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Sensitive high-performance liquid chromatographic method with fluorometric detection for the determination of heparin and heparan sulfate in biological samples: application to human urinary heparan sulfate

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Abstract

A sensitive high-performance liquid chromatographic method for the determination of unsaturated disaccharides produced from heparin and heparan sulfate is described. Heparan sulfate was depolymerized using a combination of heparin lyase I (EC 4.2.2.7), heparin lyase II and heparin lyase III (EC 4.2.2.8). Seven unsaturated disaccharides were separated under isocratic conditions within 25 min using acetonitrile–H_2O–0.2 *M* sodium phosphate buffer (pH 7.0)–3.0 *M* ammonium chloride (32:10:1:1) and were monitored by fluorescence detection using 2-cyanoacetamide as a post-column derivatizing reagent. As little as 2 pmol of a disaccharide could be detected with excitation at 346 nm and emission at 410 nm. This method was applied to the analysis of normal human urine. It was revealed that the concentration of normal human urinary heparan sulfate is 1.53±0.36 mg/mg creatinine (n=4). © 1997 Elsevier Science B.V.

Keywords: Glycosaminoglycans; Heparin; Heparan sulfate

1. Introduction

It is very important to establish a sensitive and accurate analytical method to clarify the role of heparin and heparan sulfate within bodies. Heparin and heparan sulfate have considerable heterogeneity in molecular size, disaccharide composition and sulfate content. Therefore, analysis of the unsaturated disaccharides derived enzymatically provides the most specific and perhaps the only quantitative results [1]. By degradation with bacterial heparin lyases, mainly six unsaturated disaccharides are produced from heparan sulfate, namely, ΔUA-GlcNAc, ΔUA-GlcNAc6S, ΔUA-GlcNS, ΔUA2S-GlcNS, ΔUA-GlcNS6S and ΔUA2S-GlcNS6S [2], whose proportions vary for each heparan sulfate according to the tissue and species of origin. Absorption at around 230 nm attributed to $\Delta_{4,5}$-hexuronic acid is effectively available for determination of unsaturated disaccharides by using high-performance liquid chromatography (HPLC) [3–6]. However, the detection system is often not sufficiently sensitive and sometimes requires time-consuming and tedious preparation for the micro-determination of biological samples. To improve detection limits and selectivity, we have proposed pre- [7] and post-column [8–10],

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and chemiluminescence [11] detection techniques. These detection systems allow detectable amounts to be extended to pg level for the injected sample. A fluorometric post-column method using 2-cyanoacetamide [12] was especially well suited for the determination of unsaturated disaccharides [8]. This sensitive and selective detection facilitated the determination of heparan sulfate in various biological samples.

In this study, we present a new HPLC method using fluorometric post-column derivatization that affords improvement in separation, selectivity and sensitivity for the determination of unsaturated disaccharides from heparan sulfate. We have applied this method to the analysis of human urinary heparan sulfate.

2. Experimental

2.1. Reagents and materials

Standard unsaturated disaccharides from heparan sulfate, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-threo-hex-enepyranosyluronic acid)-D-glucose (ΔUA-GlcNAc), 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-threo-hex-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔUA-GlcNAc6S), 2-acetamido-2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-threo - hex - enepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔUA2S-GlcNAc6S), 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-threo-hex-enepyranosyluronic acid)-D-glucose (ΔUA-GlcNS), 2-deoxy-2-sulfamino4-*O*-(4-deoxy-2-*O*-sulfo-α-L-threo-hex-enepyranosyluronic acid)-D-glucose (ΔUA2S-GlcNS), 2-deoxy-2-sulfamino-4-*O*-(4-deoxy - 2 - *O* -sulfo - α - L - threo-hex-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔUA2S-GlcNS6S), heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparitinase II) and heparin lyase III (heparitinase I, EC 4.2.2.8) were purchased from Sigma Chemical (USA). Bovine kidney heparan sulfate and chondroitinase ABC (EC 4.2.2.4) was obtained from Seikagaku Kogyo (Tokyo, Japan). TSKgel Amide-80 (particle size 5 μm) was obtained from TOSOH (Tokyo, Japan). All other chemicals used were of analytical reagent grade.

2.2. Apparatus and chromatographic conditions for heparan sulfate analysis

The chromatographic equipment included a high-pressure pump (L-6000, Hitach, Tokyo, Japan), a double plunger pump for the reagent solution (SPU-2.5W, Shimamura instrument, Tokyo, Japan), a sample injector with 20 μl loop (Model 7125, Reodyne, CA, USA), a fluorescence spectrophotometer (F-1050, Hitach, Tokyo, Japan), a column thermocontroller (Mini-80, Taitec, Tokyo, Japan), a chromato-integrator (D-2500, Hitach Seisakusho, Tokyo, Japan) and a dry reaction bath (DB-3, Shimamura Instrument, Tokyo, Japan).

Established HPLC conditions were as follows: a TSKgel Amide-80 column (2.0 mm I.D.×150 mm) was eluted with acetonitrile–H_2O–0.2 *M* sodium phosphate buffer (pH 7.0)–3.0 *M* ammonium chloride (32:10:1:1) at a flow-rate of 0.4 ml/min (column temperature, 60°C). To the effluent were added aqueous 1% 2-cyanoacetamide solution and 0.5 *M* sodium hydroxide at the same flow-rate of 0.25 ml/min by using a double plunger pump. The mixture passed through a reaction coil (0.5 mm I.D.×10 m) set 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 (Ex. 346 nm, Em. 410 nm). A 2 μl portion of sample solution was loaded onto the HPLC.

2.3. Preparation of human urinary heparan sulfate

A 9 ml portion of human urine sample and 600 μl of 5% hexadecylpyridinium chloride were mixed and the mixture was kept at 0°C for 4 h. After centrifugation at 2300 g for 15 min at room temperature, the precipitate was washed twice with 1.5 ml of 0.1% hexadecylpyridinium chloride. The precipitate was redissolved in 1 ml of 2.5 *M* sodium chloride. Then, insoluble materials were removed by centrifugation at 2300 g for 15 min at room temperature. To the supernatant, 11 ml of aqueous 85% (v/v) ethanol was added and glycosaminoglycans were precipitated by centrifugation at 2300 g for 15 min at 4°C after keeping the solution overnight at 0°C. The resulting precipitate was dried under reduced pressure, then redissolved in 50 μl of 0.2 *M* Tris-HCl buffer (pH

8.0). To the solution, 10 μl of an aqueous solution containing 0.1 U of chondroitinase ABC (one unit was defined as the quantity of the enzyme that catalyzes the formation of 1 μmol of unsaturated disaccharides per minute at 37°C, pH 8.0.) were added and incubated at 37°C for 3 h. Then 250 μl of ethanol was added. Heparan sulfate was precipitated by centrifugation at 2300 g for 15 min at 4°C after keeping the solution overnight at 4°C. The supernatant was used for the determination of chondroitin sulfate as described later and the precipitate was washed three times with 1 ml of aqueous 75% (v/v) ethanol at room temperature for removal of unsaturated disaccharides produced from urinary chondroitin sulfate, then the precipitate was dried under reduced pressure and redissolved in 50 μl of 0.1 *M* acetate buffer containing 10 m*M* calcium acetate (pH 7.0).

2.4. Determination of human urinary chondroitin sulfate and creatinine

Chondroitinase ABC digestion of urinary glycosaminoglycan was carried out during preparation for urinary heparan sulfate, as described above. The resulting supernatant containing unsaturated disaccharides from chondroitin sulfate was analyzed by HPLC as reported in our previous paper [9]. Briefly, the unsaturated disaccharides were separated by an amino-silica column and detected by fluorometric post-column reaction using 2-cyanoacetamide. Urinary creatinine was determined by a cation exchange HPLC method [13].

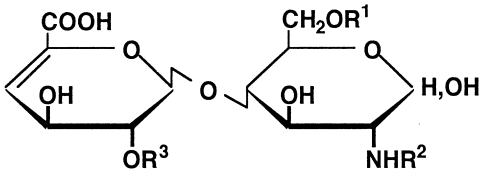
2.5. Enzymatic digestion of heparan sulfate

A 10 μl portion of sample solution containing up to 4 μg of heparan sulfate, 10 μl of 0.1 *M* acetate buffer containing 10 m*M* calcium acetate (pH 7.0) and 30 μl of an aqueous solution containing heparin lyases (4 mU of heparin lyase I, 0.4 mU of heparin lyase II and 0.4 mU of heparin lyase III, one unit was defined as the quantity of the enzyme that catalyzes the formation of 1 μmol of unsaturated disaccharides per min at 37°C, pH 7.5.) were mixed and incubated at 37°C for 12 h. A 2 μl volume of the reaction mixture was subjected to HPLC without further purification.

3. Results and discussion

3.1. Separation and detection of unsaturated disaccharides from heparan sulfate

The standard unsaturated disaccharides from heparin and heparan sulfate (Fig. 1) have been separated with various HPLC modes such as normal-phase chromatography, reversed-phase ion-pairing chromatography, anion-exchange chromatography and others [1]. Amino-bonded silica has been one of the most commonly used packings for the separation of oligosaccharides derived from heparin and heparan sulfate. However, this support is chemically unstable and its lifetime is relatively short [14]. Furthermore, elution conditions of non-, mono-, di- and tri-sulfated disaccharides are extremely different from each other. Thus, a salt gradient elution is usually employed for simultaneous determination of these disaccharides. There is a similarity between amino- and amido-bonded silica column in the



		R^1	R^2	R^3
1	ΔUA-GlcNAc	H	Ac	H
2	ΔUA-GlcNAc6S	SO_3^-	Ac	H
3	ΔUA2S-GlcNAc6S	SO_3^-	Ac	SO_3^-
4	ΔUA-GlcNS	H	SO_3^-	H
5	ΔUA2S-GlcNS	H	SO_3^-	SO_3^-
6	ΔUA-GlcNS6S	SO_3^-	SO_3^-	H
7	ΔUA2S-GlcNS6S	SO_3^-	SO_3^-	SO_3^-

Fig. 1. Structure of unsaturated disaccharides from heparin and heparan sulfate.

elution profile of unsaturated disaccharides from glycosaminoglycans. Amido-bonded silica column has the advantage of high stability compared with amino-bonded silica column, and has properties that permit the resolution of these sulfated disaccharides under isocratic conditions. Various conditions were examined to successfully determine the appropriate isocratic conditions to resolve the seven unsaturated disaccharides from heparin and heparan sulfate (Fig. 2). Disaccharides containing *N*-sulfo residues show stronger adsorption to amido-bonded silica column than those containing *O*-sulfo residues, so that ΔUA2S-GlcNAc6S elutes before ΔUA-GlcNS even though it has two *O*-sulfo residues in its structure. Fluorometric post-column method using 2-cyanoacetamide was employed for specific and sensitive detection. Calibration graphs were linear in the range 2 pmol–5 nmol as an unsaturated disaccharide and the relative standard deviations at 50 pmol were less than 3% ($n=5$).

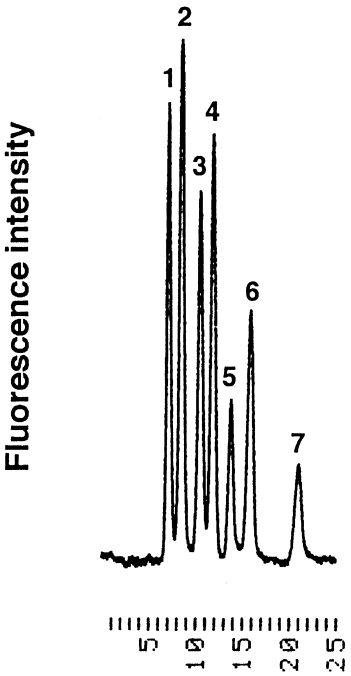


Fig. 2. Separation of unsaturated disaccharide standards from heparin and heparan sulfate. HPLC conditions: column, TSKgel Amide-80 (2.0 mm I.D.×150 mm); eluent, acetonitrile–H_2O–0.2 *M* sodium phosphate buffer (pH 7.0)–3.0 *M* ammonium chloride (32:10:1:1, v/v/v/v); column temperature, 60°C; flow-rate, 0.4 ml/min; reagent 1, 1% 2-cyanoacetamide (0.25 ml/min); reagent 2, 0.5 *M* sodium hydroxide (0.25 ml/min); reaction coil, 0.5 mm I.D.×10 m; reaction temperature, 110°C; cooling coil, 0.25 mm I.D.×3 m; excitation, 346 nm; emission, 410 nm. Sample size, 2 μl (20 ng of each disaccharide). Peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNAc6S; 3, ΔUA2S-GlcNAc6S; 4, ΔUA-GlcNS; 5, ΔUA2S-GlcNS; 6, ΔUA-GlcNS6S; 7, ΔUA2S-GlcNS6S.

3.2. Enzymatic digestion of heparan sulfate

Quantitative analyses for the unsaturated disaccharides from heparan sulfate or heparin required the exhaustive digestion of samples with a mixture of the heparin lyase I, II and III, though it must be noted that some oligosaccharide structures in heparin and heparan sulfate (for example 3-*O*-sulfated tetrasaccharides) are resistant to the digestion [15]. We reevaluated the proper amount of enzyme for the purpose of routine assay. Conventional HPLC methods with UV detection required not less than 10 mU of each enzyme. Even as low as 100 ng of heparin or heparan sulfate could be determined with sufficient signal-to-noise ratio by the HPLC method with fluorometric post-column detection, therefore, the level of the three enzymes were reduced to 0.4 mU for quantities up to 4 μg of standard heparan sulfate from bovine kidney (Fig. 3). However, additional heparin lyase I was necessary for digestion of heparin. This result indicated that it would be needed to use 4 mU of heparin lyase I for the digestion of

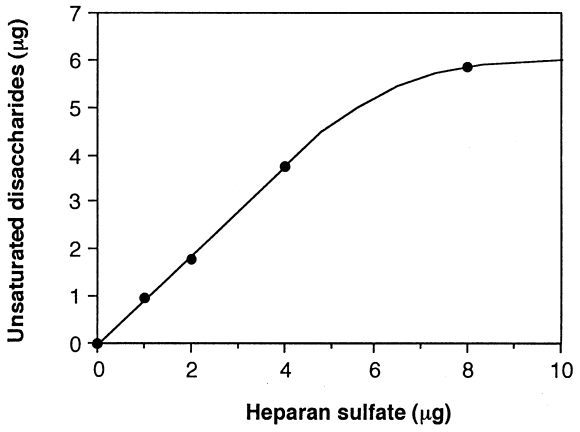


Fig. 3. Calibration graph for determination of heparan sulfate.

heparin or heparan sulfate in biological materials. Thus, we used a mixture containing 4 mU of heparinlyase I, 0.4 mU of heparin lyase II and 0.4 mU of heparin lyase III.

3.3. Analysis of human urinary heparan sulfate

The usual preparation methods for urinary heparan sulfate includes precipitation using quaternary ammonium salt such as hexadecylpyridinium chloride and followed ethanol precipitation. As a consequence, the fraction contains significant amounts of chondroitin sulfate besides heparan sulfate. Thus, we examined influence of chondroitin sulfate during enzymatic digestion with heparin lyases (Fig. 4). Fig. 4 shows that chondroitin sulfate slightly inhibits the digestion with heparin lyases, so the removal of chondroitin sulfate from the samples was accomplished by the use of anion exchange chromatography or chondroitinase digestion followed ethanol precipitation. The normal human urinary heparan sulfate was analyzed by the proposed HPLC method. Fig. 5 shows typical chromatogram of unsaturated disaccharides from normal human urinary heparan sulfate and Table 1 summarizes the compositions of disaccharide units. It was estimated that the quantity of urinary heparan sulfate corresponded to 10–20% amount of urinary chondroitin sulfate. The data were

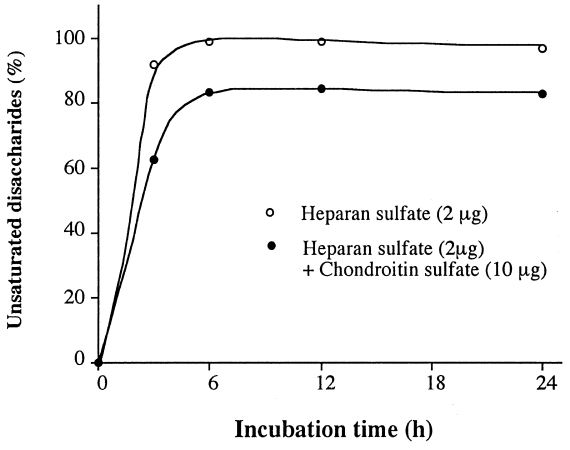


Fig. 4. Influence of chondroitin sulfate on heparin lyase digestion.

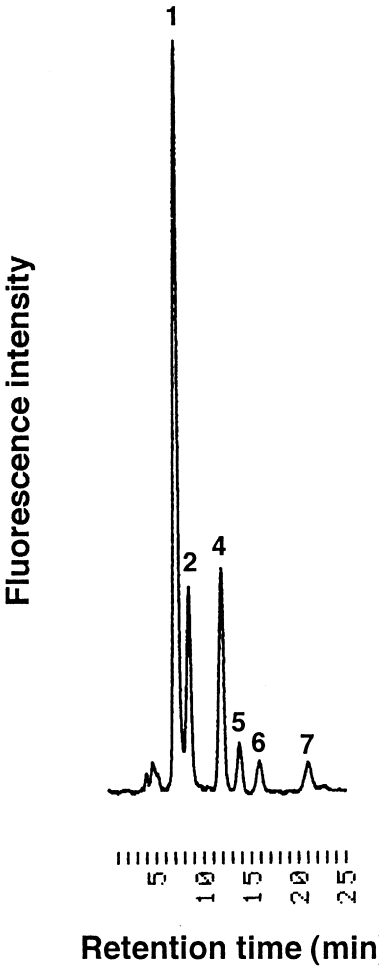


Fig. 5. Chromatogram of unsaturated disaccharides produced from human urinary heparan sulfate. Peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNAc6S; 3, ΔUA2S-GlcNAc6S; 4, ΔUA-GlcNS; 5, ΔUA2S-GlcNS; 6, ΔUA-GlcNS6S; 7, ΔUA2S-GlcNS6S.

in agreement with the reports of other investigators [16–18].

The advantages of our method are multifold. First, semi-micro columns packed with amido-bonded silica are chemically stable and provide rapid separation of unsaturated disaccharides from heparin and heparan sulfate under isocratic condition. Second, the post-column reaction using 2-cyanoacetamide allows highly sensitive and very selective detection of heparin and heparan sulfate in biological samples. This method will be applied to the measurement of

Table 1
Analysis of heparan sulfate in human urine

Case no.	Age	Sex	Unsaturated disaccharide proportion (%)						Total amount (μg/mg creatinine)
			ΔUA-GlcNAc	ΔUA-GlcNAc6S	ΔUA-GlcNS	ΔUA2S-GlcNS	ΔUA-GlcNS6S	ΔUA2S-GlcNS6S	
1	27	M	56.2	11.7	19.8	2.8	3.9	5.6	1.51
2	25	M	61.8	10.2	17.4	2.9	3.3	4.4	1.28
3	22	F	56.0	12.2	16.0	4.7	5.0	6.1	1.28
4	22	F	57.9	11.3	18.9	3.6	3.6	4.7	2.05

trace amounts of heparin and heparan sulfate in other biological fluids, such as blood plasma.

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