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Pretreatment Procedure for the Microdetermination of Chondroitin Sulfate in Plasma and Urine

Shinobu Sakai,* Jun-ichi Onose,* Haruka Nakamura,* Hidenao Toyoda,* Toshihiko Toida,*[,1] Toshio Imanari,* and Robert J. Linhardt†

**Graduate School of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263-8522, Japan; and †Department of Chemistry, Division of Medicinal and Natural Products Chemistry, Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, Iowa 52422*

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A new, simple, and rapid pretreatment method for the determination of chondroitin sulfate, dermatan sulfate, and hyaluronan from urine and blood plasma samples has been developed. Plasma proteins were first converted into small peptides by digestion using a nonspecific protease, actinase E, and the resulting small peptides were removed by centrifugal filtration. The retained, residual crude glycosaminoglycans, including chondroitin/dermatan sulfates and hyaluronan, were converted into unsaturated disaccharides through the action of chondroitin sulfate lyses. Next, these disaccharides were recovered and purified using centrifugal filtration together with ΔDi-UA2S, added as an internal standard. The filtered disaccharide mixture was analyzed by HPLC with fluorometric postcolumn derivatization using 2-cyanoacetamide as a fluorogenic reagent. This method was applied to a pharmacokinetic study of chondroitin sulfate administered intravenously to mice. The half-life of the administered chondroitin sulfates, having molecular masses from 6 to 50 kDa, varied depending on their molecular sizes. This new method should be useful for studies on the metabolic fate of exogenously administered glycosaminoglycans in small experimental animals. © 2002 Elsevier Science (USA)

Key Words: **glycosaminoglycans; microdetermination; pretreatment; urine; blood plasma.**

Glycosaminoglycans (GAGs)[2] are alternating copolymers of uronates and amino sugars. They are heterogeneous acidic polysaccharides in terms of their relative molecular mass, charge density, and physicochemical properties (1–4). The primary structures of GAGs contain different uronate residues, different hexosamine residues, and varying amounts of *O*-linked sulfo groups substituted at various positions in these residues (4). GAGs have been found in biological fluids such as blood plasma and urine (5, 6). In blood plasma, GAGs interact with biologically important proteins, including protease inhibitors, coagulation factors, lipoproteins, and complement proteins (7); cells such as lymphocytes (8), monocytes (9), and platelets (10); and the vascular endothelium (11). The presence of GAGs in human plasma has been demonstrated in several reports (12, 13); however, the structures and physicochemical properties of these GAGs have not been fully identified and characterized due to their low blood concentration and a general lack of adequate analytical methodology. Problems with recovery from biological samples are particularly evident for structurally complex GAGs, such as heparin and heparan sulfate, which have strong affinity for plasma components.

Chondroitin sulfate (CS) is a homopolymeric GAG, containing only one type of repeating disaccharide unit (→ 4GlcAβ1 → 3GalNAcβ1 →), which can be substituted with *O*-sulfo groups at various positions. Endog-

[1] To whom correspondence should be addressed. Fax: +81-43-290-2895. E-mail: toida@p.chiba-u.ac.jp.

[2] Abbreviations used: GAG, glycosaminoglycan; CS, chondroitin sulfate; 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; ΔDi-UA2S, 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose; MWavg, average molecular weight.

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enous CS, found in blood plasma, is in the form of an undersulfated CS, bound to Kuniz-type protease inhibitors. Recently, exogenous CS chains administered through oral and intravenous routes have demonstrated anti-inflammatory and chondroprotective properties (14, 15). However, the biological activities of these CS chains as medicinal agents are still unclear due to the lack of a reliable methodology for analyzing GAG chains in biological samples.

The pretreatment procedure for isolation of GAGs found in biological samples, such as blood plasma and urine, is a key requirement in the development of a rapid, accurate, and sensitive method for the determination of GAGs. Precipitation methods, used following GAG chain release from protein core by β-elimination, rely on the addition of ethanol/methanol and/or cetylpyridinium chloride. Such methods have been employed for many years to isolate GAG chains from proteoglycans in biological samples such as blood plasma and urine. Cetylpyridinium cation acts as a counterion for the GAG sulfo groups, resulting in the formation of a water-insoluble complex. While the use of cetylpyridinium has been indispensable for the analysis of GAGs in biological samples, proper sample pretreatment prior to precipitation requires considerable time, care, and technical skill, due to the complexity of the manipulations involved (16).

Recently, microfiltration devices using defined molecular cut-off membranes have become commercially available. This paper describes a simple and rapid pretreatment procedure for determination of GAG chains found in blood plasma and urine samples. A pharmacokinetic study of chondroitin sulfate in mice is used to demonstrate this newly established procedure.

MATERIALS AND METHODS

Materials

Standard unsaturated disaccharides, including 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*-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-UA2S), and chondroitin ABC (EC 4.2.2.4) and ACII *Arthro* (EC 4.2.2.5) lyases were obtained from Seikagaku Kogyo (Tokyo, Japan). CS samples (average molecular weight (MWavg) 15,000 from bovine tracheal cartilage; 6000, 16,000, and 50,000 from shark/whale cartilage) were kind gifts from Shin-Nippon Yakugyo Co. (Japan) and Seikagaku Kogyo Co., respectively. Bovine blood plasma was obtained from Boehringer Manheim. Actinase E was purchased from Kaken Pharmaceutical Co. (Tokyo, Japan). Centrifugal filters (BIOMAX-5) having a molecular weight cut-off of 5000 were purchased from Millipore Japan (Tokyo). All other chemicals were of analytical grade.

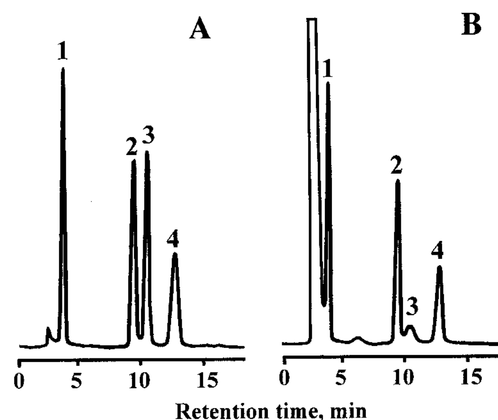

FIG. 1. Chromatograms of the unsaturated disaccharides prepared from bovine plasma sample on the postcolumn HPLC. (A) Standard unsaturated disaccharides. (B) Unsaturated disaccharides produced from bovine plasma. Peaks: 1, ΔDi-0S; 2, ΔDi-4S; 3, ΔDi-6S; 4, ΔDi-UA2S (an internal standard).

HPLC Analysis

The postcolumn HPLC system was constructed with a PU-980 intelligent HPLC pump (Jasco, Tokyo, Japan), a double-plunger pump for the fluorogenic reagent solution (SPU-2.5W; Shimamura Instrument, Tokyo, Japan), a sample injector with a 20-μl loop (Model 7725i; Reodyne, CA), a fluorescence spectrophotometer (FP-1520S; Jasco), a column thermocontroller (Mini-80; Taitech, Tokyo, Japan), a chromatointegrator (D-2500; Hitachi), and a dry reaction bath (DB-3; Shimamura Instrument). A DOCOSIL column (150 mm × 4.6-mm i.d.; Senshu Kagaku, Tokyo, Japan; 60°C) was used at a flow rate of 1.5 ml/min. Unsaturated disaccharides were eluted by the use of 0.8 mM tetrabutylammonium hydrogen sulfate and 2 mM sodium chloride in 8% acetonitrile as the eluting agent. To the effluent, aqueous 1% 2-cyanoacetamide solution and 1.0 M sodium hydroxide at the same flow rate of 0.25 ml/min were added by using a double-plunger pump. The mixture passed through a reaction coil (10 m × 0.5-mm i.d.) set in a dry reaction bath thermostated at 120°C and then through a cooling coil (3 m × 0.25 mm i.d.). The effluent was monitored fluorometrically (ex. 346 nm, em. 410 nm). A 10-μl portion of sample solution was loaded onto the HPLC. A typical chromatogram of the unsulfated and monosulfated standard disaccharides is shown in Fig. 1A.

TABLE 1

Comparison of Recovery Efficiency of Spiked Chondroitin Sulfate from Blood Plasma between the Present and the Previous[a] Methods

Sample	Disaccharide	Spiked CS (μg/ml)	Present method: Found (μg/ml)[b]	Present method: Recovery (%)	Previous method: Found (μg/ml)[b]	Previous method: Recovery (%)
Plasma		0	10.5 ± 0.4	—	9.67 ± 0.32	—
	ΔDi-0S		6.70 ± 0.58	—	5.69 ± 0.45	—
	ΔDi-4S		3.53 ± 0.23	—	3.72 ± 0.33	—
	ΔDi-6S		0.24 ± 0.02	—	0.26 ± 0.02	—
Plasma		2.0	12.4 ± 0.1[c]	95.0	11.5 ± 0.2[c]	77.0
	ΔDi-0S	0.12 ± 0.01	6.81 ± 0.72	91.7	5.73 ± 0.52	33.3
	ΔDi-6S	0.65 ± 0.03	0.85 ± 0.07	93.8	0.82 ± 0.03	86.0
Plasma		20.0	30.0 ± 0.4[c]	97.5	25.4 ± 0.3[c]	77.2
	ΔDi-0S	1.22 ± 0.00	7.87 ± 0.65	95.9	6.59 ± 0.26	73.8
	ΔDi-4S	11.8 ± 0.2	15.2 ± 0.54	98.9	12.6 ± 0.32	75.4
	ΔDi-6S	6.56 ± 0.10	6.75 ± 0.25	99.2	5.87 ± 0.24	85.5

[a] Ref. (16).
[b] $n = 5$. Mean ± SD.
[c] Because the spiked chondroitin sulfate chains contain not only -0S, -4S, and -6S disaccharide units but also disulfated disaccharide units, these data were corrected as total amounts of disaccharide units.

Procedure for Purification of Chondroitin Sulfate from Blood Plasma and Urine

The precipitation approach used was according to the method reported by Emura and Mukuda (16). The current method, involving centrifugal filtration, improves the recovery of small GAG chains and those having a low level of sulfation. Such GAG chains do not form insoluble complexes with the cetylpyridinium cation required for precipitation (16). In the current method, 40 μl of 0.05 M Tris-acetate buffer (pH 8.0) and 20 μl of 0.05 M Tris-acetate buffer (pH 8.0) containing 1% actinase E were added to a 10-μl portion of sample in a sample tube, and plasma proteins were digested at 45°C for 3 h. Next, 200 μl of 15 mM acetic acid containing 10% NaCl was added to a sample solution. The mixture was heated in a boiling water bath for 5 min and cooled in an ice bath, and then the solution was centrifuged at 2300*g* for 15 min. A 180-μl portion of the supernatant was transferred to a centrifugal filter containing 20 μl of 0.1 M NaOH on the filter. After centrifugation at 2300*g* for 20 min, a crude, retained GAG fraction was washed twice with 0.2 M Tris–HCl buffer (pH 8.0), and the retained sample was then dissolved in 20 μl of 0.2 M Tris–HCl buffer (pH 8.0). The sample was digested with 10 μl of a mixture (0.1 U each) of chondroitin sulfate ABC and ACII lyases for 3 h at 37°C. After the digestion was complete, ΔDi-UA2S was added as an internal standard, the sample was again centrifuged at 2300*g* for 20 min, and the unsaturated disaccharides passing through the filter were recovered and analyzed by postcolumn derivatization HPLC (Fig. 1B). The protein digestion by actinase E can be skipped for determination of urinary GAGs. The filter device is also useful to concentrate GAGs in urine samples prior to their analysis. In addition to CS, dermatan sulfate and hyaluronan can also be determined using this method.

TABLE 2

Recovery Efficiency of Spiked Chondroitin Sulfate from Urine

Sample	Disaccharides	Spiked CS (μg/ml)	Present method: Found (μg/ml)[a]	Present method: Recovery (%)
Urine		0	4.80 ± 0.16[b]	
	ΔDi-0S		1.61 ± 0.32	—
	ΔDi-4S		1.64 ± 0.08	—
	ΔDi-6S		1.41 ± 0.02	—
		2.0	6.67 ± 0.44[b]	93.7
Urine	ΔDi-0S	0.12 ± 0.01	1.72 ± 0.25	91.7
	ΔDi-4S	1.19 ± 0.04	2.73 ± 0.23	91.6
	ΔDi-6S	0.65 ± 0.03	2.03 ± 0.19	95.4
		20.0	23.7 ± 0.6[b]	94.0
Urine	ΔDi-0S	1.22 ± 0.00	2.79 ± 0.18	96.8
	ΔDi-4S	11.8 ± 0.2	12.2 ± 0.7	89.2
	ΔDi-6S	6.56 ± 0.10	7.93 ± 0.47	99.4

[a] $n = 3$. Mean ± SD.
[b] Both urinary and the spiked chondroitin sulfate chains contain not only Di-0S, -4S, and -6S disaccharide units but also di- and trisulfated disaccharide units; these data were corrected as total amounts of disaccharide units.

TABLE 3

Effect of Sampling Volume for Determination of Plasma Chondroitin Sulfate

Volume (μl)	Concentration (μg/ml)[a]				Relative value[b] (%)
	ΔDi-0S	ΔDi-4S	ΔDi-6S	Total	
5	5.52 ± 0.32	3.70 ± 0.24	0.23 ± 0.04	9.45 ± 0.52	93.6
10	5.76 ± 0.36	4.13 ± 0.24	0.23 ± 0.04	10.2 ± 0.6	101
25	6.15 ± 0.24	3.72 ± 0.11	0.18 ± 0.04	10.1 ± 0.4	100
50	6.18 ± 0.33	3.75 ± 0.21	0.18 ± 0.03	10.2 ± 0.4	101

[a] $n = 3$. Mean ± SD.
[b] Total value obtained from 25 μl blood plasma was designated as 100%.

Pharmacokinetic Study

Male ddY mice (25–35 g body wt) were used for the pharmacokinetic study of intravenously administered CS. Each CS sample dissolved in saline solution (10 mg/ml) was injected through the tail vein at a dose of 1.0 mg/kg body weight. Blood samples were collected from the eye socket vein at 0 to 240 min after the administration. Blood samples (about 30 μl) were collected in 0.2% disodium EDTA at the following times: 0 (before injection), 5, 10, 20, 30, 45, 60, 120, and 240 min after intravenous administration. Each collected sample was immediately centrifuged at 1500*g* for 10 min to obtain plasma, which was transferred to a polypropylene plastic vial and stored in a deep freezer until used.

RESULTS AND DISCUSSION

There are several reports of pretreatment procedures for determination of GAGs found in biological samples, such as blood plasma and urine (16–19). Because the distribution of molecular weights of GAGs found in urine samples varies substantially, the strategies for the recovery of GAGs from urine have focused on the efficient formation of insoluble complexes between GAGs and cationic substances such as cetylpyridinium cation. In blood plasma, however, the major GAG is an undersulfated CS, covalently bound to an inter-α-trypsin inhibitor (bikunin), with a GAG chain molecular mass that appears to be regulated at around 6 kDa (19). Precipitation by complex formation between this small, low-sulfated CS chain and the cetylpyridinium cation is inefficient. Thus, there appears to be ample room for improvement of the method for the recovery of undersulfated CS from plasma.

The various steps involved in the recovery of CS from plasma and its conversion to unsaturated disaccharides for quantification by HPLC have been improved in this study to afford a rapid analysis with excellent yields. Table 1 shows the comparison of the recovery of the added CS (MWavg 15,000; 2.0 and 20.0 μg/ml) from blood plasma, obtained using the current method involving a filter membrane, with the previous method (16) using ethanol/cetylpyridinium precipitation. These results clearly show that the current method is superior to the previous method in both the efficiency of CS recovery from plasma samples and the reproducibility of the procedure.

The previous procedure, established by Emura and Mukuda (16), requires a high level of skill to accurately analyze plasma CS, as well as a relatively large amount (100 μl) of plasma sample. Thus, the previous method is difficult to apply to pharmacokinetic studies of exogenous and/or endogenous GAGs such as CS. In contrast, our new method requires significantly smaller amounts (<10 μl) of plasma sample, more reasonable amounts when undertaking pharmacokinetic studies of CS in small mammals.

Our method was also applied to the determination of CS in human urine. Because of the low protein content of urine, the protease treatment can be eliminated. Table 2 shows that our method might be useful for the determination of urinary CS. The reproducibility coef-

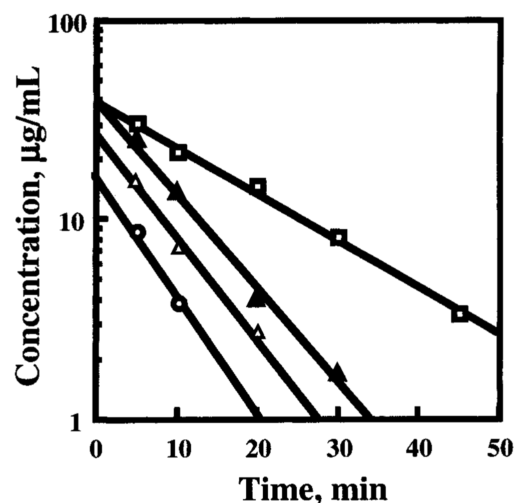

FIG. 2. Kinetics of intravenously administered chondroitin sulfates. Symbols: □, MWavg 50,000; ▲, 16,000; △, 15,000; ○, 6000.

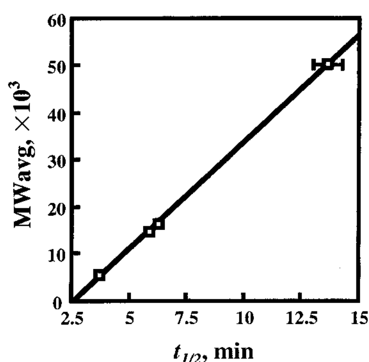

FIG. 3. Relationship between the half-life ($t_{\frac{1}{2}}$) and the molecular weight of intravenously administered chondroitin sulfates. The MWavg of chondroitin sulfate samples were 6000, 15,000, 16,000, and 50,000.

ficient of variation of the procedure and the recovery of exogenous CS (MWavg 15,000; 2.0 and 20.0 μg/ml) added to a 20-μl sample of urine were <5% ($n = 3$) and over 90%, respectively (Table 2). The current method was also superior to another method described previously (18) on the basis of both reproducibility and recovery of spiked CS standard (data not shown).

Next, the required sample volume of blood plasma was investigated. While the detection limit of analysis is controlled primarily by the sensitivity of the HPLC method using postcolumn derivatization, the analyst must also be concerned with the required plasma sample volume. Table 3 shows the results of the analysis of endogenous CS in bovine plasma using several different volumes of plasma sample. The result obtained from 5 μl plasma was almost identical to that obtained using 50 μl plasma (see Table 3). Thus, it seems that this pretreatment procedure is suitable for pharmacokinetic studies in small experimental animals being intravenously administered CS. Furthermore, the recovery of added CS (MWavg 15,000; 2.0 and 20.0 μg/ml) from plasma samples shows the current method to be superior to the previous method relying on ethanol/cetylpyridinium precipitation steps (Table 1).

The pharmacokinetics of intravenously administered CS samples with MWavg ranging from 6000 to 50,000 was determined using our new method (Fig. 2). The half-life ($t_{\frac{1}{2}}$) of the administered CS in the bloodstream shows a MW dependency. Low-molecular-weight CS shows shorter $t_{\frac{1}{2}}$ and the relationship between the $t_{\frac{1}{2}}$ and the MW is linear as depicted in Fig. 3. This is the first evidence that the molecular weight affects the half-life of CS molecule in blood plasma. Presumably, high-MW CS chains bind to plasma proteins more tightly than low-MW CS chains. It is well known that intravenously administered heparin (MWavg 20,000) can bind to plasma proteins, including antithrombin III and heparin cofactor II, prolonging its $t_{\frac{1}{2}}$ to 30–40 min. A study on specific and/or nonspecific binding of CS to proteins in blood plasma will be published elsewhere in the near future.

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