corneal KS. N.D., not detected.

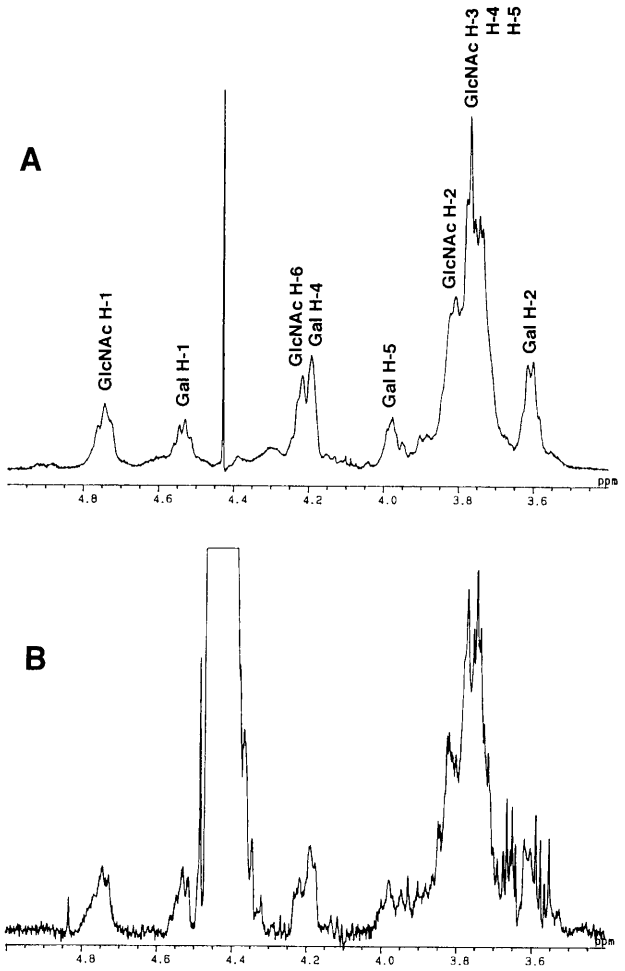

Fig. 4. 500 MHz ^{1}H-NMR Spectra of Human Urinary and Bovine Corneal KS

A, KS standard from bovine cornea. B, KS isolated from human urine.

teers was prepared from 9 ml of urine as shown in Experimental. The crude GAG was dried, then dissolved in 50 μl of water. Each 20 μl portion was digested with keratanase as described above. The levels of KS excreted to urine were constant (0.07 ± 0.015 μg/mg creatinine).

Discussion

In this report, characterization and determination of human urinary KS was described. We use UV absorption at around 230 nm attributed to unsaturated saccharides for determination of GAGs after enzymatic degradation with chondroitinases and heparin lyases. Unfortunately, KS is degraded to saturated oligosaccharides by digestion with keratanases, so we established HPLC method with post-column fluorometric detection for its sensitive analysis. Keratanase hydrolyzes β-galactosidic linkages in KS, in which non-sulfated galactosyl residues participate and then the major product from KS is disaccharide monosulfate.[23,24] The galactosidic linkage in which 6-*O*-sulfated galactose participates is resistant to the action of keratanase. Therefore, oligosaccharides such as tetra- and hexa-saccharides are usually produced by keratanase digestion. A TSKgel sugar AXI column successfully resolved the disaccharide monosulfate from

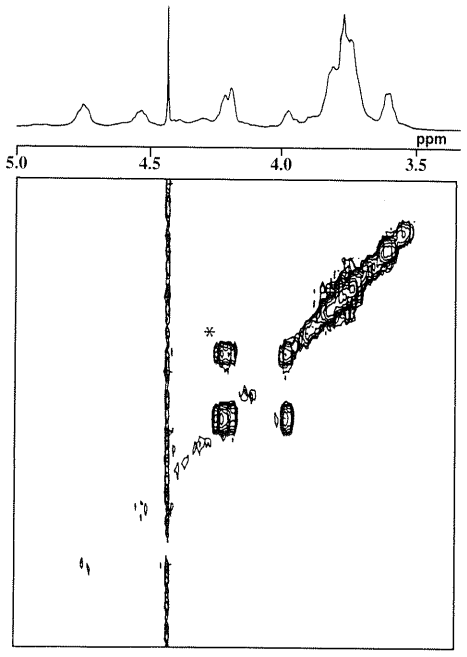

Fig. 5. 2D TQF-COSY Spectrum of Bovine Corneal KS

*, cross peak between H-5 and H-6 (×2) of GlcNAc6S.

Table 2. Chemical Shifts of KS from Human Urine and Bovine Cornea

Gal[a]	ppm	GlcNAc[a]	ppm
H-1	4.55[b]	H-1	4.75[b]
	4.44		4.74
H-2	3.62	H-2	3.82
	3.60	H-3	3.76
H-3	3.75	H-4	3.76
H-4	4.19	H-5	3.82
H-5	3.98	H-6a	4.25
H-6a	4.39	H-6b	4.21
H-6b	4.31		
		NAc-Me	2.04

a) Both residues were sulfated at C-6 positions. *b*) These signals were split by the anisotropic effects of sulfate ester.

Table 3. Analysis of Human Urinary KS

Case No.	Age (Years)	KS (μg/mg creatining)
1	24	0.056
2	25	0.055
3	23	0.075
4	24	0.060
5	23	0.089
6	36	0.084

contaminants and sensitive detection was performed by post-column derivatization. Using this rapid and sensitive method, we found the KS rich fractions during isolation of human urinary KS. Isolated KS from pooled urine was employed as a standard for determination of urinary KS. Consequently, it was possible to determine the normal human urinary KS accurately by measuring only the disaccharide monosulfate. Analyses of urinary KS from 9 ml of human urine by this method showed it to be excreted at a constant level.

Sensitive HPLC methods for determination of carbohydrate composition were used to identify the class of human urinary KS as a skeletal type (type II KS), with an *O*-glycosidic linkage between galactosamine and serine (or threonine) using μg order of KS sample. From results of the compositional analysis, molecular weight of human urinary KS was presumed to be about 5 kDa and the KS was slightly more oversulfated than corneal KS (type I KS). NMR studies further showed that urinary KS is not a proteoglycan but a polysaccharide.

There is keen general interest in KS as markers for osteoarthritis, but so far no studies have addressed the structures of KS in urine during the development of disease. A more detailed understanding of these structures is needed in the cases of lysosomal disease such as Morquio syndrome.

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