Journal of Chromatography, 496 (1989) 27–38
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4929

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC IDENTIFICATION OF DISACCHARIDES GENERATED FROM HEPARAN SULPHATE ISOMERS USING HEPARITINASES

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(First received March 15th, 1989, revised manuscript received June 20th, 1989)

SUMMARY

Specific heparan sulphate-lyases, heparitinases I and II, were used to identify unsaturated disaccharide constituents generated from heterogeneous heparan sulphate isomers. All determinations were made using high-performance liquid chromatography with a column containing a sulphonized styrene–divinylbenzene copolymer. Unsaturated disaccharides generated from variously sulphated heparan sulphate isomers after simultaneous digestion with heparitinases I and II facilitated separation of the individual disaccharides, based on sulphate groups at the specific position of the uronic acid and glucosamine residues. The simultaneous digestion with heparitinases I and II produces unsaturated disaccharides from heparan sulphate isomers with the structure of 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-amino-deoxy-D-glucose, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-deoxy-2-sulphamido-D-glucose, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-aminodeoxy-6-*O*-sulpho-D-glucose, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-deoxy-2-sulphamido-6-*O*-sulpho-D-glucose, 4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-amino-2-deoxy-6-*O*-sulpho-D-glucose and 4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-deoxy-2-sulphamido-6-*O*-sulpho-D-glucose.

0378-4347/89/$03.50

INTRODUCTION

Heparan sulphate (HS) isomers exhibit a high heterogeneity among other glycosaminoglycans (GAGs), with regard to structure, sulphate content, position of the sulphates and their repeated chain structures [1–3]. These HS isomers are present in various tissues, particularly vascular ones, and they play important roles in transporting various substances in the cell membranes [3,4]. HS isomers, as well as other sulphated GAGs, also possess physiological functions such as anticoagulant and antithrombogenic activities [5–7]. The highly heterogeneous structures of HS isomers have been elucidated using conventional electrophoretic procedures, and others [7–10].

Heparitinases I and II degrade exclusively HS isomers into constitutional unsaturated disaccharides (ΔDi-S_{HS}) but do not influence other GAGs [11–13]. Whether or not heparitinases generate ΔDi-S_{HS} from HS isomers beneficial for identifying the constituents of the HS isomers has received much attention, but success has been limited.

High-performance liquid chromatography (HPLC) is useful for characterizing and quantifying unsaturated disaccharides (ΔDi-S) generated from chondroitin sulphate (CS) and dermatan sulphate (DS) isomers with chondroitinases on silica [14–17]. HS isomers plus heparin were also resolved into three reduced ΔDi-S_{HS} by HPLC [18], but a practical application to separate ΔDi-S_{HS} in natural sources was limited. We have characterized ΔDi-S generated from the various sulphated CS and DS isomers, using HPLC with a sulphonized styrene–divinylbenzene copolymer [19–24].

We report here an analysis of a variety of HS isomers and heparin at the constitutional ΔDi-S_{HS}, after generation with heparitinases I and II, using HPLC with styrene–divinylbenzene copolymers. HS isomers were degraded with the heparitinases to various ΔDi-S_{HS}, such as unsaturated non-sulphated, mono-sulphated, di-sulphated and tri-sulphated disaccharides (ΔDi-$0S_{HS}$, ΔDi-monoS_{HS}, ΔDi-diS_{HS} and ΔDi-triS_{HS}), using HPLC.

Abbreviations

ΔDi-S_{HS}, unsaturated disaccharides from HS; ΔDi-$0S_{HS}$, unsaturated non-sulphated disaccharide of HS; ΔDi-monoS_{HS}, unsaturated mono-sulphated disaccharides from HS; Di-diS_{HS}, unsaturated di-sulphated disaccharides; ΔDi-triS_{HS}, unsaturated tri-sulphated disaccharide; ΔDi-$0S_{HS}$, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-aminodeoxy-D-glucose; ΔDi-NS_{HS}, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-deoxy-2-sulphamido-D-glucose; ΔDi-$6S_{HS}$, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-amino-deoxy-6-*O*-sulpho-D-glucose; ΔDi-di$S1_{HS}$, 4-deoxy-2-*O*-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-deoxy-2-sulphamido-6-*O*-sulpho-D-glucose; ΔDi-di$S2_{HS}$, 4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid (1→4)-2-amino-2-deoxy-6-*O*-sulpho-D-glu-

cose; ΔDi-triS_{HS}, 4-deoxy-2-*O*-sulpho-α-L-*threo*-hex-4-enepyranosyluronic acid (1 $\rightarrow$4)-2-deoxy-2-sulphamido-6-*O*-sulpho-D-glucose.

EXPERIMENTAL

Enzymes and unsaturated disaccharides

Reference ΔDi-0S_{HS}, ΔDi-monoS_{HS} (ΔDi-NS_{HS} and ΔDi-6S_{HS}) and ΔDi-diS_{HS} (ΔDi-diS1$_{HS}$, ΔDi-diS2$_{HS}$) were prepared from HS isomers of bovine kidney, using heparitinases I and II [11–13]. ΔDi-triS_{HS} was generated from heparin of bovine intestine. Heparitinases I and II, which were prepared from *Flavobacterium heparinum*, as described in the literature [11–13], specifically degrade HS isomers but not other GAGs. Heparitinase I digests HS isomers with relatively low sulphate content, whereas type II degrades compounds with higher sulphate content. Hence, simultaneous treatment with the two heparitinases generates over 95% of the HS compounds. 2-*O*-Sulphatase was prepared from *F. heparinum* [25]. Chondroitinase-ABC, prepared from *Proteus vulgaris*, and chondroitinase-AC, from *F. heparinum* [26], were also used. These enzymes, ΔDi-S_{HS}, heparin, HS, CS and DS isomers were prepared at the Tokyo Institute of Seikagaku Kogyo (Tokyo, Japan). Variously sulphated HS isomers were degraded completely with heparitinases I and II into the corresponding ΔDi-diS_{HS}. ΔDi-triS_{HS} and ΔDi-diS2$_{HS}$ possess sulphate(s) at the 2 position of uronic acid. These products were desulphated by digestion with 2-*O*-sulphatase to generate the corresponding lower sulphated ΔDi-S_{HS}, as had been found in sulphated ΔDi-S from oversulphated CS and DS isomers [24].

Heparitinases I and II (10 mU each) exclusively digested HS isomers (ca. 100 μg) in 10 m*M* acetate buffer in 1.0 μ*M* calcium acetate solution, final pH 7.0, at 37°C for 120 min. Methanol, acetonitrile, formic acid and other chemicals of HPLC grade were purchased from Wako (Osaka, Japan).

Electrophoresis of macromolecular GAGs

Electrophoretic separation of GAGs were carried out before and after the enzymic digestion on cellulose acetate strips in three buffers, as described previously [7–9,26]: 0.1 *M* pyridine–formic acid (pH 3.0) at 0.5 mA/cm for 50 min; 0.1 *M* calcium acetate at 0.5 mA/cm for 180 min; and 0.1 *M* barium acetate at 5 V/cm for 180 min. The GAGs thus separated were characterized by staining with alcian blue, then destained with 0.2 *M* acetic acid.

GAG preparation and Dowex column chromatography

Human kidney GAGs were prepared as follows. In brief, the GAGs were digested with pronase at the rate of 50 mg/g defatted dry weight, pH 7.0, for 12 h. Cold trichloroacetic acid was then added to the solution at a final concentration of 10%. The supernatants were dialysed against distilled water, and the non-dialysable GAGs were concentrated and mixed with four volumes of

ethanol to precipitate the GAGs. The GAGs were fractionated on a Dowex 1-X2 column (Cl^-) with increasing NaCl molarity, in 0.25 *M* increments, to separate the HS isomers together with other GAGs [7,8,27]. After desalting by passage through Sephadex G-10, the GAGs (100 μg as uronic acid) in 20 μl of distilled water were completely digested with 0.2 U of chondroitinase-ABC in 20 μl of Tris buffer (pH 7.8) and the AC-lyase in Tris buffer (pH 6.0) at 37°C for 120 min [7–9,25]. The undigested GAGs were precipitated by the addition of four volumes of ethanol [8,9], and the HS isomers in the precipitates were then converted with the two heparitinases into the ΔDi-S_{HS}, and analysed by HPLC.

HPLC apparatus and procedures

HPLC analyses using a sulphonized styrene–divinylbenzene copolymer were carried out as described previously [21–24]. A Model 803D HPLC apparatus (Toyosoda, Tokyo, Japan) was used as the solvent-delivery system. ΔDi-S_{HS} peaks were determined at 232 nm with a Model SS 5600 detector (Senshu Science, Tokyo, Japan). An automatic chromatographic analyser (Model CC 11, System Instruments, Tokyo, Japan) and a recorder (Type SS 250F, Sekonic, Tokyo, Japan) were used to measure retention times and peak heights or areas. To separate individual ΔDi-S_{HS}, a Shodex RS (Type DC-613) ion-exchange chromatographic column, packed with a fully porous ion-exchange resin (Na^+ form) composed of a sulphonized styrene–divinylbenzene copolymer [19,20], was purchased from Showa Denko (Tokyo, Japan). The resin, with a particle size of 6 μm, was packed into two stainless-steel columns (150 mm × 6 mm I.D.), and preceded by a guard column (46 mm × 6 mm I.D.).

The analysis of ΔDi-S_{HS} by HPLC was carried out by previously reported methods [22–24]. Each degraded product, i.e. ΔDi-S_{HS}, (0.5–10 μg aliquot per 10–20 μl), was injected onto the Shodex RS column and chromatographed at a flow-rate of 1.0 ml/min with mobile phases composed of acetonitrile–methanol–0.8 *M* ammonium formate (pH 4.5). The composition of the mobile phase was kept constant with the use of a Degasser ERC-3110 (Erma Optical Works, Tokyo, Japan). The present HPLC separation of ΔDi-S_{HS} was carried out at 70°C at 15 kg/cm^2. The eluate was monitored by UV absorbance at 232 nm, and the signals were automatically recorded at a chart speed of 2.5 mm/min to determine retention times and peak areas. On occasions, conventional HPLC on a Partisil 5-PAC silica column (Whatman Labs., Clifton, NJ, U.S.A.) was also carried out as previously described [20].

RESULTS

Electrophoresis

The digestibility of human kidney HS isomers with heparitinases I plus II and chondroitinases was estimated by comparison with the undigested GAGs,

by means of electrophoretic characterization [26,27]. GAGs from kidney tissues showed one major band corresponding to HS isomers after digestion with chondroitinases. Pretreatment of CS and DS isomers with chondroitinases was done to prepare the purified HS isomers and heparin. In the case of simultaneous digestion with specific heparitinases I and II, the single HS band disappeared. This digestibility was confirmed by separation in different buffer solutions for electrophoretic characterization (not shown in the figure). Thus, the electrophoretic findings indicate that heparitinases I and II do digest HS isomers.

High-performance liquid chromatography

With the HPLC system described, reference ΔDi-monoS_{HS}, ΔDi-diS_{HS} and ΔDi-triS_{HS} and the possible occurrence of additional peaks due to the presence of impurities were examined. The results showed that each reference ΔDi-S_{HS} gave a single peak without any contaminants (Fig. 1). Retention times of the ΔDi-S_{HS} peaks were studied as a function of various mobile phase compositions (Table I). An increasing proportion of acetonitrile resulted in better separation of the neighbouring ΔDi-0S_{HS} and ΔDi-6S_{HS} peaks, but a lower resolution of ΔDi-di$S2_{HS}$ and ΔDi-NS_{HS} and broader peaks at the later elutions. The most satisfactory mobile phase composition was estimated to be 69:11:20 (v/v) acetonitrile–methanol–0.8 *M* ammonium formate (pH 4.5), with which good separation of the peaks of representative ΔDi-S_{HS} was achieved.

The retention times of standard ΔDi-S_{HS} were characteristic, and the elution order was: ΔDi-0S_{HS}, ΔDi-6S_{HS}, ΔDi-di$S2_{HS}$, ΔDi-NS_{HS}, ΔDi-di$S1_{HS}$ and ΔDi-triS_{HS} (Table II). The individual ΔDi-S_{HS} were sufficiently resolved and the retention times were reproducible, even when the HPLC assay was performed on different days: the overall coefficient of variation was less than 1%.

The retention times of the individual ΔDi-S_{HS} generated from HS isomers of human kidney tissue were also examined. The yield of ΔDi-S_{HS} generated from the kidney HS isomers with the heparitinases was estimated to be ca. 95%. When kidney GAGs were fractionated on a Dowex 1-X2 column by increasing the NaCl molarity, the major parts of HS isomers appeared between the 0.75 and 1.25 *M* NaCl eluates. The fractionated GAG compounds were digested with chondroitinases and precipitated with ethanol, and the resulting HS isomers in the precipitates were characterized by electrophoresis. They were then digested with the heparitinases. Digestibility of the substrates was confirmed by electrophoresis, before and after generation with the enzymes [7–9,26].

The HPLC pattern showed six peaks following generation of HS isomers and heparin with the heparitinases [28]: a major peak of ΔDi-0S_{HS} plus ΔDi-6S_{HS} as a shoulder, with an intermediate peak of ΔDi-NS_{HS} (Fig. 2). Minor peaks of over-sulphated ΔDi-S_{HS}, i.e. two ΔDi-diS_{HS} (ΔDi-di$S1_{HS}$ and ΔDi-di$S2_{HS}$) and ΔDi-triS_{HS}, were detected in the kidney tissue. The proportions

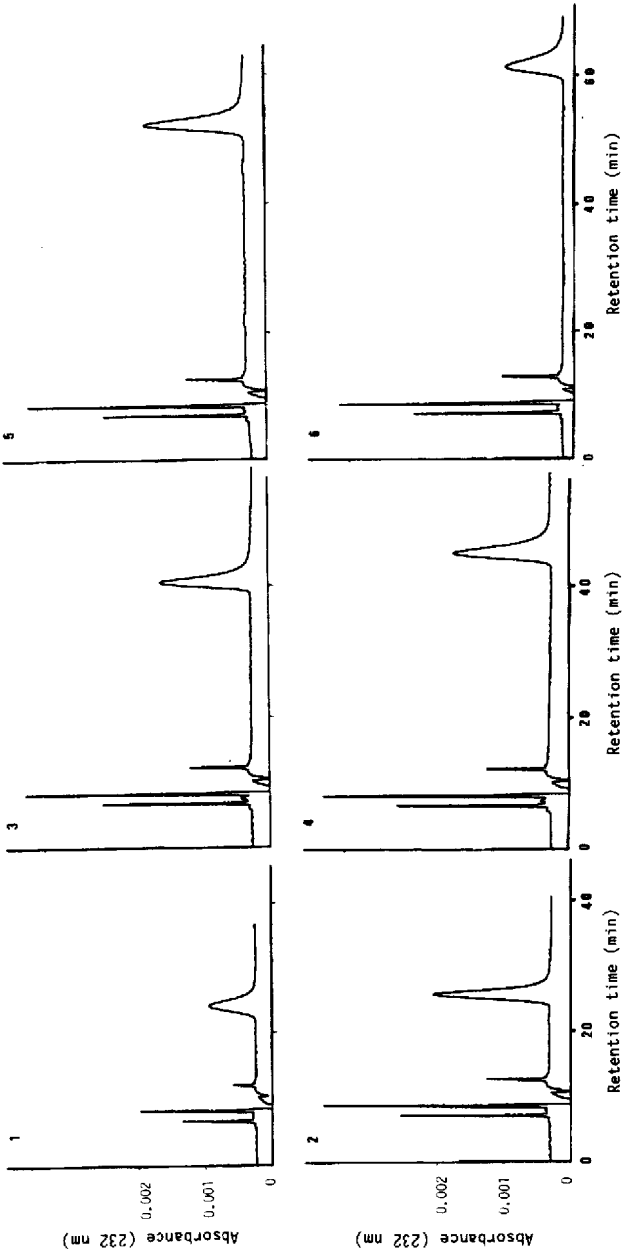

Fig. 1. Chromatogram of ΔDi-S_{HS} prepared from HS isomers and heparin by digestion with heparitinases. Each ΔDi-S_{HS} appeared as a single peak. The concentration of the HS isomers was 10 μg per 5 μl. Identification of numbers: 1 = ΔDi-0S_{HS}; 2 = ΔDi-6S_{HS}; 3 = ΔDi-diS2$_{HS}$; 4 = ΔDi-NS_{HS}; 5 = ΔDi-diS1$_{HS}$; 6 = ΔDi-triS$_{HS}$.

TABLE I

RETENTION TIMES OF ΔDi-S_{HS} ON A SHODEX-RS (TYPE DC-613) COLUMN AS A FUNCTION OF THE MOBILE PHASE COMPOSITION

The mobile phase was acetonitrile–methanol–0.8 *M* ammonium formate buffer (pH 4.5) with different ratios (v/v) All determinations were made as described in the text.

Compound	Retention time (min)						
	62.5 : 15 : 22.5	65 : 15 : 20	67.5 : 10 : 22.5	67.5 : 12.5 : 20	68 : 10 : 22	69 : 10 : 21	70 : 10 : 20
ΔDi-$0S_{HS}$	13.5	17.6	17.5	20 3	18.7	20.5	25.1
ΔDi-$6S_{HS}$	14 2	18.6	18.8	21.9	20.0	22.5	28.8
ΔDi-$diS2_{HS}$	19.3	28.9	29 1	36 5	32.0	37.0	50.5
ΔDi-NS_{HS}	21.9	12.2	31 4	39 5	34.3	39.3	52.7
ΔDi-$diS1_{HS}$	23.2	36.3	35.7	46.6	39.7	46.2	64.9
ΔDi-$triS_{HS}$	25.9	42.4	41 6	55.5	46.6	54.9	> 70.0

TABLE II

SIMPLIFIED NOMENCLATURE FOR UNSATURATED DISACCHARIDES DERIVED FROM HEPARAN SULPHATE ISOMERS AND THEIR RETENTION TIMES IN HPLC

A 5-μg amount of each ΔDi-S_{HS} was injected onto either a Shodex column or a Partisil 5-PAC column. The former HPLC was carried out as described in the text. In the latter, the mobile phase was acetonitrile–methanol–0.1 *M* ammonium formate (12·3 4, v/v), at 40°C at 58 kg/cm^2. ΔDi-S_{HS} were chromatographed in three to nine assays on different days.

Unsaturated disaccharide	Retention time (mean ± S.D.) (min)	
	Shodex-RS column	Partisil 5-PAC column
Unsaturated non-sulphated disaccharide		
ΔDi-$0S_{HS}$ = Δ-uronosyl-GlcNAc	21 95 ± 0 08	10.03 ± 0.09
Unsaturated mono-sulphated disaccharides (ΔDi-monoS_{HS})		
ΔDi-$6S_{HS}$ = Δ-uronosyl-GlcNAc-6S	24.35 ± 0.08	15.24 ± 0.26
ΔDi-NS_{HS} = Δ-uronosyl-GlcN-NS	43.35 ± 0.21	17.62 ± 0.32
Unsaturated di-sulphated disaccharides (ΔDi-diS_{HS})		
ΔDi-$diS1_{HS}$ = Δ-uronosyl-GlcN-N,6-bisS	51.85 ± 0.17	33.08 ± 0.60
ΔDi-$diS2_{HS}$ = Δ-uronosyl-2S-GlcN-NS	41.10 ± 0.15	34 68 ± 0.66
Unsaturated tri-sulphated disaccharide (ΔDi-triS_{HS})		
ΔDi-$triS_{HS}$ = Δ-uronosyl-2S-GlcN-N,6-bisS	62.55 ± 0.28	45 35 ± 0.52

of ΔDi-$diS1_{HS}$ and ΔDi-$triS_{HS}$ to ΔDi-$0S_{HS}$ increased by increasing NaCl concentration. Thus, large amounts of ΔDi-$0S_{HS}$ derived from less sulphated HS isomers were present among ΔDi-S_{HS} from human kidney HS isomers. There was a distinct peak of ΔDi-$triS_{HS}$, eluted mostly in the 1.25 *M* NaCl fraction.

Several peaks of the ΔDi-S_{HS} originally derived from the HS isomers of the human kidney could be detected after simultaneous digestion with heparitri-

TABLE III

DISTRIBUTION OF THE CONSITUTIONAL UNSATURATED DISACCHARIDES GENERATED FROM HEPARAN SULPHATES ISOMERS OF HUMAN KIDNEY TISSUES AFTER DIGESTION WITH HEPARITINASES I AND II

Values are percentages (mean ± S.D.) of total HS isomers.

Fraction	Non-sulphated (ΔDi-0S_{HS})	6-sulphated (ΔDi-6S_{HS})	2- and N-sulphated (ΔDi-diS2$_{HS}$)	N-sulphated (ΔDi-NS_{HS})	6- and N-sulphated (ΔDi-diS1$_{HS}$)	Tri-sulphated (ΔDi-triS_{HS})	Total HS isomers (total ΔDi-S_{HS})
0.75 *M* NaCl	69.08 ± 3.27	7.89 ± 1.83	3.07 ± 0 49	12.73 ± 1 25	3.68 ± 0.20	1.49 ± 0.24	97.94
1.0 *M* NaCl	53.32 ± 2.50	12.25 ± 2.25	5.13 ± 0 05	16.37 ± 0.24	7.61 ± 0.19	2.53 ± 0.24	97.21
1.25 *M* NaCl	44.76 ± 5.10	20.02 ± 4.51	2.87 ± 0 25	15.76 ± 0 68	11.57 ± 1.25	3.77 ± 0.28	98.75

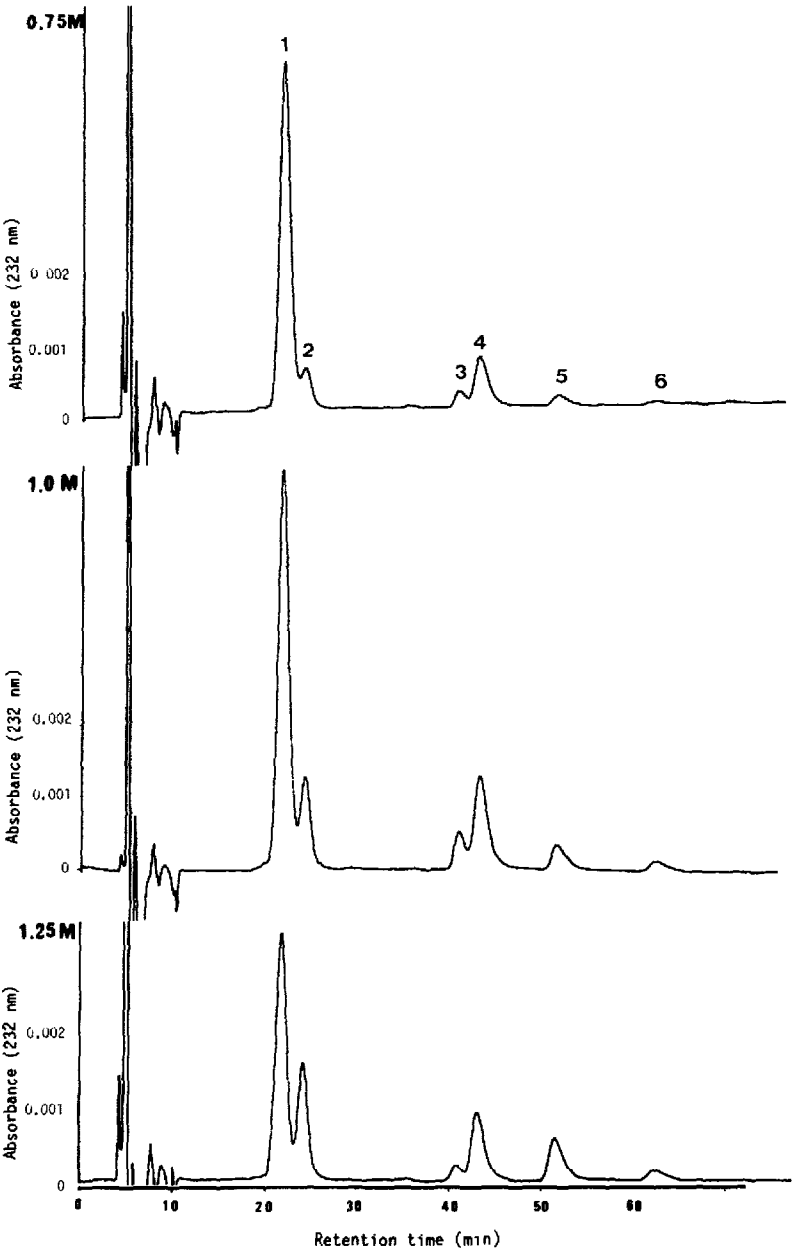

Fig. 2. HPLC patterns of ΔDi-S_{HS} digested with heparitinases I and II from HS isomers of human kidney after pre-treatment with chondroitinase-ABC. The ΔDi-S_{HS} was fractionated into 0.75 *M*, 1.0 *M* and 1.25 *M* portions on a Dowex 1-X2 column, with increasing NaCl molarity. Peak numbers 1-6 are as in Fig. 1.

nases I and II, thereby indicating that the HS isomers are heterogeneous. The appearance of a small peak of ΔDi-triS_{HS} indicates the presence of a heparin-like structure. When the molarity of NaCl was increased, the peak of ΔDi-0S_{HS} decreased, whereas increased proportions of ΔDi-diS1$_{HS}$ and ΔDi-triS_{HS} became evident. We presume that the sulphate content increases with the NaCl molarity, by which the HS isomers are eluted.

The analytical results for ΔDi-S_{HS} of human kidney GAGs with heparitinases are summarized in Table III. The HS isomers in the 1.25 *M* NaCl fraction consisted of non-sulphated, mono-sulphated, di-sulphated and tri-sulphated HS, in the approximate ratio of 45:36:15:4. In the case of the single peak of ΔDi-0S_{HS} with ΔDi-6S_{HS} at the shoulder, the proportion of the two ΔDi-S_{HS} was analysed automatically but also determined by HPLC separation on silica. The two sets of data were similar. Thus, heparitinases I and II exclusively digest HS isomers to variously sulphated ΔDi-S_{HS}, namely ΔDi-0S_{HS}, ΔDi-monoS_{HS}, ΔDi-diS_{HS} and ΔDi-triS_{HS}. Except for ΔDi-0S_{HS}, these sulphated ΔDi-S_{HS} all possess sulphate at a different NH_2 position and/or 6-glucosamine residues, with or without sulphate at position 2 of the uronic acid residues (ΔDi-diS2$_{HS}$ or ΔDi-triS_{HS}). Compounds containing the sulphates at 6-glucosamine (ΔDi-6S_{HS}, ΔDi-diS1$_{HS}$ and ΔDi-triS_{HS}) increase when the molarity of NaCl increases.

The retention times of ΔDi-S_{HS} are more reproducible with the present HPLC assay method than when a silica gel column is used. With HPLC on silica gel, the elution order for ΔDi-S_{HS} was the same as that obtained for CS and DS isomers, as reported in the literature [19,20,23]: i.e., ΔDi-0S, ΔDi-monoS, ΔDi-diS and ΔDi-triS.

DISCUSSION

Application of the heparitinases to HS isomers facilitated generation of the corresponding ΔDi-S_{HS}, and also resulted in separation and characterization of individual ΔDi-S_{HS}. Determination of the ΔDi-S_{HS} of HS isomers with heparitinases by this HPLC method makes feasible (1) differentiation of all the ΔDi-S_{HS} derived from heterogenous HS isomers and (2) clarification of quantitative changes in the HS isomers in various intercellular and cell surface matrices. The present HPLC assay using a sulphonized styrene–divinylbenzene copolymer to separate individual ΔDi-S generated from ΔDi-S_{HS} provides specific, precise and reproducible results, as was noted for ΔDi-S from CS and DS isomers, using chondroitinases [19,20,22–24].

There are only a few methods available for detecting the structures of HS isomers, and most are unsatisfactory or are not practical. Investigation of ΔDi-S_{HS} using heparitinases and this HPLC method can be made when amounts of the starting HS isomers are minute and when little information is available on HS isomers consisting of the various sulphated HS isomers. It is preferable

to use an appropriate combination of heparitinases I and II to increase the yield of degraded ΔDi-S_{HS}. The elution order of ΔDi-S_{HS} generated from HS isomers in the HPLC analysis on silica was: ΔDi-0S_{HS}, ΔDi-monoS_{HS}, ΔDi-diS_{HS} and ΔDi-triS_{HS}. In contrast, using the present HPLC system, retention times were more precise and reproducible, with a unique order of elution. Thus, the alternative application of ΔDi-S_{HS} derived from HS isomers with the heparitinases on the two HPLC systems can be used to confirm the compositional structure by determination of the different order of the retention times of ΔDi-S_{HS}.

The present HPLC assay using the heparitinases is useful for characterizing various sulphated HS isomers present in different matrices. The reproducibility required for the determination of individual ΔDi-S_{HS} is excellent. ΔDi-S_{HS} resulting from digestion with heparitinases make feasible the estimation of the constitution of original HS macromolecular compounds.

ACKNOWLEDGEMENT

This study was supported in part by Grants-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1 A. Linker and P. Hovingh, Biochemistry, 11 (1972) 563
2 M Hook, U Lindahl and P. Iverius, Biochem. J., 137 (1974) 33.
3 A Linker and P. Hovingh, in N.M. McDuffie (Editor), Heparin: Structure, Cellular Functions and Clinical Applications, Academic Press, New York, 1979, pp. 3-24.
4 P.M. Kraemer, Biochemistry, 10 (1971) 1437.
5 B.J. Grossman and J.A. Cifonelli, J. Lab. Clin. Med., 59 (1962) 1020.
6 K. Izuka and K. Murata, Atherosclerosis, 16 (1972) 217.
7 K. Murata, K. Izuka and K. Nakazawa, Atherosclerosis, 29 (1978) 95.
8 K. Murata, Connect Tissue Res., 4 (1976) 131.
9 K. Murata, Y. Ochiai and K Akashio, Gastroenterology, 89 (1985) 1248.
10 A. Linker and P. Hovingh, Carbohydr. Res., 29 (1973) 41.
11 M.E. Silva and C.P. Dietrich, Biochim. Biophys. Res. Commun., 56 (1974) 965.
12 M.E. Silva and C.P. Dietrich, J. Biol. Chem., 250 (1975) 6841.
13 N. Ototani, M. Kikuchi and Z. Yoshizawa, Carbohydr. Res., 88 (1981) 291.
14 G.L. Lee, J.E. Evans and H. Tickelmann, J. Chromatogr., 146 (1978) 439.
15 G.L. Lee and H. Tickelmann, Anal. Biochem., 94 (1979) 231.
16 A. Hjerpe, C.A. Antonopoulos and B. Engeldt, J. Chromatogr., 171 (1979) 339.
17 D.C. Seldin, N. Seno, K.N. Austin and R.L. Stevens, Anal. Biochem., 141 (1984) 291.
18 G.L Lee, D.W. Liu, J W. Pav and H. Tieckelmann, J. Chromatogr., 212 (1981) 65.
19 K. Murata and Y. Yokoyama, Anal. Biochem., 146 (1985) 327.
20 K. Murata and Y. Yokoyama, Anal. Biochem., 149 (1985) 261.
21 K. Murata and Y. Yokoyama, J. Chromatogr., 374 (1986) 37.
22 K. Murata and Y. Yokoyama, J Chromatogr., 415 (1986) 51.
23 K. Murata and Y. Yokoyama, J. Chromatogr., 425 (1986) 231
24 K. Murata and Y. Yokoyama, J. Biochem. Biophys. Methods, 15 (1987) 23.

25 M.W. McLean, J.S Bruce, W.F. Long and F.B. Williamson, Eur. J. Biochem., 145 (1984) 607.
26 K. Murata, in R.L. Whistler and S N. BeMiller (Editors), Methods of Carbohydrate Chemistry, Vol. 8, Academic Press, New York, 1980, pp. 81–88.
27 K. Murata, Connect. Tissue Res., 4 (1976) 131.
28 K. Murata, in G. Crepaldi (Editor), Atherosclerosis, Vol. 8, Elsevier, Amsterdam, 1989, pp. 39–42.