CHROMBIO. 3507

LIQUID CHROMATOGRAPHIC ASSAY FOR CONSTITUENT DISACCHARIDES OF HYALURONIC ACID AND CHONDROITIN SULPHATE ISOMERS

KATSUMI MURATA* and Y. YOKOYAMA

Department of Medicine and Physical Therapy, University of Tokyo School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 (Japan)

SUMMARY

An improved high-performance liquid chromatographic (HPLC) method for unsaturated disaccharides prepared from hyaluronic acid and various chondroitin sulphate and dermatan sulphate isomers was developed, which involves an ion-exchange resin prepared from a sulphonated styrene–divinylbenzene copolymer. The retention times of the individual unsaturated disaccharides were unique and reproducible, the disaccharides appearing in the following order: unsaturated non-sulphated disaccharide derived from hyaluronic acid, then unsaturated 6-sulphated, non-sulphated and 4-sulphated disaccharides from chondroitin sulphate isomers. Unsaturated disulphated disaccharide G had a much shorter retention time than the unsaturated non-sulphated disaccharide derived from hyaluronic acid. The contents of these individual unsaturated disaccharides could be determined with similar sensitivities on the basis of their ultraviolet absorbance. Selective and unique retention times and good resolutions were found for various unsaturated disulphated and trisulphated disaccharides. The proposed method can be used to determine various chondroitin sulphate and dermatan sulphate isomers in addition to hyaluronic acid in amounts as small as 100 ng to 8 μg. The practicality of this method was verified by its application to the separation and determination of the different types of chondroitin sulphate and dermatan sulphate isomers derived from human arteries in the presence of appreciable amounts of hyaluronic acid.

INTRODUCTION*

Glycosaminoglycans (GAGs) isolated from intercellular matrices exhibit polymorphism due to differences in chain length constituents and sulphate content [1–3]. GAGs can be degraded to the respective constituent disaccharide units by specific GAG-lyases [4,5]. This procedure is applicable to both the identification and quantitation of the disaccharides of chondroitin sulphate (Ch-S) and dermata sulphate (DS) isomers [4,5]. The disaccharide products generated from Ch-S and DS components with chondroitinases and chondrosulphatases are usually separated by paper or thin-layer chromatography, and then quantified spectrophotometrically.

During the last decade, high-performance liquid chromatography (HPLC) has been used for the identification of unsaturated disaccharides (ΔDi-S) obtained on digestion of Ch-S and DS isomers with chondroitinases [6–8]. Hyaluronic acid (HA) is present in small amounts in various tissues and organs [9], and it participates in the regulation of the metabolism of Ch-S isomers [2,3,9]. On the other hand, chondroitin constitutes a non-sulphated part of repeated disaccharide chains of Ch-S isomers and occurs as a minor component of under-sulphated Ch-S in body fluids [10].

The unsaturated non-sulphated disaccharide (ΔDi-0S) from chondroitin, unsaturated monosulphated disaccharide (ΔDi-monoS), commonly present as unsaturated 4- and 6-sulphated disaccharides (ΔDi-4S, ΔDi-6S), unsaturated disulphated disaccharide (ΔDi-diS) and saturated trisulphated disaccharide (DitriS) were separated by HPLC using silica columns [6–8]. Recently, we reported the analysis of different over-sulphated ΔDi-S of known GAG composition by HPLC using a unique resin, viz. a sulphonated styrene–divinylbenzene copolymer [11]. By this method, the ΔDi-0S compounds, ΔDi-0S$_{HA}$ from HA and ΔDi-0S from chondroitin, can be separated from each other [12], which is not possible using conventional silica gel HPLC columns. Nevertheless, it is still difficult to separate all ΔDi-S. For example, ΔDi-0S$_{HA}$ cannot be distinguished from ΔDi-6S, as these neighbouring ΔDi-S compounds show low resolution [13].

This paper reports a new HPLC method for the separation of ΔDi-0S$_{HA}$ generated from HA from all other ΔDi-S generated from various Ch-S and DS iso-

---

*Abbreviations used: GAGs, glycosaminoglycans; Ch-S, chondroitin sulphate; DS, dermatan sulphate; HA, hyaluronic acid; HPLC, high-performance liquid chromatography; ΔDi-S, unsaturated disaccharides; ΔDi-0S, unsaturated non-sulphated disaccharides; ΔDi-diS, unsaturated disulphated disaccharides; ΔDi-triS, unsaturated trisulphated disaccharides; ΔDi-0S$_{HA}$, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose; 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-sulpho-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; ΔDi-diS$_B$, 2-acetamido-2-deoxy-3-O-(2-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4-sulpho-D-galactose; ΔDi-diS$_D$ or ΔDi-diS$_G$, 2-acetamido-2-deoxy-3-O-(2-sulpho-β-D-gluco-4-enepyranosyluronic acid)-6-O-sulpho-D-galactose; ΔDi-diS$_E$ or ΔDi-diS$_H$, 2-acetamido-2-deoxy-3-O-(β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-O-sulpho-D-galactose; ΔDi-triS, 2-acetamido-2-deoxy-3-O-(2-sulpho-β-D-gluco-4-enepyranosyluronic acid)-4,6-bis-O-sulpho-D-galactose. ΔDi-diS$_D$, ΔDi-diS$_G$, ΔDi-diS$_E$ and ΔDi-diS$_H$ have epimeric structures in the configuration of the hexuronic acid in the parent polymers.

mers. It is now possible to separate all ΔDi-S, i.e., ΔDi-0S, ΔDi-monoS, ΔDi-diS and ΔDi-triS, in the presence of appreciable amounts of ΔDi-0S$_{HA}$ generated from HA. All these ΔDi-S, including ΔDi-diS and ΔDi-triS in the Ch-S and DS compounds, can be differentiated and quantitation is possible because of their unique elution order. Our modified HPLC method proved to be useful for the separation and quantitation of ΔDi-S from the Ch-S and DS components in the presence of ΔDi-0S$_{HA}$ generated from HA of human arteries.

EXPERIMENTAL

Reference unsaturated disaccharides and enzymes
   ΔDi-0S$_{HA}$ was prepared by generation from HA with chondroitinase-ABC or -AC. Three ΔDi-S, ΔDi-0S, ΔDi-6S and ΔDi-4S, were prepared from non-sulphated chondroitin, chondroitin 4-sulphate (Ch-4S) and chondroitin 6-sulphate (Ch-6S) of bovine nasal cartilage and shark cartilage by degradation with either chondroitinase-ABC or -AC. Standard GAGs such as HA, chondroitin, Ch-4S and Ch-6S, and Ch-S-lyases, chondroitinase-ABC (EC 4.2.2.4) from *Proteus vulgaris* [1,4] and chondroitinase-AC (EC 4.2.2.5) from *Arthrobacter aurescens* [1,14] were obtained from the Tokyo Institute of Seikagaku Kogyo (Tokyo, Japan).
   An over-sulphated DS, prepared from human kidney [15], has the same ΔDi-S constituents as chondroitin sulphate H (Ch-S$_H$) from hagfish notochord [16]. Ch-S$_D$ [17] from shark cartilage was kindly provided by Dr. T. Furuhashi and Ch-S$_E$ from squid cartilage by Professor N. Seno [18]. Ch-S$_G$, prepared from human aorta [19], contains ΔDi-diS$_G$ as a constituent [20]. ΔDi-triS was synthesized by sulphation of Ch-S$_D$ of shark cartilage. These over-sulphated Ch-S and DS isomers were degraded completely with chondroitinase-ABC to the corresponding ΔDi-diS, which were then separated from the other ΔDi-S either by chromatography with a different sodium chloride concentration followed by desalting with Sephadex G-10 or by high-voltage electrophoresis [20]. The ΔDi-diS thus prepared were detected as single spots at 232 nm after either descending paper chromatographic characterization or high-voltage electrophoresis [1,5,20].
   These ΔDi-S compounds were eluted with a small amount of distilled water and adjusted to a concentration of 25 μg per 100 μl. The ΔDi-S contents were determined by means of the borate carbazole reaction [21]. All the ΔDi-S showed UV absorption with a maximum at 232 nm [17]. As starting materials for the solvent system, methanol, acetonitrile, formic acid, potassium phosphate and other chemicals of HPLC grade were purchased from Wako (Tokyo, Japan).

HPLC apparatus
   The HPLC system employed was a Model 803D (Toyosoda, Tokyo, Japan) solvent delivery system. A Model C-5600 detector (Senshu Science, Tokyo, Japan) equipped with a Model SS-250F flat mini-pen type integrator recorder (Sekonic, Tokyo, Japan) was used for monitoring peaks of ΔDi-S. A Shodex-RS (Type DC-613) column (Showa Denko, Tokyo, Japan; Showa Denko America, New York, NY, U.S.A.; Showa Denko Europe, Düsseldorf, F.R.G.), containing a fully porous

cation-exchange resin in the Na$^+$ form made from sulphonated sty-rene–divinylbenzene copolymers, was used to separate the ΔDi-S isomers as described previously [11,12]. The resin, with a particle size of 10 μm, was pre-packed into two coupled stainless-steel columns (150 $\times$ 6 mm I.D.), connected to a guard column (70 $\times$ 6 mm I.D.).

HPLC procedure

The ΔDi-S compounds (0.25–10 μg in 10-μl aliquots of solutions) prepared as described above were chromatographed at a flow-rate of 1.0 ml/min with a solvent system consisting of acetonitrile–methanol–0.8 M ammonium formate (pH 4.5) (65:15:20, v/v/v). The composition of the mobile phase was systematically changed so as to obtain the optimal conditions, by the procedure mentioned above, for the separation of ΔDi-S, i.e., ΔDi-diS$_G$, ΔDi-0S$_{HA}$, ΔDi-6S, ΔDi-0S and ΔDi-4S. HPLC analyses were carried out at 70°C at a pressure of 15 kg/cm^2 for 30 min. The eluate was detected at 232 nm, and the chromatograms were recorded at a chart speed of 2.5 or 5 mm/min to determine the retention times, peak heights and areas. At 0.08 a.u.f.s., 0.25 μg of ΔDi-S could be detected well without appre-ciable baseline noise.

ΔDi-S compounds were analysed by HPLC using a Whatman Partisil-10 PAC mono-cyano-substituted silica column [11].

RESULTS

HPLC

The ΔDi-S investigated were prepared from various macromolecular Ch-S and DS polymers. Following their purification, they were subjected to the HPLC. The origin of additional peaks possibly due to the presence of impurities and/or the use of high temperatures was examined for reference ΔDi-S including ΔDi-0S$_{HA}$ by this HPLC method. The results showed that each ΔDi-S gave a characteristic single peak with a retention time different to that of any impurity.

The retention time of each ΔDi-S showed good reproducibility under the con-ditions used. The numbers of theoretical plates (N) for representative ΔDi-S peaks with various mobile phase compositions were determined and are sum-marized in Table I. The best separation of ΔDi-0S$_{HA}$, ΔDi-6S, ΔDi-0S and ΔDi-4S was obtained using an acetonitrile–methanol–0.8 M ammonium formate (pH 4.5) mobile phase composition of 65:15:20 (v/v/v). Satisfactory separation of the peaks of neighbouring ΔDi-0S$_{HA}$, ΔDi-6S and ΔDi-0S was achieved with this mobile phase. The difference in N values between ΔDi-0S$_{HA}$ and ΔDi-6S was greater than with other mobile phases. An increasing proportion of acetonitrile in the solvent improved the resolution of ΔDi-0S$_{HA}$ and ΔDi-6S.

The representative ΔDi-S were separated in a unique elution order (Fig. 1). The retention times (mean $\pm$ S.D.) of ΔDi-diS$_G$, ΔDi-0S$_{HA}$, ΔDi-6S, ΔDi-0S and ΔDi-4S were 8.61 $\pm$ 0.10, 9.42 $\pm$ 0.12, 10.41 $\pm$ 0.13, 11.58 $\pm$ 0.15 and 14.79 $\pm$ 0.15 min, respectively. ΔDi-diS$_G$ had the same retention time as ΔDi-diS$_D$, and ΔDi-diS$_E$ the same as ΔDi-diS$_H$. ΔDi-diS$_G$ and ΔDi-diS$_D$ had shorter retention times than ΔDi-0S$_{HA}$, whereas those of ΔDi-diS$_E$ and ΔDi-diS$_H$ were longer than that of ΔDi-

TABLE I

NUMBERS OF THEORETICAL PLATES (N) WITH DIFFERENT MOBILE PHASE BUFFER COMPOSITIONS (BY VOLUME) ON A SHODEX-RS (DC-613) COLUMN

N of the unsaturated disaccharides (ΔDi-S) from GAGs depends on the molarity of ammonium formate in the mobile phase. N was calculated by the peak width at half-height method, using $N = 5.54 \cdot (t_R/W_{\frac{1}{2}})$, where t_R = retention time and $W_{\frac{1}{2}}$ = peak width at half-height. The N values are averages for four different chromatograms.

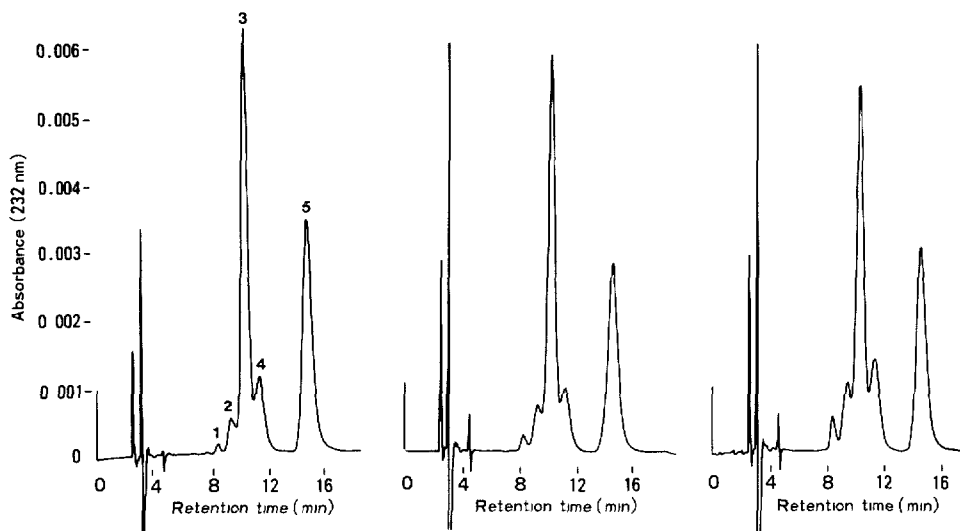| Unsaturated disacharide	N				
	0.75 M	0.80 M	0.90 M	1.00 M	1.25 M
Acetonitrile-methanol-ammonium formate, pH 4.5 (60:15:20, v/v/v)					
ΔDi-0S$_{HA}$	—	—	535	557	596
ΔDi-6S	558	—	605	650	717
ΔDi-0S	458	—	694	694	723
ΔDi-4S	702	—	946	979	1030
Acetonitrile-methanol-ammonium formate, pH 4.5 (65:15:20, v/v/v)					
ΔDi-0S$_{HA}$	728	742	813	837	—
ΔDi-6S	848	873	936	954	—
ΔDi-0S	922	967	985	992	—
ΔDi-4S	1397	1463	1525	1526	—

Fig. 1. Representative chromatograms of ΔDi-S (14-15 μg as uronic acid) obtained using a Shodex-RS resin column showing the separation of standard ΔDi-diS$_D$ or ΔDi-diS$_G$, ΔDi-0S$_{HA}$, ΔDi-6S, ΔDi-0S and ΔDi-4S at concentrations corresponding to those present in the various arteries. The ratios of ΔDi-diS$_G$, ΔDi-0S$_{HA}$ and ΔDi-0S are approximately 1:3.6 (left), 1.5:4·5 (middle) and 2.5:5:7 (right) and those of ΔDi-6S and ΔDi-4S are 6·4, respectively. Peaks: 1 = ΔDi-diS$_G$; 2 = ΔDi-0S$_{HA}$; 3 = ΔDi-6S; 4 = ΔDi-0S; 5 = ΔDi-4S.

4S. The $\varDelta$Di-diS and $\varDelta$Di-triS could be clearly differentiated from $\varDelta$Di-0S, $\varDelta$Di-4S and $\varDelta$Di-6S; the retention times of $\varDelta$Di-diS$_B$ and $\varDelta$Di-diS$_E$ or $\varDelta$Di-diS$_H$ were 12.42 ± 0.12 and 18.38 ± 0.21 min, respectively. Surprisingly, the retention time of $\varDelta$Di-triS was 13.43 ± 0.19 min, which was between those of $\varDelta$Di-diS$_B$ and $\varDelta$Di-4S.

To achieve the efficient separation of each $\varDelta$Di-S and to evaluate the linearity of response and the reproducibility of the method, 10-μl aliquots containing 0.25–8 μg of the representative $\varDelta$Di-S isomers, i.e., $\varDelta$Di-0S$_{HA}$, $\varDelta$Di-6S, $\varDelta$Di-0S and $\varDelta$Di-4S, were chromatographed under the same conditions. The peak areas of individual $\varDelta$Di-0S and $\varDelta$Di-monoS were plotted against the corresponding amounts of the compounds. The responses for different contents of the $\varDelta$Di-S compounds obtained with this HPLC system were closely related to the uronic acid content, from 0.5 to 8 μg, determined by the borate carbazole method [21], and a linear relationship was found between the integrator response and the content of each $\varDelta$Di-S. The within-day coefficient of variation for $\varDelta$Di-S was less than 0.14% ($n=12$) and approximately 0.18% over a five-day period. The recoveries of individual $\varDelta$Di-S were more than 96%.

On the other hand, the elution order of $\varDelta$Di-0S, $\varDelta$Di-monoS and $\varDelta$Di-diS on silica gel [11] was found to be $\varDelta$Di-0S, $\varDelta$Di-6S, $\varDelta$Di-4S, $\varDelta$Di-diS$_D$ or $\varDelta$Di-diS$_G$, $\varDelta$Di-diS$_B$ and $\varDelta$Di-diS$_E$ or $\varDelta$Di-diS$_H$, and finally $\varDelta$Di-triS.

Analysis of $\varDelta$Di-S in human arterial GAGs

This HPLC method was applied to the analysis of $\varDelta$Di-S prepared from GAGs from the three different layers of the human aorta and other arteries [19,22]. The arterial GAGs were digested with chondroitinase-ABC in 0.1 M Tris buffer (pH 8.0) and with the AC-lyase in 0.1 M acetate buffer (pH 6.0) for 120 min each to obtain the constituent $\varDelta$Di-S. The heparan sulphates and other compounds in the buffers not digested by the ABC-lyase, which accounted for approximately one third of the total GAGs, were precipitated by the addition of 80% ethanol. The $\varDelta$Di-S in the supernatant were passed through a membrane filter to remove insoluble substances and then analysed by HPLC. Arterial GAGs from equivalent amounts of the same sample were determined in duplicate. The proportion of each $\varDelta$Di-S in duplicate determinations was close to the others and the recoveries exceeded 94%.

Fig. 2 shows the separation of five $\varDelta$Di-S (elution order: $\varDelta$Di-diS$_G$, $\varDelta$Di-0S$_{HA}$, $\varDelta$Di-6S, $\varDelta$Di-0S and $\varDelta$Di-4S) of the GAGs prepared from various parts of human arteries. These results show that the present HPLC method using a Shodex-RS (DC-613) column is applicable to the separation of $\varDelta$Di-S isomers derived from human intercellular GAGs in the presence of HA. The peaks of $\varDelta$Di-0S$_{HA}$, $\varDelta$Di-6S, $\varDelta$Di-0S and $\varDelta$Di-4S showed the same values following digestion with either chondroitinase-ABC or -AC. Subtraction of the peak area of $\varDelta$Di-4S derived from GAGs digested with chondroitinase-AC from that obtained by the ABC-lyase gave the amount of the DS component [1,22,23]. The peak of $\varDelta$Di-diS had the same retention time as the standard $\varDelta$Di-diS$_G$, and it appeared after digestion with chondroitinase-ABC but not after digestion with the AC-lyase. Thus, the $\varDelta$Di-diS component of over-sulphated DS present in human arteries was proved
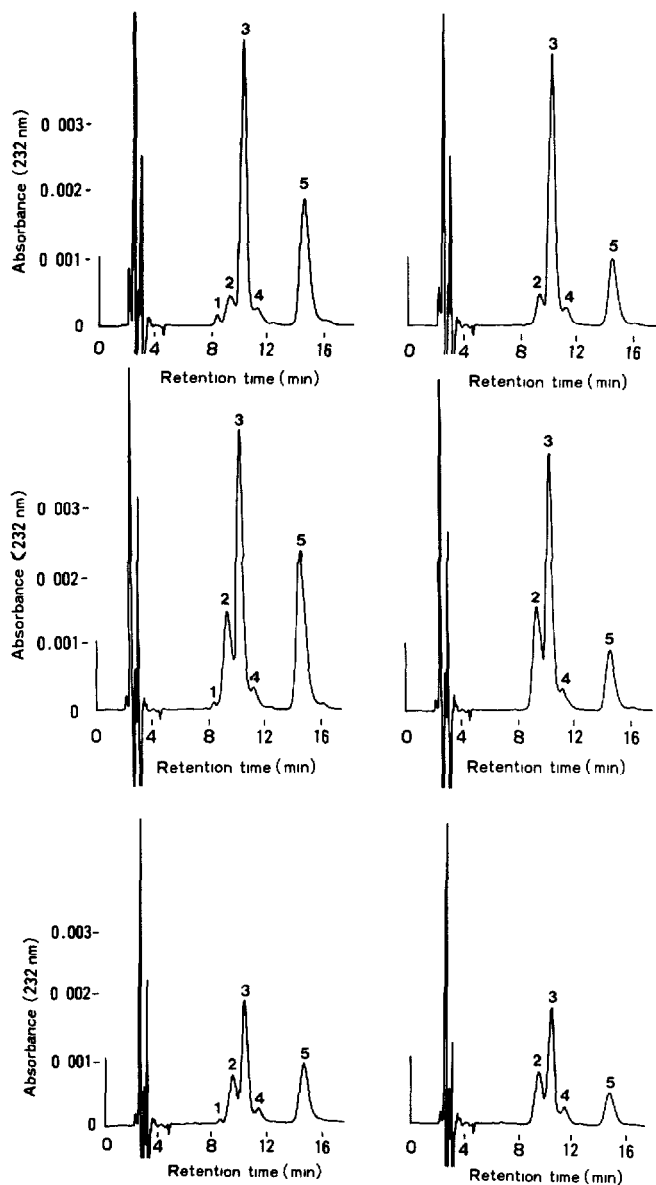
Fig. 2. HPLC of ΔDi-S derived from GAGs of human aortic tissues on digestion with chondroitinase-ABC and -AC. Note that the main ΔDi-S are ΔDi-6S and ΔDi-4S followed by an intermediate amount of ΔDi-0S$_{HA}$ and smaller amounts of ΔDi-diS$_G$ and ΔDi-0S. The amount of ΔDi-4S after digestion with the ABC-lyase was greater than that with the AC-lyase. ΔDi-diS$_G$ could be detected after digestion with chondroitinase-ABC but not with the AC-lyase. Left, treated with chondroitinase-ABC; right, treated with chondroitinase-AC. Top, normal aortic intima (approximately 11 μg as uronic acid); middle, atherosclerotic aortic intima (11 μg); bottom, aortic media (5 μg). Peaks: 1 = ΔDi-diS$_G$; 2 = ΔDi-0S$_{HA}$; 3 = ΔDi-6S; 4 = ΔDi-0S; 5 = ΔDi-4S.

TABLE II

PROPROTIONS OF HYALURONIC ACID, CHONDROITIN SULPHATE AND DERMATAN SULPHATE ISOMERS PREPARED FROM DIFFERENT LAYERS OF HUMAN AORTA AND CEREBRAL AND CORONARY ARTERIES

The percentages of hyaluronic acid and chondroitin sulphate and dermatan sulphate isomers of human arterial GAGs were determined as unsaturated disaccharide units by HPLC on a Shodex-RS (DC-613) column with the mobile phase acetonitrile–methanol-0.8 M ammonium formate (pH 4 5) (65 15:20, v/v/v). The values for individual GAGs were calculated from the corresponding peak areas followed by correction based on a calibration graph for each unsaturated disaccharide as described previously [11]. The values are the means of three determinations with different samples.

Glycosaminoglycan	Unsaturated disaccharide	Aortic intima				Cerebral artery	Coronary artery	
		Normal	Sclerotic	Media	Adventitia		Normal	Sclerotic
Chondroitin sulphate G	ΔDi-diS$_G$	2	1	2	2	2	3	3
Hyaluronic acid	ΔDi-0S$_{HA}$	6	15	21	31	2	19	16
Chondroitin 6-sulphate	ΔDi-6S	53	42	41	26	43	38	40
Chondroitin	ΔDi-0S	4	4	6	4	3	5	4
Chondroitin 4-sulphate	ΔDi-4S	19	14	16	16	14	15	11
Dermatan sulphate	ΔDi-4S	16	24	14	21	36	20	26

to be ΔDi-diS$_G$, as had been determined in previous studies [11,19,20,23].

The results from the ΔDi-S prepared from GAGs of various human arterial tissues are summarized in Table II, which shows that the major ΔDi-S from human arteries were ΔDi-6S and ΔDi-4S, followed by small amounts of ΔDi-diS$_G$, ΔDi-0S$_{HA}$ and ΔDi-0S. These data confirm those obtained previously by paper chromatography [19,22–24]. The present HPLC data show that the proportions of HA components are smaller in cerebral arteries than in coronary arteries and the aorta. An increase in DS was found with increasing severity of atherosclerosis.

DISCUSSION

This HPLC method for the analysis of ΔDi-S compounds gave specific, precise and highly reproducible results. The elution order of ΔDi-S with the proposed HPLC method using a highly cross-linked sulphonated polystyrene–divinylbenzene copolymer differs greatly from that obtained with the conventional systems involving silica gel. The specific mode of partitioning or absorption and/or both of the newly devised sulphonated polystyrene–divinylbenzene copolymers may be responsible for the separation of ΔDi-0S$_{HA}$ from ΔDi-6S.

The method was found to be especially effective in the separation of disaccharide isomers. The elution order of representative ΔDi-0S, ΔDi-monoS and ΔDi-diS was found to be similar to those obtained with 0.5 M ammonium formate–acetonitrile–methanol [11]. The present method can be used to separate ΔDi-0S$_{HA}$ from ΔDi-6S with the unique elution order of ΔDi-diS, although it shows delayed retention times and slightly lower sensitivity than those obtained with the previous 0.5 M ammonium formate solvent system.

ΔDi-S show similar responses to equivalent amounts of uronic acid and hence

can be analysed more precisely than with conventional methods using silica [6–8].

With the described HPLC method, $\varDelta$Di-0S, $\varDelta$Di-monoS, $\varDelta$Di-diS and $\varDelta$Di-triS can be analysed in less than 20 min. The present system also has advantages over silica HPLC, viz., a unique elution pattern and higher reproducibility with respect to the determination of different $\varDelta$Di-S compounds, i.e., $\varDelta$Di-0S and $\varDelta$Di-monoS, and $\varDelta$Di-diS and $\varDelta$Di-triS could be separated in the presence of $\varDelta$Di-0S$_{HA}$. Hence the present method provides even more information when used in conjunction with HPLC on silica gel.

The fact that $\varDelta$Di-diS$_G$ and $\varDelta$Di-diS$_D$ and also $\varDelta$Di-diS$_E$ and $\varDelta$Di-diS$_H$ had the same retention times on the new column and on silica gel indicates that Ch-S$_G$ or Ch-S$_D$ and Ch-S$_E$ or Ch-S$_H$ have epimeric structures. The $\varDelta$Di-diS and $\varDelta$Di-triS and also $\varDelta$Di-0S$_{HA}$ were found not only to have characteristic retention times but also to show satisfactory sensitivity. This is true for non-sulphated or under-sulphated Ch-S and also HA. The retention times of $\varDelta$Di-diS$_G$ and $\varDelta$Di-diS$_D$ were much lower than that of $\varDelta$Di-0S$_{HA}$, whereas both $\varDelta$Di-diS$_E$ and $\varDelta$Di-diS$_H$ appeared after $\varDelta$Di-4S. The peak of $\varDelta$Di-triS was detected later than that of $\varDelta$Di-diS$_B$. Although over-sulphated Ch-S or DS isomers are present in relatively small amounts compared with other major Ch-S isomers in various intercellular matrices, good resolution should be possible with the new HPLC procedure when identifying and measuring specific over-sulphated Ch-S isomers. The chondro-sulphatase application to the new HPLC system for obtaining desulphated $\varDelta$Di-S prepared from the parent sulphated $\varDelta$Di-S following digestion with chondroiti-nases is especially beneficial when there is little information on the starting GAGs which consist of variously sulphated Ch-S or DS isomers.

The results obtained for GAGs in various human arteries proved that, with the modified system, it is possible to detect as little as 0.25 μg of $\varDelta$Di-diS$_G$, and $\varDelta$Di-0S$_{HA}$ from HA and $\varDelta$Di-0S from chondroitin in the presence of large amounts of $\varDelta$Di-6S and and $\varDelta$Di-4S. Hence analysis with the present HPLC system provides a practical means for the identification and determination of $\varDelta$Di-monoS and $\varDelta$Di-diS generated from different Ch-S isomers in the presence of a small amount of $\varDelta$Di-0S$_{HA}$ or $\varDelta$Di-0S prepared from non-sulphated macromolecular compounds, i.e., HA and chondroitin, in various intercellular matrices.

ACKNOWLEDGEMENTS

REFERENCES

1 K. Murata, in R.L. Whistler and J.N. BeMiller (Editors), Methods in Carbohydrate Chemistry, Academic Press, New York, 1980, pp. 81–88.
2 H. Muir, Biochem. Soc. Trans., 11 (1983) 613–622.
3 D. Heinegård and M. Paulsson, in K.A. Piez and A.H. Reddi (Editors), Connective Tissue Biochemistry, Elsevier, Amsterdam, 1984, pp. 277–327.

4   S. Suzuki, H. Saito, T. Yamagata, K. Anno, N. Seno, Y. Kawai and T. Furuhashi, J. Biol. Chem., 243 (1968) 1523–1535.

5   K. Murata and Y. Yokoyama, Atherosclerosis, 45 (1982) 53–65.

6   G.J.-L. Lee, J.E. Evans and H. Tieckelmann, J. Chromatogr., 146 (1978) 439–448.

7   G.J.-L. Lee, D.-W. Liu, J.W. Pav and H. Tieckelmann, J. Chromatogr., 212 (1981) 65–73.

8   D.C. Seldin, N. Seno, K.F. Austin and R.L. Stevens, Anal. Biochem., 141 (1984) 291–300.

9   W.D. Comper and T.C. Laurent, Physiol. Rev., 58 (1978) 255–313.

10   J.R.E. Fraser, T.C. Laurent, A. Engström-Laurent and U.G.B. Laurent, Clin. Exp. Pharmacol. Physiol., 11 (1984) 17–25.

11   K. Murata and Y. Yokoyama, Anal. Biochem., 146 (1985) 327–335.

12   K. Murata and Y. Yokoyama, J. Chromatogr., 374 (1986) 37–44.

13   A.H. Hjerpe, C.A. Antonopoulos and B. Engfeldt, J. Chromatogr., 245 (1982) 365–368.

14   K. Hiyama and S. Okada, J. Biol. Chem., 250 (1975) 1824–1828.

15   K. Murata and Y. Horiuchi, Renal Physiol., 1 (1978) 48–55.

16   K. Anno, N. Seno, M.B. Mathews, T. Yamagata and S. Suzuki, Biochim. Biophys. Acta, 237 (1971) 173–177.

17   S. Suzuki, J. Biol. Chem., 235 (1960) 3580–3588.

18   Y. Kawai, N. Seno and K. Anno, J. Biochem., 60 (1966) 317–321.

19   K. Izuka and K. Murata, Atherosclerosis, 16 (1972) 217–224.

20   T. Harada, K. Murata, T. Fujiwara and T. Furuhashi, Biochim. Biophys. Acta, 177 (1969) 676–679.

21   T. Bitter and H.M. Muir, Anal. Biochem., 4 (1962) 330–334.

22   K. Murata, K. Nakazawa and A. Hamai, Atherosclerosis, 21 (1975) 93–103.

23   K. Murata, Stroke, 16 (1985) 687–694.

24   K. Murata, Connect. Tissue Res., 6 (1978) 131–138.