ELSEVIER

Journal of Chromatography A, 830 (1999) 197–201

JOURNAL OF CHROMATOGRAPHY A

Short communication

Rapid and sensitive analysis of disaccharide composition in heparin and heparan sulfate by reversed-phase ion-pair chromatography on a 2 μm porous silica gel column

Hidenao Toyoda, Hiromi Yamamoto, Nobuko Ogino, Toshihiko Toida, Toshio Imanari*

Faculty of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi, Inage-ku, Chiba 263-8522, Japan

Received 30 July 1998; received in revised form 5 October 1998; accepted 13 October 1998

Abstract

A rapid and sensitive method was developed for the analysis of disaccharide composition in heparin (HP) and heparan sulfate (HS) by reversed-phase ion-pair chromatography on a 2 μm porous silica gel column. HP and HS were digested with heparin lyase I, II and III in combination, and the produced unsaturated disaccharides were separated within 15 min. Calibration graphs were linear in the range 1 ng–1 μg with the fluorometoric post-column detection using 2-cyanoacetamide.

Keywords: Carbohydrates; Heparin; Heparan sulfate; Disaccharides

1. Introduction

Heparin (HP) and heparan sulfate (HS) are composed of different proportions of the same disaccharide building blocks. The both types of polysaccharides may either have or lack biological activity with reference to the sugar sequence. Thus, compositional analysis of the disaccharides (Fig. 1) derived enzymatically from HP and HS is effectively used as a definitive analytical technique. There are various HPLC methods such as normal-phase chromatography, anion-exchange chromatography and reversed-phase ion-pair chromatography (RP-IPC) for the separation of the disaccharides [1]. RP-IPC has a lot of potential for the analysis of oligosaccharides from HP and HS [2,3], since the columns for reversed-phase chromatography are improved constantly.

Alkylsilylated silica gel has been widely employed for separating biological samples. In general, the particle sizes utilized for high-performance liquid chromatography are around 3 and 5 μm. In the last decade, many researchers have emphasized the advantages of the use of small particles 1.5–3 μm in diameter [4–6]. These columns, based on non-porous materials, allow fast separation and good resolution for proteins and DNAs [7–9]. However, these columns show a low capacity for low-molecular-mass compounds because of the small surface area.

Recently, new reversed-phase chromatographic columns based on 2 μm porous silica gel have

*Corresponding author Tel.: +81-43-290-2894; fax: +81-43-290-3021.

0021-9673/99/$ – see front matter
PII: S0021-9673(98)00854-1

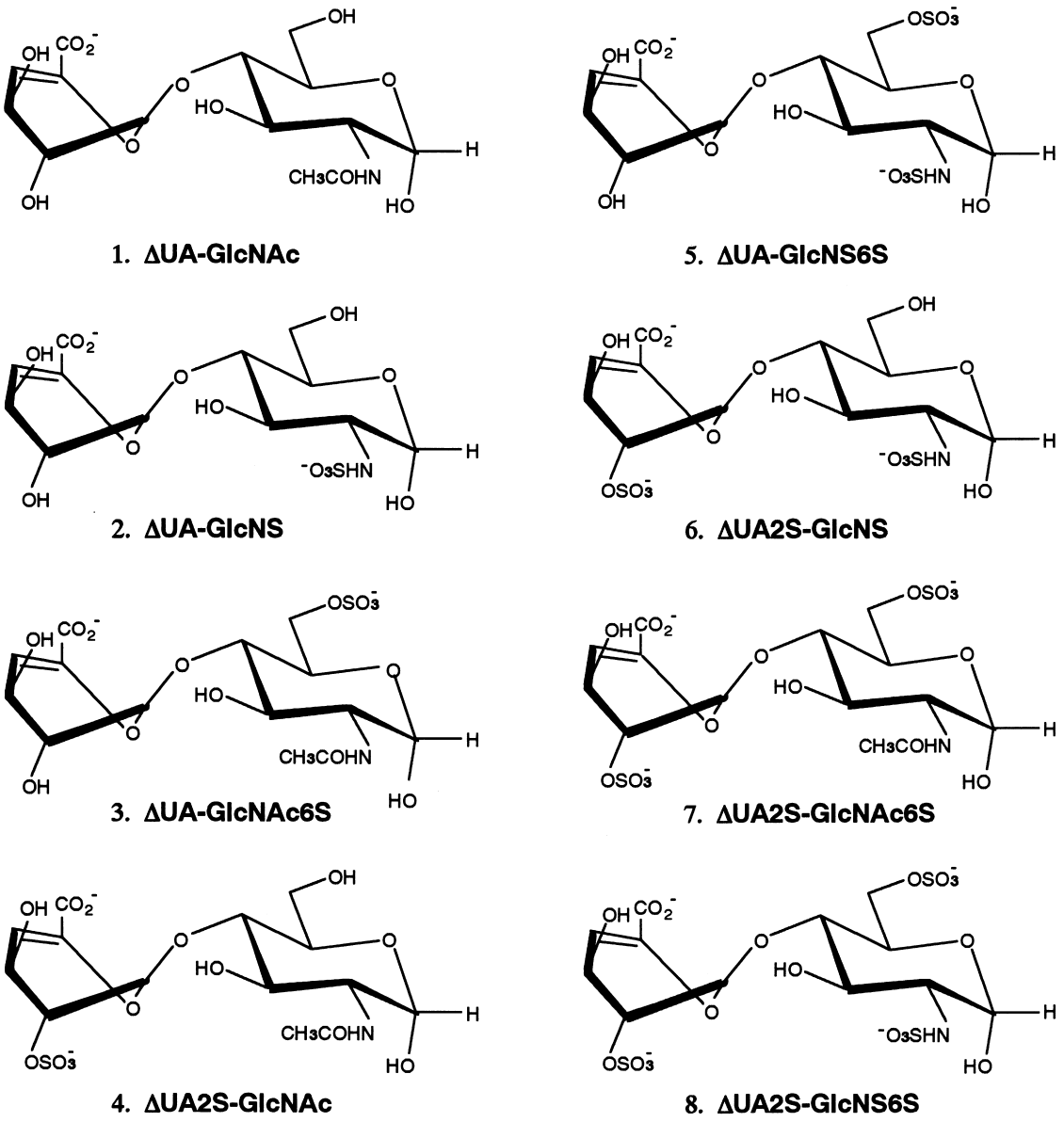

Fig. 1. Structure of unsaturated disaccharides from heparin and heparan sulfate.

become commercially available [10]. In this paper, we report a rapid and sensitive method for analysis of disaccharide composition in heparan sulfate and heparin by reversed-phase ion-pair chromatography using a 2 μm porous silica gel column.

2. Experimental

2.1. Materials and chemicals

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-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-*threo*-hex-enepyranosyluronic acid)-D-glucose (ΔUA-GlcNS), 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)-D-glucose (ΔUA2S-GlcNAc), 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-enepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔUA-GlcNS6S), 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-enepyranosyluronic acid)-D-glucose (ΔUA2S-GlcNS), 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-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), heparin lyase III (heparitinase I, EC 4.2.2.8), HS (from bovine kidney), HP (from bovine intestinal mucosa) and low-molecular-weight HP (LMHP, from porcine intestinal mucosa) were purchased from Sigma (USA). Tetrabutylammonium hydrogensulfate (TBA) was purchased from Wako (Osaka, Japan). TSKgel Super-ODS (100 mm×4.6 mm I.D., particle size 2 μm), TSKgel Super-Octyl (100 mm×4.6 mm I.D., particle size 2 μm) and TSKgel Super-Phenyl (100 mm×4.6 mm I.D., particle size 2 μm) were obtained from Tosoh (Tokyo, Japan). All other chemicals used were of analytical reagent grade.

2.2. HPLC apparatus

The chromatographic equipment included two L-6000 high-pressure pump controlled by L-5000 LC controller (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). A gradient was applied for 11.5 min at a flow-rate of 1 ml/min on a TSKgel Super-Octyl (100 mm×4.6 mm I.D., particle size 2 μm, 50°C). Gradient program was as follows: eluent A, 1.2 m*M* TBA in 4% acetonitrile; eluent B, 0.1 *M* cesium chloride in 4% acetonitrile; gradient, 0–3 min (1–15% B), 3–3.5 min (15–50% B), 3.5–8.5 min (50–75% B), 8.5–11.5 min (90% B) and then equilibrated with 1% B for 10 min. To the effluent were added aqueous 1% 2-cyanoacetamide solution and 0.25 *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 (10 m×0.5 mm I.D.) set in a dry reaction bath thermostated at 120°C and a following cooling coil (3 m×0.25 mm I.D.). The effluent was monitored fluorometrically (excitation 346 nm, emission 410 nm). A 5 μl portion of sample solution was loaded to the HPLC.

2.3. Enzymatic digestion of HP and HS

To a 10 μl portion of sample solution containing 1 μg of HP or HS was added 10 μl of 0.1 *M* acetate buffer (pH 7.0) containing 10 m*M* calcium acetate and 30 μl of an aqueous solution containing heparin lyase I (2 U), heparin lyase II (0.2 U) and heparin lyase III (0.2 U) and the mixture was incubated at 37°C for 12 h [11].

3. Results and discussion

We optimized the elution conditions on reversed-phase columns by using gradient elution with acetonitrile Fig. 2(A) and NaCl Fig. 2(B). The best resolution was obtained on a TSKgel-Octyl column with NaCl gradient. The separation of ΔUA-GlcNAc6S and ΔUA2S-GlcNAc was incomplete by 6% or higher acetonitrile concentration and ΔUA2S-GlcNS6S was adsorbed on the column by 2% or lower, on the NaCl gradient. The k' values for the unsaturated disaccharides were also analyzed as a function of concentration of TBA (0.1 m*M*–10 m*M*). We found that 1.2 m*M* or further were the optimal condition for the separation of monosulfated disaccharides, and resolution of disulfated disaccharides became worse at concentrations exceeding 1.4

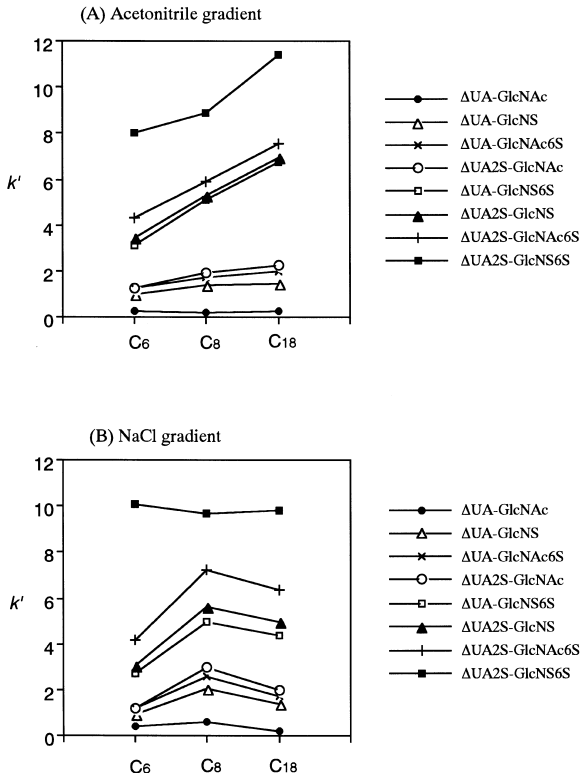

Fig. 2. Capacity ratios of the unsaturated disaccharides as functions of gradient elution with acetonitrile (A) and NaCl (B). C_6, TSKgel Super-Phenyl; C_8, TSKgel Super-Octyl; C_{18}, TSKgel Super-ODS.

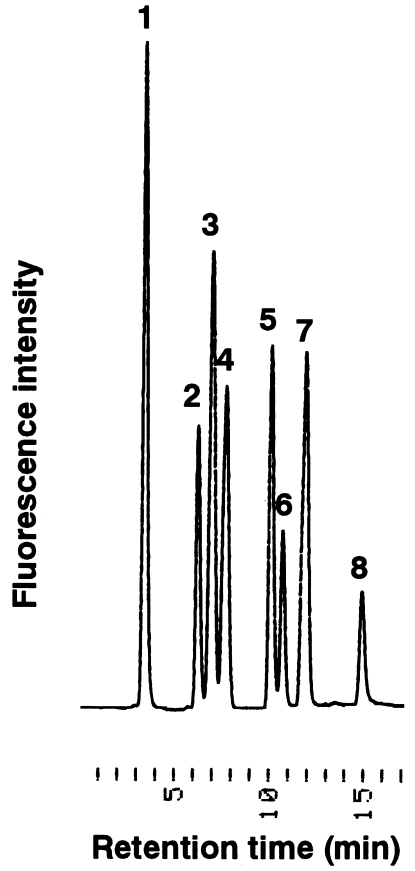

Fig. 3. Typical chromatogram of unsaturated disaccharides. HPLC conditions as in Experimental. Peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNS; 3, ΔUA-GlcNAc6S; 4, ΔUA2S-GlcNAc; 5, ΔUA-GlcNS6S; 6, ΔUA2S-GlcNS; 7, ΔUA2S-GlcNAc6S; 8, ΔUA2S-GlcNS6S. Sample size, 10 μl (50 ng of each sugar).

m*M*. Thus it became apparent that using the TSKgel-Octyl column, the optimal elution condition was 1.2 m*M* TBA in 4% acetonitrile with NaCl gradient. Unsaturated disaccharides eluted from the column were detected fluorometrically with 2-cyanoacetamide [11,12] as a post-column reagent. Without significant change in k', baseline became more stable by using CsCl instead of NaCl under the gradient elution. Though analytical times were over 30 min in previous papers [2,3], the separation of the disaccharides was improved to be less than 15 min (Fig. 3). Calibration graphs for the unsaturated disaccharides were linear in the range 1 ng–1 μg and the relative standard deviations at 5 ng were less than 3% (n=5). HP and HS were digested enzymatically with heparin lyases as described in the Experimental section. The analyses gave well separated peaks, as seen in Fig. 4.

In conclusion, this paper has demonstrated an high-resolution and rapid separation of mixtures of disaccharides from HP and HS using RP-IPC on a octylsilyl silica gel with particle size of 2 μm. Furthermore, the post-column fluorometric detection with 2-cyanoacetamide allows highly sensitive and very selective detection of the disaccharides. The RP-IPC method might provide a way to reduce analytical time even as compared with the capillary electrophoresis [13–15].

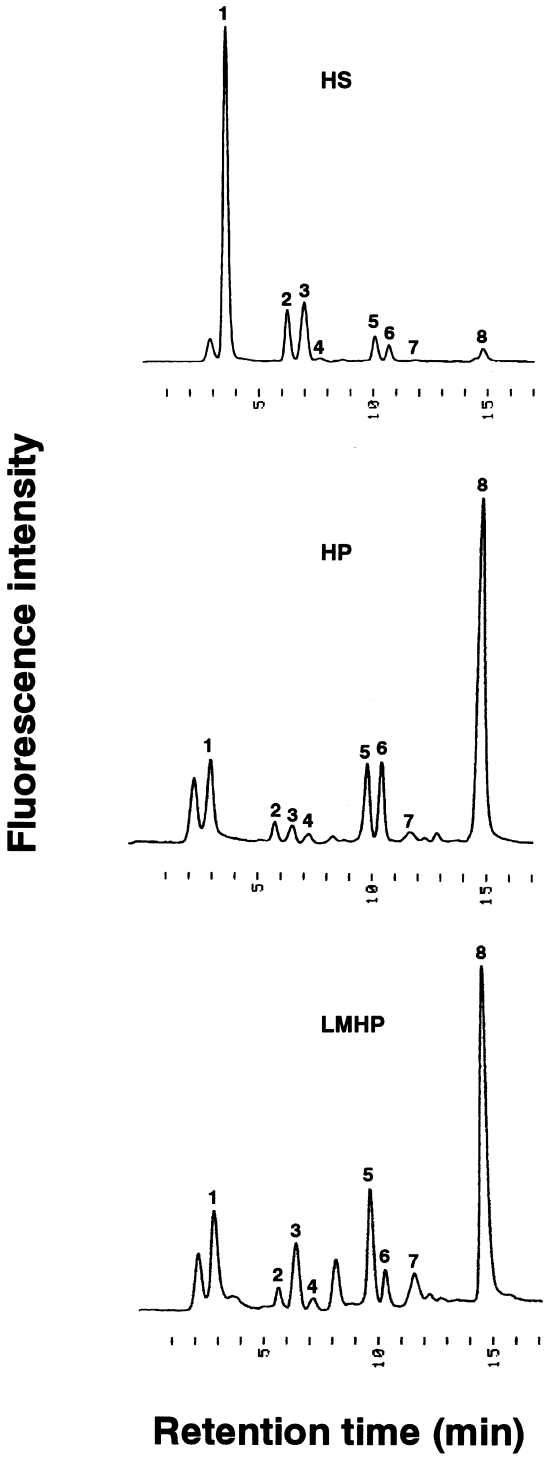

Fig. 4. Chromatograms of unsaturated disaccharides produced from HP and HS after enzymatic digestion with heparin lyase I, II and III.

References

[1] T. Imanari, T. Toida, I. Koshiishi, H. Toyoda, J. Chromatogr. A 720 (1996) 275–293.
[2] N.K. Karamanos, P. Vanky, G.N. Tzanakakis, T. Tsegenidis, A. Hjerpe, J. Chromatogr. A 765 (1997) 169–179.
[3] Y. Guo, H.E. Conrad, Anal. Biochem. 168 (1988) 54–62.
[4] H. Giesche, K.K. Unger, U. Esser, U. TrŸdinger, J.N. Kinkel, J. Chromatogr. 465 (1989) 39–57.
[5] K. Kalghatgi, C. Horvath, J. Chromatogr. 443 (1988) 343–354.
[6] Y. Kato, T. Kitamura, A. Mitsui, T. Hashimoto, J. Chromatogr. 398 (1987) 327–334.
[7] J.K. Duncan, A.J.C. Chen, C.J. Siebert, J. Chromatogr. 397 (1987) 3–12.
[8] Y. Kato, T. Kitamura, S. Nakatani, T. Hashimoto, J. Chromatogr. 483 (1989) 401–405.
[9] Y. Kato, T. Kitamura, A. Mitsui, Y. Yamasaki, T. Hashimoto, T. Murotsu, S. Fukushige, K. Matsubara, J. Chromatogr. 447 (1988) 212–220.
[10] H. Moriyama, M. Anegayama, K. Komiya, Y. Kato, J. Chromatogr. A 691 (1995) 81–89.
[11] H. Toyoda, T. Nagashima, R. Hirata, T. Toida, T. Imanari, J. Chromatogr. B 704 (1997) 19–24.
[12] S. Honda, Y. Matsuda, M. Takahashi, K. Kakehi, Anal. Chem. 52 (1980) 1079–1082.
[13] N.K. Karamanos, P. Vanky, G.N. Tzanakakis, A. Hjerpe, Electrophoresis 17 (1996) 391–395.
[14] R. Malsch, J. Harenberg, D.L. Heene, J. Chromatogr. A 716 (1995) 259–268.
[15] A. Pervin, A. Al-Hakim, R.J. Linhardt, Anal. Biochem. 221 (1994) 182–188.