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Review

High-performance liquid chromatographic analysis of glycosaminoglycan-derived oligosaccharides

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Abstract

High-performance liquid chromatography of glycosaminoglycan (GAG)-derived oligosaccharides has been employed for the structural analysis and measurement of hyaluronan, chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate and heparin. Recent developments in the separation and detection of unsaturated disaccharides and oligosaccharides derived from GAGs by enzymatic or chemical degradation are reviewed.

Contents

1. Introduction

Glycosaminoglycans (GAGs) are negatively charged polysaccharides, which are classified on the basis of structure into several groups such as hyaluronan (HA), chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparan sulphate (HS) and heparin (HP). These are composed of alternating uronic acid and N-acetylhexosamine residues, with the exception of

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KS. Sulphate groups are attached to the limited position of hydroxy or amino groups on GAGs and contribute to their polyanionic properties.

In tissues, GAG chains are covalently linked to the protein core, and these glycoconjugates are termed proteoglycans (PGs). PGs occur mainly in the extracellular matrix or on the cell surface, and play important roles [1]. Recently, the biological significance of GAG chains in PGs has been elucidated precisely for HS/HP [2] and over-sulphated DS [3] with respect to anticoagulant activities. Further, the roles of CS chains in thrombomodulin [4] and inter-α-trypsin inhibitor [5,6] are investigated and discussed.

In metabolic pathways of GAGs, low-molecular-mass GAGs and PGs were shown to be excreted into urine. Thus the urinary GAGs have long been studied for their metabolism and diagnosis of inheritable diseases which are widely known as mucopolysaccharidosis [7].

In the analysis of GAGs in biological materials, the most common and available pretreatment procedures are the cetylpyridinium chloride (CPC) precipitation method and ethanol precipitation method, since GAGs have a strongly negative charge and large molecular size ($M_r > 10\,000$) [8,9]. Although the crude GAGs are collected easily, the separation and identification of several types of GAGs are very difficult. A useful and common method for qualitative analysis or identification is cellulose acetate membrane electrophoresis [8,9]. Biological studies on GAGs have been focused on the structure, biosynthesis and metabolism, and many analytical methods such as paper chromatography, liquid chromatography, gas chromatography, affinity chromatography and electrophoretic analysis have been developed and applied to the quantitative and qualitative analysis of GAGs [8,9]. Recently, capillary electrophoretic methods have made great progress and been applied to the GAGs. In the near future, this newly developed tool will be used for the quantitative analysis of biological samples [10–15]. However, the applicability of HPLC in the analysis of biological materials is generally accepted, and precise and various conditions for HPLC separation and detection systems have been established for the analysis of GAGs. Especially HPLC methods can be used widely for the purification and measurement of the molecular sizes of intact GAGs.

To elucidate the physiological functions and structural characteristics, it is useful to analyse the oligosaccharides that are enzymatically and chemically derived from GAGs. The oligosaccharides are separated by ion-exchange chromatography, reversed-phase ion-pair chromatography, partition chromatography, gel permeation chromatography and reversed-phase chromatography with precolumn derivatization, based on the differences in anion charge, polarity and molecular size.

In this paper, the selection of references to be reviewed was restricted to those describing HPLC/LC of GAG-derived oligosaccharides, which was used and devised for structural analysis and measurements of GAG chains.

2. Analysis of GAGs as their unsaturated disaccharides

GAGs have considerable heterogeneity concerning their molecular size, disaccharide composition and sulphate content. Therefore, analysis of the unsaturated disaccharides derived enzymatically from GAGs provides the most practical or perhaps the only quantitative approach. The method is called disaccharide compositional analysis or disaccharide mapping, and is effectively used as a definitive analytical technique for structural studies on GAGs. UV absorption at around 230 nm attributed to $\Delta^{4,5}$-hexuronic acid can be utilized for the detection and determination of the unsaturated disaccharides.

2.1. Determination of HA, CS and DS

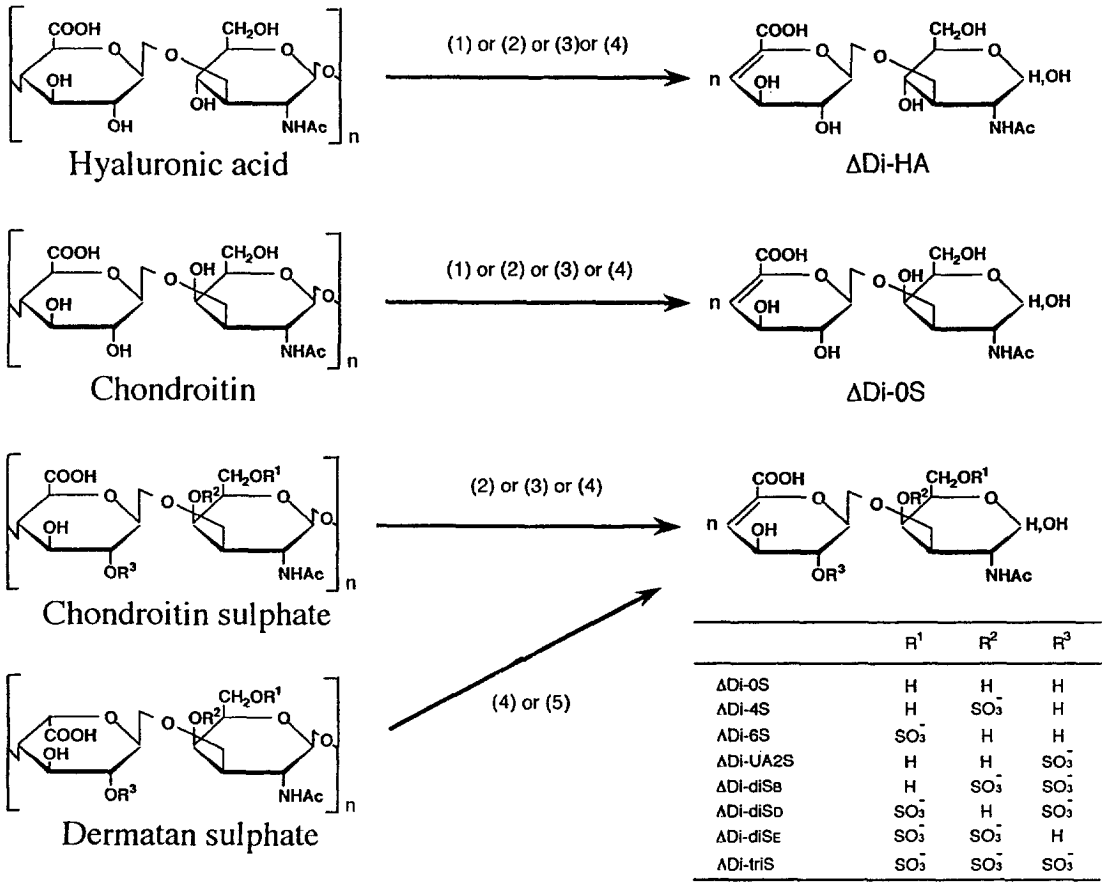
Since HA, CS and DS are composed of alternating β1–3 hexuronidic and β1–4-N-acetylhexosaminidic bonds, these structural isomers are decomposed into oligosaccharides with some common enzymes. Accordingly, the de-

termination of HA, CS and DS is discussed together.

2.1.1. Enzymatic digestion of HA, CS and DS

Several enzymes are commercially available for the degradation of HA, CS, and DS to their unsaturated disaccharides. The substrate specificity of these enzymes and products are summarized in Fig. 1.

Quantification of DS is usually carried out with the difference in the analytical values of chondroitinase ABC and AC digestions, since DS is not attacked by chondroitinase AC [18–21]. Chondroitinase ACII shows a mixed action pattern, initially endolytic followed by exolytic, whereas the action of chondroitinase ACI is endolytic [21], so that ACII does not efficiently act on DS/CS copolymers. In view of this difference, the appropriate approach should be employed to measure DS content. Chondroitinase ACII is normally selected for the measurement of DS content. When the molecular size of CS is relatively small, the analytical results obtained with chondroitinase ABC would be lower than those obtained with chondroitinase ACII [27,28], because the former does not act on a disaccharide unit nearest the linkage region [29,30]. Thus, cooperative digestion by chondroitinase ABC and ACII instead of chondroitinase ABC alone gives a more accurate estimate



	R^1	R^2	R^3
ΔDi-0S	H	H	H
ΔDi-4S	H	SO_3^-	H
ΔDi-6S	SO_3^-	H	H
ΔDi-UA2S	H	H	SO_3^-
ΔDi-diSB	H	SO_3^-	SO_3^-
ΔDi-diSD	SO_3^-	H	SO_3^-
ΔDi-diSE	SO_3^-	SO_3^-	H
ΔDi-triS	SO_3^-	SO_3^-	SO_3^-

Fig. 1. Enzymatic digestion of hyaluronic acid, chondroitin sulphate and dermatan sulphate. (1) Hyaluronidase SD (from *Streptococcus dysgalactiae*) (EC 4.2.2.–) [16,17]. (2) Chondroitinase AC I (from *Flavobacterium heparinum*) (EC 4.2.2.5) [18,19]. (3) Chondroitinase AC II (from *Arthrobacter aurescens*) (EC 4.2.2.5) [20,21]. (4) Chondroitinase ABC (from *Proteus vulgaris*) (EC 4.2.2.4) [22,23]. (5) Chondroitinase B (from *Flavobacterium heparinum*) (EC 4.2.2.–) [24–26].

Table 1
HPLC conditions for the determination of unsaturated disaccharides from HA, CS and DS

Ref.[a]	Compound[b]	Column[c] (particle size in μm, length in cm × I.D. in mm)	Eluent[b] (flow-rate in ml/min)	Analysis time (min)	Method of detection[b]	Enzyme[b]	Application[b]
[32]	0, 6, 4, B	LiChrosorb SI-100 (5, 25 × 4.6)	Dichloromethane–MeOH–0.5 *M* ammonium formate (pH 4.8), 60:34:6 (2.0)	30	254 nm	ABC, ACI	Urine from MPS
[33]	0, 6, 4	Partisil-10 PAC (10, 25 × 4.6)	MeCN–MeOH–0.5 *M* ammonium formate (pH 4.8), 60:15:25 (2.0)	10	254 nm	ABC, ACII	C4, DS, C6
		LiChrosorb NH_2 (10, 25 × 4.6)	MeOH–0.5 *M* ammonium formate (pH 4.8), 35:65 (2.0)	8	254 nm		
[34]	0, 6, 4	μ Bondapak-C_{18} (10, 30 × 4.0)	0.035 *M* TBA hydroxide adjusted to pH 7.54 with 0.01 *M* phosphate (2.5)	25	232 nm	ABC	C4, C6
[35]	0, 6, 4	μ Bondapak Carbohydrate (10, 30 × 4.0)	0.2 *M* sodium acetate at pH 5.0 (0.5) or 0.02 *M* sodium sulphate in 0.01 *M* acetate buffer at pH 5.0 (0.5)	20	231 nm	ABC	C4, C6
[36,38]	0, 6, 4, B, E	Partisil-10 PAC (10, 25 × 4.6)	MeCN–MeOH–0.5 *M* ammonium formate (pH 4.8), 60:20:20 (2.0)	10	254 nm	ABC, AC	C4, C6, urine from MPS
[37]	r4, r6	Partisil 10 SAX (10, 25 × 4.6)	40 m*M* potassium dihydrogenphosphate containing 15% MeOH (1.0)	40	Rad		
[63]	r0, r6, r6	Partisil-10 PAC (10, 25 × 4.6)	MeCN–MeOH–0.5 *M* ammonium formate (pH 6.0), 69:14:17 (1.5)	16	254 nm	ABC, ACII	C4, C6
[39]	0, 6, 4, E	Partisil 10 SAX (10, 25 × 4.6)	(0–7 min) 7.5 m*M* potassium phosphate buffer (pH 6.5) (5.0) (7–17 min) gradient elution [39]	17	232 nm	ABC, AC	C4, DS, C6, urine from MPS
[40]	0, 6, 4, diS	Hypersil APS (25 × 4.6)	0.1 *M* sodium sulfate containing 0.05 *M* AcONa at pH 5.0 (0.7)	10	231 nm	AC	C6
[41]	H, 0	Hypersil APS (25 × 4.6 with 10 × 4.6)	9 m*M* sodium dihydrogenphosphate buffer (pH 2.55) (1.1)	15	231 nm	ABC, AC	HA, C0
[73]	diS, 6, 4.0	μ Bondapak-C_{18} (10, 30 × 3.9)	MeOH–8 m*M* potassium phosphate buffer (pH 6.0), 1:30 (0.4 or 0.6)	30	Pre, 2-AP Ex. 310 nm, Em. 375 nm	ABC, AC	C4, DS, C6, urine from MPS
[42]	0, 6, 4, diS	Partisil-10 PAC (10, 25 × 4.6)	70% MeCN–MeOH (3:1) and 30% 0.5 *M* ammonium acetate–acetic acid (pH 5.3) (1.0)	20	232 nm	ABC, AC	C4, DS, C6, dDS, CD,
	T	Partisil-10 PAC (10, 25 × 4.6)	65% MeCN–MeOH (3:1) and 30% 0.5 *M* ammonium acetate–AcOH (pH 5.3) (1.0)	15	232 nm		CE, CH, CK, CT
[43]	D, 6, 0, 4, E	Shodex RS (DC-613) (10, 15 × 6.0) 70°C	MeCN–MeOH–0.5 *M* ammonium formate (pH 4.5), 65:15:20 (1.0)	10	232 nm	ABC, ACII	Coronary arteries
[44]	D, U, 6, 0, B, 4, E	Shodex RS (DC-613) (10, 15 × 6.0 with 7 × 6.0) 70°C	MeCN–MeOH–0.5 *M* ammonium formate (pH 4.5), 65:15:20 (1.0)	15	232 nm	ABC, ACII, 4S, 6S	C4, C6, CB, CD, CE, CG, CH
[45]	H, 0	Shodex RS (DC-613) (10, 15 × 6.0 with 7 × 6.0) 70°C	MeCN–MeOH–0.5 *M* ammonium formate (pH 4.5), 65:15:20 (1.0)	10	232 nm		
[74]	diS, 6, 4, 0	ERC-ODS-1771 (20 × 6.0) 18°C	MeOH–6.5 m*M* potassium phoshate buffer (pH 6.05), 1:100 (0.6)	45	Pre, 2-AP Ex. 310 nm, Em. 375 nm	ABC, ACII, 4S, 6S	Urine from MPS
[46]	H, 0, 6, 4	Partisil-5 PAC (5, 25 × 4.6)	MeCN–MeOH–[0.5 *M* Tris–0.1 *M* boric acid–3.6 m*M* sulphate pH 8.0], 52:12:36 (1.2)	25	229 nm	ABC	Urine from MPS
[70]	H, 0, U, 6, 4, D, B, E	TSKgel NH_2-60 (5, 25 × 4.6)	MeCN–0.1 *M* acetate buffer (pH 5.6), 90:20 (1.0)	90	Pre, Dns		
[47]	0, 6, 4	Zorbax NH_2 (6, 25 × 4.6)	4% MeOH, 60% 0.5 *M* ammonium formate (pH 5.5) (0.9)	16	232 nm		

[48]	0, 6, 4	Separon SIX NH_2 (10, 25 × 4.6) 50°C	10 m*M* sodium sulphate and 1 m*M* AcOH (2.2)	4	232 nm		
[49]	D, H, 6, 0, 4	Shodex RS (DC-613) (10, 15 × 6.0 with 7 × 6.0) 70°C	MeCN–MeOH–0.8 *M* ammonium formate (pH 4.5), 65:15:20 (1.0)	16	232 nm	ABC, AII	Aortic tissues
[50]	H, 6, 0, B, T, 4, E	Shodex RS (DC-613) (6, 15 × 6.0 with 4.6 × 6.0) 70°C	MeCN–MeOH–0.8 *M* ammonium formate (pH 4.5), 65:15:20 (1.0)	20	232 nm	ABC, ACI, cB, 4S, 6S, 2S	Kidney
[71]	0, 6, 4	TSKgel NH_2-60 (5, 25 × 4.6)	MeCN–0.1 *M* acetate buffer (pH 5.6), 90:20 (1.0)	35	Pre, Dns	ABC	Urine
[51]	0, 6, 4	TSKgel NH_2-60 (5, 25 × 3.0)	10 m*M* ammonium formate (pH 5.0) containing 10 m*M* sodium sulphate in 4% MeCN (0.5)	20	Pos, 2-CA Ex. 346 nm, Em. 410 nm	ABC	Plasma
[52]	H, 0, U, 6, 4, D, B, E, T	TSKgel Amide-80 (5, 25 × 4.6) 70°C	MeCN–MeOH–0.5 *M* ammonium formate (pH 4.8), 70:5:25 (0.5)	80	232 nm	ABC	HA, C0, C4, DS, C6
[53]	(H, 0), (U, 6), 4, D, (B, E), T	LiChrosorb NH_2 (25 × 2.6) 40°C	Linear gradient from 16 to 800 m*M* sodium dihydrogenphosphate over 60 min (1.5)	30	230 nm	ABC, 6S, 2S	CS, DS
[54]	0, (U, 6), 4, D, (B, E), T	Polyamine-bound silica PA03 (25 × 4.6)	Linear gradient from 16 to 530 m*M* sodium dihydrogenphosphate over 60 min (1.0)	60	232 nm	ABC, 2S, 4S, 6S	EHS-tumour
[55]	4, 6	Ion Pac AS5A (5, 15 × 4.0)	12 m*M* sodium carbonate and 6 m*M* sodium hydrogencarbonate (1.0)	15	*Conductivity*	ABC	C4, DS, C6
	4, 6	Spherisorb SAX (5, 25 × 4.6)	0–0.15 *M* NaCl (adjusted to pH 3.5) gradient over 27 min	27	232 nm		
[81]	0, 6, 4	TSKgel NH_2-60 (5, 25 × 3.0)	10 m*M* ammonium formate (pH 5.0) containing 10 m*M* sodium sulphate in 4% MeCN (0.5)	20	Pos, 2-CA	ABC	Urine, serum
	D, B, E	TSKgel NH_2-60 (5, 25 × 3.0)	10 m*M* ammonium formate (pH 5.0) containing 25 m*M* sodium sulphate in 4% MeCN (0.5)	35	Ex. 346 nm, Em. 410 nm		
	T	TSKgel NH_2-60 (5, 5 × 4.0)	10 m*M* ammonium formate (pH 5.0) containing 60 m*M* sodium sulphate in 4% MeCN (0.5)	20			
[27,56]	H, 0, 6, 4, D, B, E	TSKgel NH_2-60 (5, 25 × 4) 30°C	MeCN–0.1 *M* Tris–HCl containing 0.1 *M* boric acid and	90	Pos, 2-CA	ABC, ACII	Urine
56]	H, 0		10 m*M* sodium sulphate (pH 7.0 with conc. HCl), 64:36 (0.5)	30	Ex. 346 nm, Em. 410 nm	SD	Urine from pregnant women
[79]	H, 0, U	TSKgel NH_2-60 (5, 15 × 4.6)	MeCN–0.1 *M* acetate buffer (pH 6.0), 80:20 (1.0)	40	Pre Dns with Chem	SD	Plasma
[57]	(H, 0), (U, 6), 4, D, B, E, T	Partisil-5PAC (5, 25 × 4.6)	MeCN–MeOH–[0.5 *M* Tris–0.1 *M* boric acid–23.4 m*M* sulphate (pH 8.0)], 48:14:38 (1.2)	40	229 nm	ABC	HA, C4, DS, C6, CD, CE
	H, 0, U, 6, 4	Partisil-5PAC (5, 25 × 4.6)	MeCN–MeOH–0.5 *M* Tris–0.1 *M* boric acid—23.4 m*M* sulphate (pH 8.0)], 52:12:36 (1.2)	26	229 nm		Kidney
[80]	H, 0, U, 4, D, B, E, T	TSKgel Amide-80 (5, 25 × 4.0) 50°C	MeCN–0.15 *M* acetate buffer (pH 5.0), 80:20 (0.8)	55	Pre, Dns with Chem	ABC, ACII	Mast cells
	H, 0, U, 6, 4	TSKgel NH_2-60 (5, 25 × 4.6) 45°C	MeCN–0.1 *M* acetate buffer (pH 5.0), 80:20 (1.0)	50			
[72]	H, 0, 6, 4	TSKgel NH_2-60 (5, 25 × 4.6)	MeCN–0.1 *M* acetate buffer (pH 5.6), 76:20 (1.0)	40	Pre, Dns Ex. 350 nm, Em. 530 nm	ACII	Perio-*dontium*
[75]	B, D, E, 4, 6, H, 0	CHEMCO $3C_{18}$-H (3, 10 × 6.0) 40°C	MeOH–66.7 m*M* sodium phosphate buffer (pH 7.0), 2.7:100 (1.0)	20	Pre, 2-AP Ex. 320 nm, Em. 400 nm	ABC, AC	Skin
[59]	4	Phase Separations C_6 (5, 15 × 1.0)	Solvent A: 3.3 m*M* TPAOH adjusted to pH 4.0 with formic acid Solvent B: 3.3 m*M* TPAOH–*MeCN* (10:90), pH 4.0 0–3 min, 0% B; 3–20 min, 0–40% B; 40–50 min, 40% B (0.05)	30	MS	ABC	DS

(Continued on p. 280)

Table 1 (*continued*)

Ref.[a]	Compound[b]	Column[c] (particle size in μm, length in cm × I.D. in mm)	Eluent[b] (flow-rate in ml/min)	Analysis time (min)	Method of detection[b]	Enzyme[h]	Application[b]
[68]	B, 4, E, 0, 6, H, D	Carbonex (7, 10 × 4.6) 50°C	4 m*M* sodium carbonate containing 0.5 m*M* sodium hydrogencarbonate in 3% MeCN (0.5)	50	Pos, 2-CA Ex. 335 nm, Em. 395 nm		
[76]	B, D, E, 6, 4, 0, H	InertsilPH (25 × 2.6) 50°C	Linear gradient from 6 to 31% of MeCN–water (1:1) in 20 m*M* phosphate buffer (pH 7.5) containing 5% MeCN	26	Pre, PMP 245 nm MS	ABC, ACII	Skin
[60]		Spherisorb Hexyl reversed-phase column (5, 25 × 4.6)	Solvent A: 3.3 m*M* TPAOH adjusted to pH 4.0 with formic acid Solvent B: 3.3 m*M* TPAOH–MeCN (10:90), pH 4.0 0–3 min, 0% B; 3–23 min, 0–50% B; 23–30 min, 50% B (1.0)		MS		
[61]	6, 4	Nucleosil SB 5 (5, 12.5 × 4.6) with Hypersil ODS RP-C_{18} (5, 12.5 × 4.6)	0.22 *M* NaCl (0.8)	20	232 nm	ABC, ACII	Plasma Serum
	H, 0	Hypersil APS (5, 25 × 4.6) with Hypersil ODS RP-C_{18} (5, 1.5 × 4.6)	2.5 m*M* disodium hydrogenphosphate (pH 3.0) containing 15 m*M* NaCl (0.8)	20			
[62]	H, 0	Econosphere NH_2 5U (5, 25 × 4.6)	5 m*M* sodium dihydrogenphosphate buffer (pH 2.55) (1.1)	10	231 nm	ABC, AC, cB	CS, DS,
	r0, r4, r6, rU, r3		50 m*M* sodium dihydrogenphosphate buffer (pH 2.50) (0.7)	25		4S, 6S	squid
	r0, (r4, r6, rU), r3, rN		50 m*M* sodium dihydrogenphosphate buffer (pH 5.0) (0.7)	25			skin CS,
	6, 4, N, D, B, E, NT, (T, 3T)		50 m*M* sodium sulphate–10 m*M* AcONa (pH 5.0) (1.5)	40			sea urchin
[131]	r0, rH, r4, rU, r6, rB, rE, rD, rT	CarboPac PA1 (10, 25 × 4.6)	Solvent A: 0.1 *M* NaOH Solvent B: 0.5 *M* TFA in 0.1 *M* NaOH 0–12 min, 3% B; 12–32 min, 3–26% B, 32–42 min, 26% B; 42–62 min, 26–60% B; 62–72 min, 60% B; 72–82 min, 60–100% B (1.0)	80	PAD		
			Solvent A: 0.1 *M* NaOH Solvent B: 1.0 *M* NaCl in 0.1 *M* NaOH 0–12 min, 2% B; 12–62 min, 2–60% B; 62–70 min, 60–100% B; 70–85 min, 100% B (1.0)	85	232 nm		

[a] References are listed through the table in chronological order.

[b] The names of carbohydrates are given in the order of elution. H, ΔDi-HA; 0, ΔDi-0S; 4, ΔDi-4S; 6, ΔDi-6S; U, ΔDi-UA2S; B, ΔDi-diS_B; D, ΔDi-diS_D; E, ΔDi-diS_E; T, ΔDi-triS (Fig. 1). 3, Δdi-mono3S; N, Δdi-monoNS; NT, Δdi-tri(2,6,N)S; 3T, Δdi-tri(3,4,6)S [62]. 0-0, ΔUA-GlcNAc; 2-0, ΔUA2S-GlcNAc; 0-6, ΔUA-GlcNAc6S; 2-6, ΔUA2S-GlcNAc6S; 0-N, ΔUA-GlcNS; 2-N, ΔUA2S-GlcNS; 0-N6, ΔUA-GlcNS6S; 2-N6, ΔUA2S-GlcNS6S (Fig. 4). diS, unsaturated disulphated disaccharides. The r at the beginning of abbreviations indicates that the hemiacetal group is reduced to the primary hydroxyl group. MeOH, methanol; MeCN, acetonitrile. TPA, tetrapropylammonium; TBA, tetrabutylammonium; TFA, sodium trifluoroacetate. Pre, precolumn; Pos, postcolumn; Rad, radiolabelled; 2-CA, 2-cyanoacetamide; 2-AP, 2-aminopyridine; Dns, dansylhydrazine; PMP, 1-phenyl-3-methyl-5-pyrazolone; Chem, chemiluminescence; MS, mass spectrometry; PAD, pulsed amperometric detection. ABC, chondroitinase ABC; AC, chondroitinase AC; cB, chondroitinase B; 4S, chondro-4-sulphatase; 6S, chondro-6-sulphatase; 2S, glycuronate-2-sulphatase; H1, heparin lyase I; H2, heparin lyase II; H3, heparin lyase III. C4, chondroitin 4-sulphate; DS, dermatan sulphate; C6, chondroitin 6-sulphate; dDS, disulphated dermatan sulphate [42]; CG, chondroitin sulphate G [44]; CD, chondroitin sulphate D; CE, chondroitin sulphate E: CH, chondroitin sulphate H; CK, chondroitin sulphate K; CT, trisulphated chondroitin sulphate. MPS, mucopolysaccharidoses; EHS-tumour, Engelbreth–Holm–Swarm mouse tumour.

[c] Suppliers of columns: LiChrosorb, Merck; Partisil, Whatman; μBondapak, Waters; Hypersil, Shandon; Shodex, Showa Denko; REC-ODS, Eruma Kogaku; TSKgel, Tosoh; Zorbax, DuPont; Separon SIX, Laboratórní Přístroje; Polyamine-bound silica PA03, YMC; Ion Pac, Dionex; Spherisorb, Phase Separations; CHEMCO C_{18}, Chemco; Carbonex, Tonen; Inertsil, GL Sciences; Nucleosil, Macherey–Nagel; Econosphere, Alltech; CarboPac PA1, Dionex.

for the determination of DS in biological materials containing CS and DS. Chondroitinase B, which catalyses the eliminative cleavage of N-acetylgalactosaminide linkages to L-iduronic acid units in DS [24–26], could be favourable in such a case. However, careful analyses are required because the commercially available chondroitinase B is expensive, unstable and occasionally contains significant amounts of chondrosulphatases as contaminant.

The conditions of enzymatic digestion differ slightly within each laboratory. For details, the reader should refer to reviews [8,9,31,134] and/or the references contained in Table 1. Thus, our procedures will hereafter be described as an example.

For chondroitinase ABC and/or ACs digestion, a 20-μl portion of sample containing up to 100 μg of GAGs, 10 μl of 0.2 *M* Tris–acetate buffer (pH 8.0) and 10 μl of aqueous solution containing 0.05 U of each enzyme are mixed, then the mixture is incubated at 37°C for 3 h (1 U is defined as the amount of the enzyme that catalyses the formation of 1 μmol of unsaturated disaccharides from chondroitin sulphate per minute at 37°C). For hyaluronidase SD digestion, a 20-μl portion of sample containing up to 100 μg of GAGs, 10 μl of 0.2 *M* sodium phosphate buffer (pH 6.2) and 10 μl of aqueous solution containing 0.05 U of enzyme are mixed, then the mixture is incubated at 37°C for 3 h. For chondroitinase B digestion, a 20-μl portion of sample containing up to 50 μg of GAGs, 10 μl of 0.2 *M* Tris–acetate buffer (pH 8.0) and 10 μl of aqueous solution containing 0.05 U of enzyme are mixed, then the mixture is incubated at 37°C for 3 h. Each reaction tube is heated in a boiling water-bath for 30 s to stop the digestion.

2.1.2. Separation of the unsaturated disaccharides derived from HA, CS and DS

The unsaturated disaccharides have been separated with various HPLC modes such as normal-phase chromatography, reversed-phase ion-pair chromatography and anion-exchange chromatography [32–63,131] (Table 1). In 1979, an amino-bonded silica column was used for the separation of the unsaturated disaccharides produced from CS [33,35]. Since that time, amino-bonded silica has been one of the most commonly used packings for the separation of oligosaccharides derived from GAGs, although it is chemically unstable [64] and its lifetime is relatively short. In the methods, the elution conditions for oversulphated disaccharides (ΔDi-diS_B, ΔDi-diS_D, ΔDi-dS_E and ΔDi-triS; abbreviations as in Fig. 1) are very different from those for non- and monosulphated disaccharides, and thus a salt gradient elution has been employed for simultaneous determination [53,54]. Moreover, complete separations of ΔDi-UA2S and ΔDi-6S (or ΔDi-4S). ΔDi-diS_B and ΔDi-diS_E are frequently difficult on commercially available amino-bonded silica columns. In this case, it is necessary for identification of peaks to make a comparison between the two chromatograms with or without enzymatic digestion by chondro-4- or 6-sulphatases [65–67], which catalyse the hydrolysis of the sulphate ester at positions 4 or 6 of the N-acetylgalactosamine in the unsaturated disaccharides, respectively.

Chondroitinases digest both HA and CS, ΔDi-HA and ΔDi-0S mostly being produced from the samples. These disaccharides differ only by being C-4 epimers of the hexosamine part (Fig. 1), and separation of the disaccharides from each other is difficult to achieve. Several attempts to separate the saccharides using an isocratic system have been reported, as follows: separation on an anion-exchange column under acidic condition [41,61]; on a sulphonated styrene–divinylbenzene copolymer column [45]; on an amido-bonded silica column [52]; on a graphitized carbon column [68]; and separation as their borate complexes (Fig. 2) on an anion-exchange column [27,46,56,57]. For the determination of HA, digestion with hyaluronidase (EC 4.2.2.1) purified from *Streptomyces hyalurolyticus* [69] is sufficiently effective to avoid the difficulty described above. This enzyme is specific for HA and yields $\Delta^{4,5}$-unsaturated tetra- and hexasaccharides as the products. Then the unsaturated oligosaccharides are submitted to HPLC. This subject will be discussed further in the next section (see Section 3.1 and Table 4).

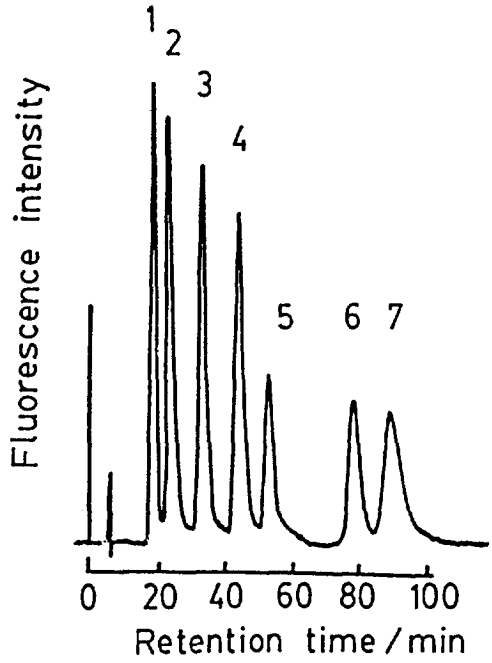


Fig. 2. Typical chromatogram of standard unsaturated disaccharides. Column, TSKgel NH_2-60 (250 mm × 4.0 mm I.D.); eluent, 52% acetonitrile in aqueous buffer (0.1 *M* boric acid, 0.1 *M* Tris and 10 m*M* sodium sulphate adjusted to pH 7.0 with concentrated HCl); column temperature, 30°C; flow-rate, 0.5 ml/min; reagent 1, 1% 2-cyanoacetamide (0.25 ml/min); reagent 2, 0.3 *M* sodium hydroxide (0.25 ml/min); reaction coil, 10 m × 0.5 mm I.D.; reaction temperature, 100°C; cooling coil, 2 m × 0.25 mm I.D.; detection, fluorescence (excitation 346 nm, emission 410 nm); sample size, 10 μl (100 ng of each sugar). Peaks: 1 = ΔDi-HA; 2 = ΔDi-0S; 3 = ΔDi-6S; 4 = ΔDi-4S; 5 = ΔDi-diS_D; 6 = ΔDi-diS_B; 7 = ΔDi-diS_E. Reproduced from Ref. [27], with permission.

2.1.3. Detection of the unsaturated disaccharides

The most common detection method is UV absorption at around 230 nm, with routinely detectable amounts in the nanogram range for the injected sample. However, the detection system is not sensitive enough and sometimes requires time-consuming and tedious preparation for the micro-determination of biological samples.

To improve the detection limits and specificity, pre- and postcolumn and chemiluminescence detection techniques have been investigated. Dansylhydrazine [70–72], 2-aminopyridine [73–75] and 1-phenyl-3-methyl-5-pyrazolone (PMP) [76] are used as precolumn reagents. A postcolumn reagent for reducing sugars, 2-cyanoacetamide [77], has been employed for fluorimetric [27,51,56] or UV detection [78]. HPLC with chemiluminescence detection has been reported using dansylhydrazine as a precolumn reagent, and bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) and hydrogen peroxide as energy transfer reagents [79,80]. These detection systems allow detectable amounts to be extended to the picogram level. Conveniently, the separation of ΔDi-HA and ΔDi-0S is effected by precolumn derivatization techniques.

A postcolumn method using 2-cyanoacetamide is especially well suited for unsaturated disaccharides with fluorimetric detection. The relative fluorescence intensities of unsaturated disaccharides were about 300–400 times stronger than those of neutral monosaccharides (Table 2) [51]. As a result of basic studies of the postcolumn method, this selective and sensitive detection technique has facilitated the determination of GAGs in various biological samples such as plasma and serum [51,81,82] (Fig. 3), blood cells

Table 2
Comparison of fluorescence intensities formed from various carbohydrates

Carbohydrate	Relative molar fluorescence intensity (Ex. 346 nm)/(Em. 410 nm)
D-Glucose	1
D-Galactose	1
D-Glucosamine·HCl	7
D-Galactosamine·HCl	4
D-Glucuronic acid	7
D-Galacturonic acid	19
N-Acetyl-D-glucosamine	22
N-Acetyl-D-galactosamine	61
ΔDi-0S	398
ΔDi-4S	436
ΔDi-6S	456

Detection was performed with fluorimetric postcolumn labelling using 2-cyanoacetamide. Reproduced from Ref. [51] with permission.

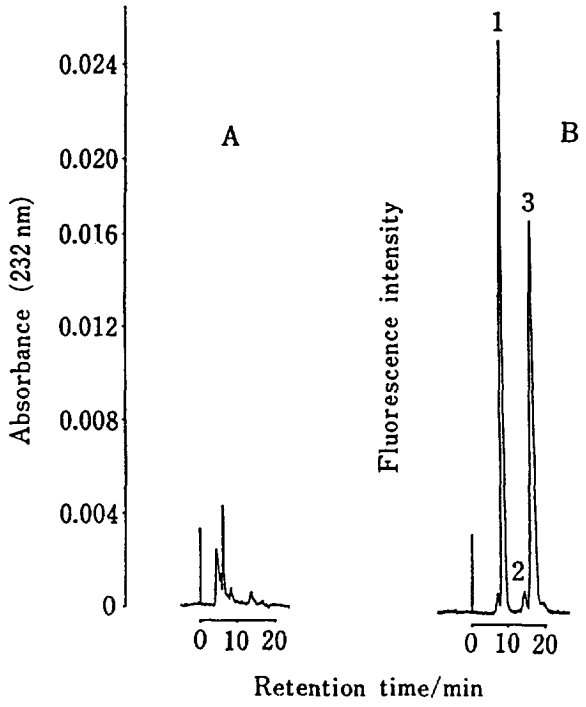


Fig. 3. Chromatograms of unsaturated disaccharides produced from rabbit plasma GAGs. Column, TSKgel NH_2-60 (250 mm × 3.0 mm I.D.); eluent, 20 m*M* ammonium formate buffer (pH 5.0) containing 10 m*M* sodium sulphate in 4% acetonitrile; flow-rate, 0.5 ml/min; sample size, 10 μl; amount injected, 25% of the enzymatically digested products from CS in 100 μl of plasma. (A) UV detection at 232 nm. (B) Fluorimetric detection as follows: to the eluate were added 0.125 *M* sodium tetraborate–sodium hydroxide buffer (pH 10.8) (0.2 ml/min) and aqueous 1% 2-cyanoacetamide solution (0.2 ml/min). The mixture passed through a reaction coil (10 m × 0.5 mm I.D.) set in a dry reaction bath at 100°C and a following cooling coil (10 m × 0.25 mm I.D.). The effluent was monitored by the spectrofluorimeter (excitation 346 nm, emission 410 nm). Peaks: 1 = ΔDi-0S; 2 = ΔDi-6S; 3 = ΔDi-4S. Reproduced from Ref. [51], with permission.

[83,84], urine [27,81,85], culture medium [86] and tissues [87].

Recently, HPLC with on-line connection to mass spectrometry (MS) has been introduced for the characterization of sulphated disaccharides and oligosaccharides derived from GAGs [59, 60]. In addition, conductivity detection is possible [55], although its sensitivity and specificity are inferior to those of UV detection at 232 nm.

2.2. Determination of HS and HP

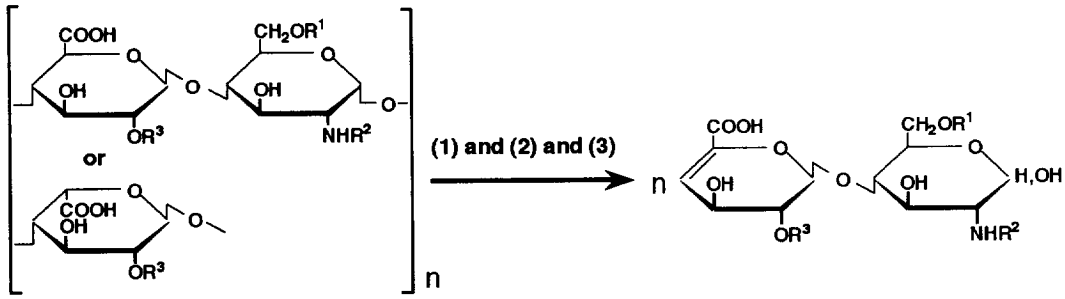
HS is one of the most complex mammalian polysaccharides. A disaccharide repeat of glucosamine and hexuronic acid makes up the basic backbone structure [88–90]. A high sulphate content and anticoagulant activity are characteristic of HP, which is believed to originate from mast cells. This highly sulphated GAG contains the same structural units as those found in HS as described above. Usually HP contains more sulphate groups and iduronate residues than HS does.

In this section, HPLC methods for determination of the unsaturated disaccharides derived from HS and HP by the specific enzymes are described.

2.2.1. Enzymatic digestion of HS and HP

Heparin lyases, isolated from *Flavobacterium heparinum* [91–93,95–97], also known as *Cytophaga HPa* [94], are very important enzymes of a class of polysaccharide lyases (EC 4.2.2) that degrade certain glycosaminoglycans. Recently, heparin lyase I (heparinase, EC 4.2.2.7), heparin lyase II (heparitinase II, no assigned EC number) and heparin lyase III (heparitinase I, EC 4.2.2.8) have been purified to homogeneity [93,132,133] and are commercially available from Seikagaku American, Grampian Enzymes and Sigma. Quantitative analyses for the unsaturated disaccharide composition of HP and HS indicate that the thorough digestion of samples using a mixture of the three lyases is required. However, it must be noted that some oligosaccharide structures in HP and HS are resistant to the digestion [127]. The main products of heparin lyases are summarized in Fig. 4.

Studies by a large number of researchers have demonstrated that the principal cleavage site by heparin lyase I is →4)-α-D-GlcNp2S6S(or 6OH)(1→4)-α-L-IdoAp2S(1→ and by heparin lyase III is →4)-α-D-GlcNp2S(or 2Ac)6S(or 6OH)-(1→4)-β-D-GlcAp(1→. Using defined oligosaccharide substrates, it was shown that heparin lyase I also tolerates 3-O-sulphation and/or 6-O-sulphation on the α-D-2-deoxy-2-aminoglucopyranose residue [96].



Heparan sulphate and heparin

Unsaturated disaccharides

(1), Heparin lyase I (Heparinase, Heparinase I), (from *Flavobacterium heparinum*) (EC 4.2.2.7).

(2), Heparin lyase II (Heparitinase II, Heparinase II), (from *Flavobacterium heparinum*) (no EC No.).

(3), Heparin lyase III (Heparitinase I, Heparinase III), (from *Flavobacterium heparinum*) (EC 4.2.2.8).

		R^1	R^2	R^3
I	ΔUA-GlcNAc	H	Ac	H
II	ΔUA2S-GlcNAc	H	Ac	SO_3^-
III	ΔUA-GlcNAc6S	SO_3^-	Ac	H
IV	ΔUA2S-GlcNAc6S	SO_3^-	Ac	SO_3^-
V	ΔUA-GlcNS	H	SO_3^-	H
VI	ΔUA2S-GlcNS	H	SO_3^-	SO_3^-
VII	ΔUA-GlcNS6S	SO_3^-	SO_3^-	H
VIII	ΔUA2S-GlcNS6S	SO_3^-	SO_3^-	SO_3^-

Fig. 4. Enzymatic digestion of heparan sulphate and heparin.

The primary specificity of heparin lyase II was shown to be broad-based in its ability to degrade HP, HS and chemically modified HP chains [91,97,100]. Heparin lyase II acts on linkages to either α-L-idopyranosyluronic or β-D-glucopyranosyluronic acid residues [92]. Studies using heparin lyase II on defined oligosaccharide substrates agreed with the results from the polymeric substrate studies of Moffat et al. [91]. These studies also demonstrated that heparin lyase III could tolerate a 6-O-sulphate group on the α-D-deoxy-2-aminoglucopyranose moiety, in contrast to a report by Nader et al. [97].

The evaluation of the substrate specificity of each heparin lyase has been very difficult owing to the many contrasting observations made by researchers in this field. It has been pointed out by Lohse and Linhardt [93] that some of these anomalous observations are probably the result of contaminating impurities in preparations of the heparin lyases studied.

2.2.2. Separation of the unsaturated disaccharides derived from HS and HP

A simple and reliable HPLC method for the separation of the unsaturated disaccharides derived from HS and HP was described by Murata and co-workers [98,99]. It uses a sulphonated styrene–divinylbenzene copolymer resin column (150 mm × 6 mm I.D.) at 70°C and isocratic elution with acetonitrile–methanol–0.8 *M* ammonium formate (pH 4.5). Several HPLC systems have been described elsewhere [53,68,98–104] (Table 3).

Anion-exchange HPLC [53,100,101] and reversed-phase ion-pair HPLC [101–103] have been used to separate unsaturated disaccharides and large oligosaccharides prepared by enzymatically depolymerizing HS and HP with heparin lyases. Six unsaturated disaccharides (I, III, V, VI, VII and VIII) shown in Fig. 4 were eluted and separated using an HPLC system with a Dionex CarboPac PA1 column [104]. Eight un-

Table 3
HPLC conditions for the determination of unsaturated disaccharides from HS and HP

Ref.[a]	Compound[b]	Column[c] (particle size in μm, length in cm × I.D. in mm)	Eluent[b] (flow-rate in ml/min)	Analysis time (min)	Method of detection[b]	Enzyme[b]	Application[b]
[101]	0-0, 0-6, 0-N	Partisil 10 PAC (10, 25 × 4.6)	MeCN–MeOH–0.5 *M* ammonium acetate (pH 6.5), 60:20:20 (1.0)	12	254 nm		
	2-N6	Partisil 10 ODS (10, 25 × 4.6)	MeOH–0.005 *M* TBA phosphate (pH 7.0), 10:90 (1.0)	10			
[63]	r0-0, r0-6, r0-N	Partisil 10 PAC (10, 25 × 4.6)	MeCN–MeOH–0.5 *M* ammonium acetate (pH 4.5), 60:20:20 (1.5)	12	254 nm		
[103]	0-0, 0-N, 0-6, 0-N6	Jasco SC-02 (25 × 4.6)	10 m*M* TBA phosphate (pH 7.0) containing 30% MeOH (1.0)	25	232 nm	H1, H2, H3	Kidney, lung
	2-N, 0-N6	Jasco SC-02 (25 × 4.6)	35 m*M* TBA phosphate (pH 5.3) (1.0)	20			
	0-N, 0-N6, 2-N6	Jasco SC-02 (25 × 4.6)	10 m*M* TBA phosphate (pH 7.0) containing 47% MeOH (1.0)	15			
[100]	2-N6, unsaturated oligosaccharides	Spherisorb SAX (5, 25 × 4.6)	Linear gradient from 0.2 to 1.1 *M* NaCl (pH 3.5) over 75 min (1.5)	75	232 nm	H1	Porcine mucosa
[98,99]	0-0, 0-6, 2-6, 0-N, 0-N6, 2-N6	Shodex RS (DC-613) (6, 15 × 6 with 4.6 × 6) 70°C	MeCN–MeOH–0.8 *M* ammonium formate (pH 4.5), 69:11:20 (1.0)	70	232 nm	H2, H3	Kidney
[53]	0-0, 0-6, 0-N, 0-N6, 2-N, 2-N6	LiChrosorb NH_2 (25 × 2.6) 40°C	Linear gradient from 16 to 800 m*M* sodium dihydrogenphosphate over 60 min (1.5)	30	230 nm	H1, H2, H3	Kidney, intestine, lung
[102]	0-0, 0-N, 0-6, 2-0, 0-N6, 2-N, 2-6, 2-N6	Spherisorb ODS2 (5, 25 × 4.6) 20°C	40% (v/v) MeOH containing 10 m*M* TBA phosphate (pH 6.7) (1.0)	20	226 nm	H2	HP
[104]	0-0, 0-N, 0-6, 2-6, 2-N, 2-N6	CarboPac PA1 (10, 25 × 4.0) 40°C	Gradients: 1, from 51 to 170 m*M* LiCl (0–5 min); 2, from 170 to 570 m*M* LiCl (5–8 min); 3, from 0.57 to 1.14 *M* LiCl (8–15 min); 4, from 1.14 to 2.10 *M* LiCl (15–20 min); 5, from 2.10 to 2.19 *M* LiCl (20–24 min)	25	230 nm	H2, H3	HP
[68]	2-6N, 2-6, 0-N6, 0-0, 0-N, 0-6	Carbonex (7, 10 × 4.6) 50°C	4 m*M* sodium carbonate containing 0.5 m*M* sodium hydrogencarbonate in 3.5% MeCN (0.5)	50	Pos, 2-CA Ex. 346 nm, Em. 410 nm		
Toyoda, 1992[d] (see Fig. 5)	0-0, 0-6, 2-6, 0-N, 2-N, 0-N6, 2-N6	TSKgel Amide-80 (5, 25 × 3) 60°C	MeCN–MeOH–20 m*M* phosphate buffer (pH 6.0) containing 0.3 *M* ammonium chloride, 65:5:25 (0.5)	45	Pos, 2-CA Ex. 346 nm, Em. 410 nm	H1, H2, H3	Urine

[a,b] See Table 1.
[c] Suppliers of columns: Partisil, Whatman; Jasco SC-02, Japan Spectroscopic; LiChrosorb, Merck; Spherisorb, Phase Separations; Shodex, Showa Denko; CarboPac PA1, Dionex; Carbonex, Tonen; TSKgel, Tosoh.
[d] Unpublished data.

saturated disaccharides, including two novel species (II and IV in Fig. 4), were resolved by reversed-phase HPLC using an ODS column following heparin lyase II digestion of intact pig mucosal HP and the chemically modified HPs [102]. Fig. 5 shows a typical chromatogram of the unsaturated disaccharide standards derived from HS and HP using a TSKgel Amide-80 column. Further, the utility of a graphitized carbon column for the separation of the unsaturated disaccharides derived from HS and HP has been demonstrated [68]. Because of the stability of the graphitized carbon column in an alkaline medium, pulsed amperometric detection can be used with such a column.

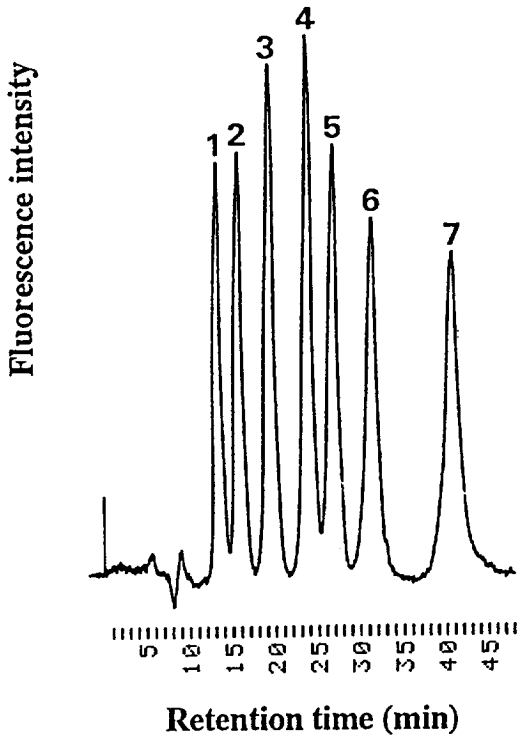


Fig. 5. Typical chromatogram of standard unsaturated disaccharides derived from heparan sulphate and heparin. Column, TSKgel Amide-80 (250 mm × 3.0 mm I.D.); column temperature, 60°C; eluent, acetonitrile–methanol–20 m*M* sodium phosphate buffer (pH 6.0) containing 0.3 *M* ammonium chloride (65:5:25, v/v/v); flow-rate, 0.5 ml/min; reagent 1, 1% 2-cyanoacetamide (0.25 ml/min); reagent 2, 0.5 *M* sodium hydroxide (0.25 ml/min); reaction coil, 10 m × 0.5 mm I.D.; reaction temperature, 110°C; cooling coil, 2 m × 0.25 mm I.D.; detection, fluorescence (excitation 346 nm, emission 410 nm); sample size, 10 μl. Peaks: 1 = ΔUA-GlcNAc (25 ng); 2 = ΔUA-GlcNAc6S (25 ng); 3 = ΔUA2S-GlcNAc6S (50 ng); 4 = ΔUA-GlcNS (50 ng); 5 = ΔUA2S-GlcNS (100 ng); 6 = ΔUA-GlcNS6S (50 ng); 7 = ΔUA2S-GlcNS6S (100 ng). H. Toyoda and T. Imanari, unpublished data.

2.2.3. Detection of the unsaturated disaccharides

Owing to the UV absorbance of the unsaturated disaccharides at around 230 nm, there have been many reports on simple HPLC systems for the determination of glycosaminoglycans as their unsaturated disaccharides [53,68,98–104] (Table 3). However, accurate analyses are often difficult because of interfering chromatographic peaks from strongly UV-absorbing trace contaminants in solvents and samples. Even chemically derived oligosaccharides have been detected at low wavelength, although the sensitivity of this technique is not greater than that of refractive index detection. Wavelengths used for the detection of other oliogsaccharides containing each unit are 190–195 nm for 2-acetamido-2-dehydroxyhexose units and 210–220 nm for hexuronic acids.

To enhance the detectability, methods based on postcolumn derivatization have been developed. In these methods, the unsaturated disaccharides are separated by using normal HPLC techniques, and only the detection system is altered. Highly sensitive postcolumn HPLC using 2-cyanoacetamide as a postcolumn reagent has been examined (Fig. 5).

3. Analysis of oligosaccharides derived from GAGs

Studies on GAG analysis are proceeding in the following fields: (1) identification of different types of GAGs, (2) measurement of molecular size of GAGs and (3) structural analysis of unique domains on GAGs. In these fields, HPLC contributes to the separation of oligosaccharides derived from GAG chains.

3.1. Identification of different types of GAGs

Sequential enzymatic digestion is required for the identification of different types of GAGs. Linhardt et al. [55] reported reversed-phase ion-pair HPLC and ion-exchange HPLC with conductivity detection. In their study, oligosaccharides were derived from standard GAGs with enzymatic digestion, followed by conductivity detection. Whitfield et al. [105] applied pulsed amperometric detection (PAD) to the chromatographic identification of GAGs containing iduronic acid such as HP, HS and DS. These GAGs were simultaneously degraded and desulphated into oligosaccharides containing iduronic acid with 1.0 *M* HCl at 100°C for 4 h. PAD is suitable for carbohydrates that are peculiar to polyalcohols.

Several types of hyaluronidase are commercially available. Each hyaluronidase degrades HA in a hydrolytic or eliminative fashion, so that the resultant oligosaccharides are diverse, including saturated and unsaturated oligosaccharides. For HA determination, convenient HPLC methods with enzymatic degradation were established. The determinations of unsaturated tetrasaccharides and hexasaccharides, which were obtained from HA by *Streptomyces* hyaluronidase (EC 4.2.2.1) digestion, were achieved by normal-phase HPLC [106] [column, Zorbax Sil (DuPont)], reversed-phase ion-pair HPLC [107,108] (column, ODS (Radial Pak C_{18} cartridge (Waters), Ultrasphere ODS (Beckman); counter ion, tetrabutylammonium], gel permeation HPLC [109] [column, sulphated polystyrene–divinylbenzene gel, Shodex Ionpak KS-802 (Showa Denko)] and HPLC on an amino-phase chemically bonded silica column [47] [column, Zorbax NH_2 (DuPont)] with UV detection at 232 nm (Table 4). These procedures were applicable to biological materials such as synovial fluid and articular cartilage. Further, a combination of these chromatographic separations with postcolumn derivatization may develop into a highly sensitive method [111,112]. Some reports have been published on the determination of saturated oligosaccharides from HA [110,113]. Kakehi et al. [113] reported precolumn HPLC using 1-(4-methoxy)phenyl-3-methyl-5-pyrazolone (PMPMP) as a derivatizing reagent. In their work, porcine skin HA was digested by sheep testis hyaluronidase, and the resultant oligosaccharides, saturated di-, tetra- and hexasaccharides, were submitted to precolumn derivatization. The derivatives were developed on a Cosmosil $5C_{18}$-Ar column and detected with UV detection at 249 nm. The detection limit was less than 0.5 μg.

It is well known that the KS level in biological fluids changes drastically in patients with certain diseases such as rheumatism. KS is an essential component of cornea and cartilage, and also has a polydisperse structure. The cleavage of KS to oligosaccharides was achieved with keratanase (from *Pseudomonas* sp., EC 3.2.1.103), keratanase II (from *Bacillus* sp., EC 3.2.1.–), and *endo*-β-galactosidase (from *Escherichia freundii*, EC 3.2.1.103) [114].Keratanase and *endo*-β-galactosidase hydrolyse the β-galactoside bond between non-sulphated galactose and N-acetylgalactosamine, whereas keratanase II hydrolyses the β-glucosaminide bond in the KS chain. Gel permeation HPLC [TSKgel G4000, G3000 and G2500 columns (Tosoh) assembled in series] with UV-detection was applied to the separation of oligosaccharides obtained from bovine cornea KS and shark cartilage KS by enzymatic digestion [115].

3.2. Measurement of molecular size of GAGs

The determination of the molecular size of GAGs using LC has been hampered by a lack of readily available standards. So far, intact GAGs and some artificially synthesized polymers such as dextran sulphates have been employed as molecular size standards. Wasteson [116,117] first showed, using conventional gel permeation column chromatography, that monodisperse GAGs were suitable as standards for assigning size distributions to unknown GAG samples. Melrose and Ghosh [118] prepared relatively monodisperse GAGs from commercially available bovine tracheal chondroitin sulphate A. This type of molecular size standard may be ideal for the determination of the molecular size of GAG

Table 4
HPLC conditions for the determination of unsaturated tetra- and hexasaccharides from HA

Ref.[a]	Column[b] (particel size in μm, length in cm × I.D. in mm)	Eluent[c] (flow-rate in ml/min)	Analysis time (min)	Method of detection[c]	Application
[106]	Zorbax SIL (15 × 4.6)	MeCN–MeOH–0.5 *M* ammonium formate (pH 6.0), 10:6:3 (0.3)	40	232 nm	
[47]	Zorbax NH_2 (6, 25 × 4.6)	4% MeOH, 60% 0.5 *M* ammonium formate at pH 5.5 (0.9)	16	232 nm	
[108]	Ultrasphere ODS (5, 25 × 4.6)	Solvent A, 10 m*M* TBA hydroxide, 8 m*M* phosphoric acid in 20% MeCN (pH 7.6) Solvent B, 10 m*M* TBA hydroxide, 6 m*M* phosphoric acid in 60% MeCN (pH 7.5) Gradient from 0 to 5% B over 18 min (1.0)	18	232 nm	Cartilage, nucleus pulposus, annulus fibrosus, skin, aorta, cervix, cockscomb, synovial fluid, vitreous humor
[111]	TSKgel NH_2-60 (5, 15 × 4)	10 m*M* ammonium formate (pH 5.0) containing 6.5 m*M* sodium sulphate in 4% MeCN (0.5)	30	Pos, 2-CA Ex. 346 nm, Em. 410 nm	Plasma
[109]	Shodex Ionpak KS-802 (30 × 8) 80°C	0.2 *M* NaCl (1.0)	12	232 nm	Synovial fluid
[107]	Radial Pak C_{18} (4, 10 × 8)	MeCN–0.01 *M* TBA phosphate, 17:83, adjusted to pH 7.35 with phosphoric acid (1.3)	12	232 nm	Synovial fluid

[a] See footnote a in Table 1.
[b] Suppliers of columns: Zorbax, DuPont; Ultrasphere, Beckman; TSKgel, Tosoh; Shodex, Showa Denko; Radial Pak, Waters.
[c] See footnote b in Table 1.

chains using fast protein liquid chromatography (FPLC) on Superose 6 and 12. Volpi and Bolagnani [119,120] also emphasized the usefulness of HPLC for the determination of molecular size of intact GAGs or oligosaccharides from GAGs using GAG molecular size standards. Oligosaccharides from GAGs were applied on Protein Pak 125 and 300 columns (Waters) assembled in series. This method achieved the fractionation of HP oligosaccharides with molecular size in the range M_r 1600–11 600, which were obtained from beef intestinal mucosal heparin by a controlled chemical depolymerization process induced by free radicals in the presence of copper salt. Ferrari et al. [121] applied high-performance size exclusion chromatography (TSKgel G4000SW and TSKgel G2000SW columns (Tosoh) assembled in series) for the separation and determination of the molecular size of DS oligosaccharides, which have antithrombotic activity.

3.3. Structural analysis of unique domains on GAGs

HS, HP and DS are polydisperse GAGs. Some physiologically active substances are confirmed to have an affinity for certain characteristic domains on GAGs. Incidentally, these domains seemed to be made by transferases involved in a GAG synthesis, and thus the proportion of the active domains on GAG chain is extremely low. Therefore, a step in the structural analysis of active domain is to purify the desired oligosaccharide from the many oligosaccharides derived from GAGs. The oligosaccharides from HS, HP and/or DS are a mixture of isomers that differ in molecular size, kind of uronic acid, degree of sulphation and sulphation position. To separate these isomers, the mixture of oligosaccharides was commonly submitted successively to chromatographic separation according to molecular size, ion charge and isomeric structure.

Habuchi et al. [122] elucidated that the domain on HS having an affinity against bFGF was IdoA(2S)-rich hexadecasaccharides. In this work, oligosaccharides having an affinity against bFGF were isolated by affinity chromatography, gel permeation chromatography and ion-exchange chromatography on a Mono-Q column. The disaccharide component of the active oligosaccharide was determined by enzymatic digestion followed by HPLC on a Partisil-10 SAX column.

Guo and Conrad [123] presented the reversed-phase ion-pair HPLC (column, Hi-Chrom 5S ODS; counter ion, tetrabutylammonium) of HP oligosaccharides derived from porcine intestinal mucosa heparin by nitrous acid (pH 1.5). Prior to HPLC analysis, the mixture of oligosaccharides was fractionated into di-, tetra- and hexasaccharides by gel permeation chromatography. Each fraction was subjected to reversed-phase ion-pair HPLC, and almost all peaks of disaccharides and tetrasaccharides were assigned to the isomers, which were identified.

The enzymatic digestions of GAGs prepared from tissue PGs yield several types of oligosaccharides, which are derived from the non-reducing terminal, the reducing terminal or the oligosaccharide-repeating region. The digestion of CS/DS by highly purified chondroitin ABC lyase (protease-free chondroitinase ABC; Seikagaku Kogyo) yielded unsaturated tetrasaccharides from the disaccharide-repeating region [124], and unsaturated tri-, tetra- and hexasaccharides from the linkage region between GAG chain and core protein [125,126], in addition to abundant unsaturated disaccharides. The digestion of HP by a mixture of heparinase and heparitinases yielded unsaturated oligosaccharides resistant to these enzymes [127]. For the isolation of these oligosaccharides, ion-exchange HPLC on a PA03 amino-bonded silica column (YMC, Kyoto, Japan) or a CarboPak PA1 column (Dionex) was applied. The isolated oligosaccharides were submitted to structural analysis by NMR spectroscopy.

KS differs compositionally from the other GAGs, since KS is decorated at non-reducing terminal and/or reducing regions by sialic acid. For the structural analysis of reducing and non-reducing terminal domains on KS, Dickenson et al. [128–130] used HPLC. They achieved the separation of oligosaccharides produced from KS

by keratanase digestion with anion-exchange HPLC on a Nucleosil 5SB column.

Recently, detailed studies on GAGs have suggested the physiological functions of GAG chains. With respect to clinical applications, these findings encourage the synthesis of artificially decorated GAGs and the search for natural GAGs having complex structures. These investigations have increased the necessity for chromatographic techniques for oligosaccharides, which are applicable for structural studies on these GAGs.

4. Conclusion

There are several different types of GAGs, each of which have heterogeneity with respect to molecular size, disaccharide composition and sulphate content. Biological and structural studies have focused on GAGs and/or PGs, and some convenient and effective methods have been developed and used for studies on the compositional qualitative and quantitative analysis of GAG chains. Among the methods, HPLC is the most convenient and effective for purification, molecular size determination and structural and quantitative analysis. As described above, unsaturated disaccharides produced enzymatically from GAGs have been analysed by HPLC for the identification and determination of GAGs.

Recently, for CS, DS, HA, HS and HP, unsaturated disaccharide analysis has almost been established by HPLC with digestion by chondroitin sulphate lyases and heparin lyases, which are commercially available. However, HPLC methods for the determination of KS are still under investigation. Therefore, more specific enzymes for the production of oligosaccharides from GAGs have become increasingly important for HPLC analysis.

Moreover, new advances in the HPLC of the unsaturated disaccharides and oligosaccharides from GAGs are expected in three areas. The first areas is in the development of more durable and stable stationary phases.

The second is in the rapid development of practical and preparative HPLC for GAG-derived oligosaccharides. Early preparative systems used large, expensive columns with low resolving power, and were not extensively applied in GAG research. New research is showing that some GAGs can be separated on the milligram scale using an FPLC system and a mono-Q column.

The third area is in combined techniques and other methods that provide qualitative and quantitative information about sample constituents, such as high-performance liquid affinity chromatography. The use of specific lectin- and monoclonal antibody-based stationary phases for analytical and preparative applications is now being considered.

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