

# Handbook of Glycomics

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# Glycosaminoglycans

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## Introduction

Glycosaminoglycans (GAGs) are a family of highly sulfated, complex, polydisperse linear polysaccharides that display a variety of important biological roles. Based on the difference of repeating disaccharide units comprising GAGs, they can be categorized into four main groups: heparin (HP)/heparan sulfate (HS), chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA) (Figure 3.1). GAGs are found in all animals from *Caenorhabditis elegans* to human, in the extracellular matrix and basement membranes as a structural scaffold [1].

HP and HS are mixtures of sulfated linear polysaccharides having a molecular weight (MW) range from 5000 to 40000 with an MW average of 10000 to 25000. The major repeating disaccharide sequence of heparin (75–90%) is [ $\rightarrow$ 4]-2-O-sulfo- $\alpha$ -L-idopyranosyluronic acid (IdoA<sub>p</sub>) (1 $\rightarrow$ 4)-2-deoxy-2-N-,6-O-sulfo- $\alpha$ -D-glucopyranosylamine (GlcN<sub>p</sub>) (1 $\rightarrow$ ), while minor sequences contain  $\beta$ -D-glucopyranosyluronic acid (GlcA<sub>p</sub>) residues, a reduced content of sulfo groups as well as N-acetylation. HP proteoglycan (PG) is O-linked to serine residues of the core protein serglycin and is found intracellularly in the granules of mast cells [2]. HS is O-linked to serine residues of a number of core proteins resulting in a number of PGs, including glypican and syndecan. HS has the similar structure with heparin, primarily containing nonsulfated disaccharide [ $\rightarrow$ 4]  $\beta$ -D-GlcA<sub>p</sub> (1 $\rightarrow$ 4)- $\alpha$ -D-GlcN<sub>p</sub>Ac (1 $\rightarrow$ ) and monosulfated disaccharides, such as [ $\rightarrow$ 4]  $\beta$ -D-GlcA<sub>p</sub> (1 $\rightarrow$ 4)-6-sulfo- $\alpha$ -D-N-GlcN<sub>p</sub>Ac].

HP, commonly used as a clinical anticoagulant, has other biological activities with potential clinical applications, such as effects on lipoprotein lipase, effects on smooth muscle proliferation, inhibition of complement activation, anti-inflammatory activity, angiogenic and antiangiogenic activities, anticancer activity, and antiviral activity [2–8]. HS has a number of important biological activities. It has polysaccharide components that bind antithrombin, supporting blood flow within blood vessels [5]. It is also

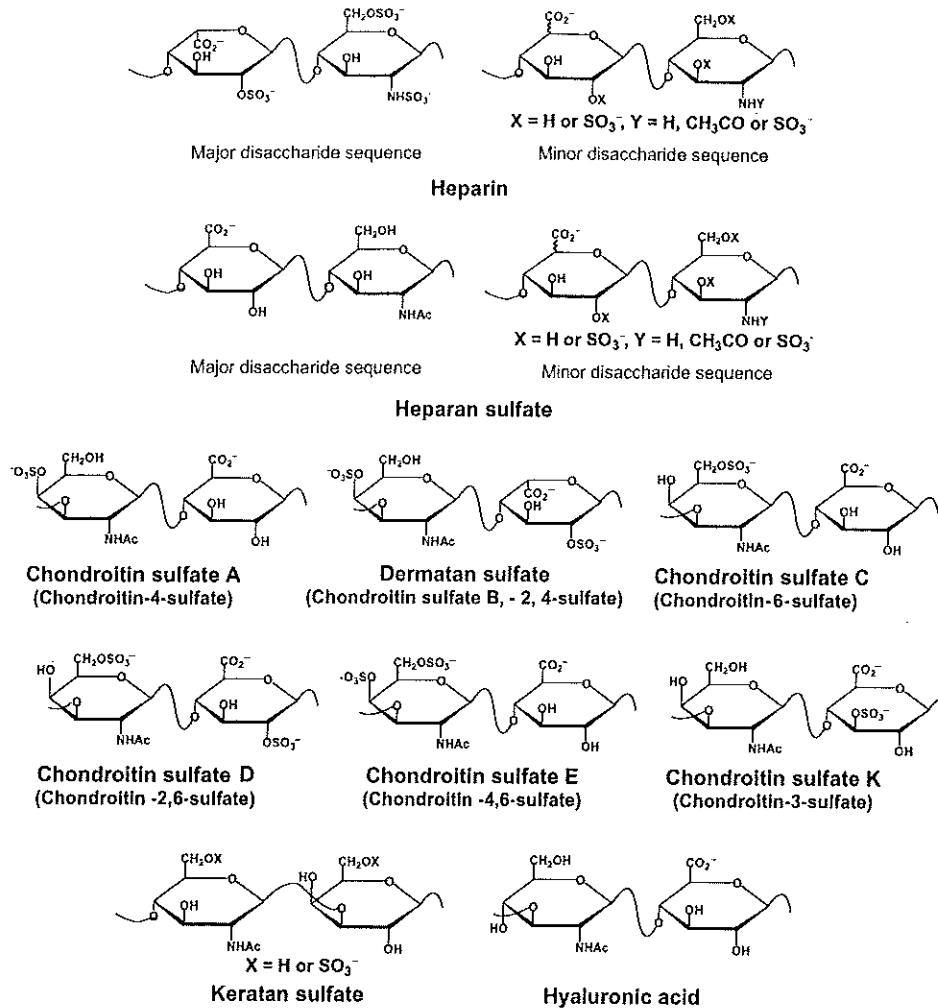


FIGURE 3.1 Major and minor disaccharide sequences of heparin (HP)/heparan sulfate (HS) and major disaccharide sequences of chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS), and hyaluronan (HA).

important in cell–cell interactions involving adhesion proteins, cell–cell communication involving chemokines and cell signaling involving growth factors [8–10].

The CS/DS family of GAGs is comprised of alternating 1→3, 1→4 linked 2-amino, 2-deoxy- $\alpha$ -D-galactopyranose (GalN<sub>p</sub>Ac) and uronic acid ( $\beta$ -D-GlcA<sub>p</sub> in CS and  $\alpha$ -L-IdoA<sub>p</sub> in DS) residue [11]. The molecular weights of CS/DS range from 2 to 50kDa. There are multiple forms of CS named A, B (also known as DS), C, D, E, and K, differing based on sulfo group substitution and the type of uronic acid that each contains (Figure 3.1) [11]. This family is the most common type of GAG found in extracellular matrix PGs. CS is important in cell–cell interaction and communication, and DS exhibits important venous antithrombotic activity [11–13]. CS and DS also have intriguing functions in infection, inflammation, neurite outgrowth, growth factor signaling, morphogenesis, and cell division [13–15].

KS has two different forms, KS-I and KS-II, originally designated based on differences between KS from cornea and cartilage. Currently, the term KS-I includes

all Asn-linked KS molecules, and KS-II is used to refer to all KS linked to protein through GalNAc-O-Ser/Thr [16,17]. A third type of KS linkage (mannose-O-Ser) has been identified that has been called KS-III [18]. KS is comprised primarily of 6-O-sulfo-GlcN<sub>p</sub>Ac and galactose (Gal) (which may contain 6-O-sulfo groups) [17]. KS-PGs are the major class of PG in the corneal stroma and are thought to play an important role in corneal structure and physiology, particularly in the maintenance of corneal transparency [19]. KS-containing molecules have been identified in numerous epithelial and neural tissues in which KS expression responds to embryonic development, physiological variations, and to wound healing [17]. Evidence has also been presented supporting functional roles of KS in cellular recognition of protein ligands, axonal guidance, cell motility, and in embryo implantation [17].

HA is a homocopolymer made of repeating disaccharide units of [ $\rightarrow$ 3]-D-GlcN<sub>p</sub>Ac(1 $\rightarrow$ 4)- $\beta$ -D-GlcA<sub>p</sub>(1 $\rightarrow$ ) [20]. It is an unsulfated GAG with very high MW of up to 2000 kDa. HA is widely distributed in cartilage, skin, eye, and most body liquids. It is not only an important structural component of extracellular matrices but also interacts instructively with cells during embryonic development, healing processes, inflammation, and cancer [21].

GAGs perform a variety of biological functions and play an important role in a number of different diseases. Their activities are mainly triggered by interactions with a wide range of proteins [4]. Experiments have shown that specific sequences within heparin and HS act as protein binding sites [22]. This suggests that polysaccharides may work as informational molecules and suggests the importance of developing analytical techniques to sequence GAG molecules [23,24]. The primary structure determination of biopolymers, such as proteins and nucleic acids, are commonly solved by automated sequencing of amino acid residues and nucleotide residues, respectively. In contrast with current state-of-the-art methods, the sequencing of polysaccharides with a high level of structural complexity is still extremely challenging.

## Isolation of GAGs

The long linear chain and high density of negative charges in GAGs distinguish this class of glycans from the short-branched oligosaccharides of glycoproteins and glycolipids. The high density of negatively charged structure of GAGs is the basis for their physical separation. This property facilitates GAG isolation/purification by strong anion-exchange (SAX) chromatography and precipitation with cetylpyridinium chloride and methanol/ethanol [25]. GAGs are generally attached to core proteins but free chains can accumulate because of proteolysis and endoglycosidic cleavage of the PG. GAGs can be integrated in the extracellular matrix by non-covalent interactions or be associated with the cell surface by hydrophobic interaction or by ionic binding with other cell surface molecules. GAGs can also be sequestered in intracellular compartments, such as in storage or secretory granules, or in pre-lysosomal or endosomal compartments [26]. Fragmentation of matrix PGs often occurs and gives rise to GAG chains attached to short peptides [25]. The principle of GAG isolation is similar to the methods for PG extraction except that proteolysis is used for GAG isolation. Chaotropic reagents, such as 4 M guanidine hydrochloride or 8 M

Table 3.1 Glycosaminoglycans (GAGs) isolated from different animal tissues, cell cultures, and biological fluids

	Sources	GAGs	Ref.
Organ tissues	Porcine intestine, bovine lung	HP, HS	27
	Bovine/porcine kidney, pancreas, lung livers	HS	28
	Dromedary intestine	HP/HS	29
	Human liver	HS	30
	Human amyloid A and fibrils	HS, DS	31
	Brains from rat, monkey, chicken, sheep, rabbit	HA, CS, HS	32
	Human non-epithelial tumors	CS, HA	33
	Chick embryo	CS	34
	Human placenta	DS	35
	Bovine, human cartilage	CS, KS	36
	Mouse organs	HS, CS	37
Cell culture	Stem cells	HS	38
	Chinese hamster ovary (CHO) cells	HS	39
Biological fluids	Plasma, urine	HP, CS, HA	40,41
	Human follicular fluid	HS	42
	Bovine follicular fluid	HS, CS	43
Insects	Drosophila		44
	Mosquitoes		45
Fish/marine/molluscs	Zebra fish	HS, CS	46
	Clams	HS, CS	47
	Eel skin	DS, KS	48
	Africa giant snail	Acharan sulfate	49

HP, heparin; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; KS, keratan sulfate; HA, hyaluronan.

urea, are effective in recovering of GAGs (or PGs) from cell matrix compartments. These reagents denature proteins and dissociate most non-covalent interactions. Detergents, such as Triton X-100, 2% of CHAPS, are often used to dissociate hydrophobic interactions [26]. The general procedures for the isolation/purification GAGs include: (i) sample pre-treatment, such as homogenization, freeze-drying and de-fatting, (ii) proteolysis, (iii) high-salt or chaotropic reagent/detergent extraction; (iv) anion-exchange chromatography; and (v) methanol or ethanol precipitation. GAGs have been recovered from a variety of different tissues, cell culture samples, and biological fluids (Table 3.1).

The following detailed procedure provides an example for isolation of GAGs from large organ or tissue samples (i.e., pig liver, bovine brain, human liver):

1. Wash the tissue in cold phosphate buffered saline (PBS) at 4°C.
2. Cut tissue into small pieces (2 × 2 cm).
3. Freeze-dry, and weigh the dried tissue.
4. Grind dry tissue into powder.

5. Remove fat by extracting the tissues with three solvent mixtures: chloroform:methanol (2:1, 1:1, 1:2 (v:v)) each left overnight at room temperature, dry defatted tissue in fume hood. Weigh the dry, defatted tissue.
6. Suspend defatted sample (5–10% (w/v)) in water and proteolyze at 55°C with 10% (w/w) of a non-specific protease such as actinase E (20 mg/mL) for 18 h.
7. Add 8 M urea containing 2% CHAPS to supernatant.
8. Remove insoluble residue by centrifugation (5000 rpm, for 30 min).
9. Equilibrate a SAX column with 8 M urea, 2% CHAPS at pH 8.3, load sample and wash the column with 3 column volumes of 200 mM aqueous NaCl. GAGs are then released by washing the column with 1 column volume of 16% NaCl.
10. Methanol precipitate (80% vol methanol) the released GAGs dissolved in 16% NaCl at 4°C overnight. Recover the precipitated GAGs by centrifugation (5000×g for 30 min).

## Purification of Individual GAGs

Individual GAGs can be purified by: (i) selective enzymatic digestion with polysaccharide lyases; (ii) selective degradation with nitrous acid; (iii) SAX chromatography purification; and (iv) selective precipitation.

Polysaccharide lyases cleave specific glycosidic linkages present in acidic polysaccharides and result in depolymerization [50]. These enzymes act through an eliminase mechanism resulting in unsaturated oligosaccharide products that have UV absorbance at 232 nm. This class of enzymes includes heparin lyases (heparinases), heparan sulfate lyases (heparinases or heparitinases), chondroitin lyases (chondroitinases), and hyaluronate lyases (hyaluronidases) (Table 3.2). These enzymes can be used, alone or in combinations, to degrade the undesired GAGs, or to confirm the presence of GAGs in a sample as well as to distinguish between different GAGs. Chondroitin lyase ABC can depolymerize all forms of CS as well as HA, chondroitin lyase AC can depolymerize CS-A and CS-C and HA but not DS, and chondroitin lyase B can only degrade DS (CS-B). Heparin lyase III degrades HS but does not act on HP or other GAGs while heparin lyase I degrades HP but only acts in a very limited extent on HS. Each lyase enzyme has different optimal buffer and reaction conditions and can be inhibited by the presence of other GAGs [50]. Enzyme activity should be assayed prior to using it in an experiment to ensure it is active and has been stored properly.

A unique structural feature of HP and HS is that a large proportion of their GlcN<sub>p</sub> residues contain *N*-sulfo groups (85–90% in heparin; 30–60% in a typical heparan sulfate) and the remainder is modified with *N*-acetyl groups [50]. The glycosidic bonds of these *N*-sulfo GlcN<sub>p</sub> residues can be cleaved rapidly at room temperature with nitrous acid at pH 1.5 to afford a mixture of oligosaccharides. Nitrous acid susceptibility distinguishes HP and HS from all other GAGs (i.e., CS, DS, HA, and KS) as these contain only *N*-acetyl substituted amino sugars, which are not cleaved by nitrous acid [51].

GAGs also differ based on their average charge densities and these differences can be exploited to identify and separate different GAG classes by using anion-exchange

Table 3.2 Polysaccharidases used in glycosaminoglycan analysis

Enzyme	Source	Action pattern	Specificity
Chondroitin lyase ACI	<i>Flavobacterium heparinum</i>	CS (endo)	$\rightarrow 3)\alpha\text{-D-GalNpAc}(4\text{S}/\text{OH}, 6\text{S}/\text{OH})(1 \rightarrow 4)\text{-}\beta\text{-D-GlcA}_p(1 \rightarrow$ Primary to disaccharide products
Chondroitin lyase ACII	<i>Arthrobacter aureescens</i>	CS (exo)	$\rightarrow 3)\alpha\text{-D-GalNpAc}(4\text{S}/\text{OH}, 6\text{S}/\text{OH})(1 \rightarrow 4)\text{-}\beta\text{-D-GlcA}_p(1 \rightarrow$ Primary to disaccharide products
Chondroitin lyase ABC	<i>Proteus vulgaris</i>	CS/DS/HA (endo and exo)	$\rightarrow 3)\alpha\text{-D-GalNpAc}(4\text{S}/\text{OH}, 6\text{S}/\text{OH})(1 \rightarrow 4)\text{HexAp}(1 \rightarrow$ Primary to disaccharide products
Chondroitin lyase ABC	<i>Bacteroides thetaitaomicron</i>	CS/DS/HA (exo)	$\rightarrow 3)\alpha\text{-D-GalNpAc}(4\text{S}/\text{OH}, 6\text{S}/\text{OH})(1 \rightarrow 4)\text{HexAp}(1 \rightarrow$ Primary to disaccharide products
Chondroitin lyase B	<i>Flavobacterium heparinum</i>	DS (endo)	$\rightarrow 3)\alpha\text{-D-GalNpAc}(4\text{S}/\text{OH})(1 \rightarrow 4)\text{-}\alpha\text{-L-IdoAp}(1 \rightarrow$ Primary to disaccharide products
Heparin lyase I	<i>Flavobacterium heparinum</i>	Hp (endo)	$\rightarrow 4)\alpha\text{-D-GlcNpS}(6\text{S}/\text{OH})(1 \rightarrow 4)\text{-}\alpha\text{-L-IdoAp}2\text{S}(1 \rightarrow$ Primary to disaccharide products
Heparin lyase II	<i>Flavobacterium heparinum</i>	Hp/HS (endo)	$\rightarrow 4)\alpha\text{-D-GlcNp}(S, \text{Ac})(6\text{S}/\text{OH})(1 \rightarrow 4)\text{-}\alpha\text{-L-IdoAp}/\text{-}\beta\text{-D-GlcA}_p(2\text{S}/\text{OH})(1 \rightarrow$ Primary to disaccharide products
Heparin lyase III	<i>Flavobacterium heparinum</i>	HS (endo)	$\rightarrow 4)\alpha\text{-D-GlcNp}(\text{Ac}/\text{S})(\text{OH}/6\text{S})(1 \rightarrow 4)\text{-}\beta\text{-D-GlcA}_p\text{-}\alpha\text{-L-IdoAp}(1 \rightarrow$ Primary to disaccharide products
Hyaluronate lyase	<i>Streptomyces hyalurolyricus</i>	HA (endo)	$\rightarrow 3)\alpha\text{-D-GlcNpAc}(1 \rightarrow 4)\text{-}\beta\text{-D-GlcA}_p(1 \rightarrow$ Primary to disaccharide products
Hyaluronidase	Bovine testicular	HA/CS (endo)	$\rightarrow 3)\alpha\text{-D-GlcNpAc}(1 \rightarrow 4)\text{-}\beta\text{-D-GlcA}_p(1 \rightarrow$ Primary to saturated tetrasaccharide products

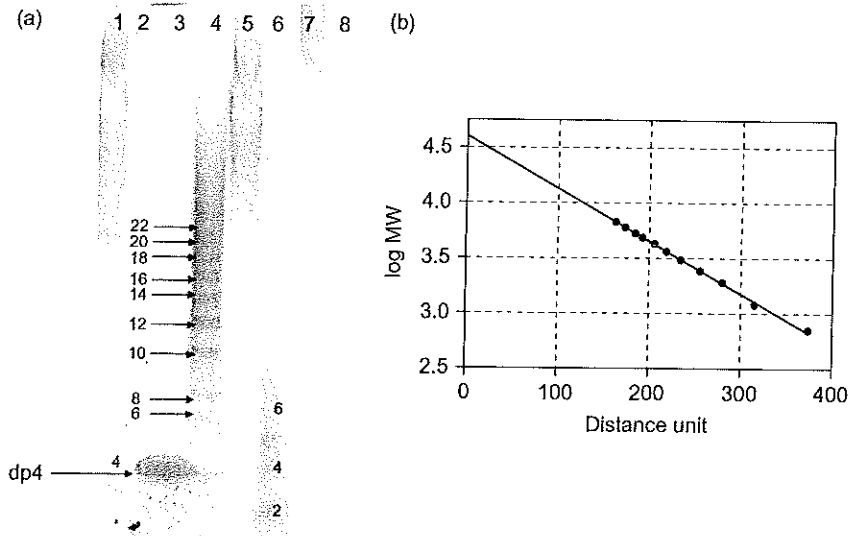
chromatography or selective precipitation. GAGs with different charge densities can resolve, at least partially, into separate peaks with a continuous salt gradient release. Many different types of ion exchangers are now available in high-performance liquid chromatography (HPLC) columns and membrane cartridges. For example, KS and DS were purified from a GAG-containing mixture (extracted from eel skin) by anion-exchange chromatography on a DEAE Sephacel column eluted with 2 column volumes of 0.5 M NaCl, 1 M, 1.2 M, 1.4 M, and 1.6 M NaCl [48].

## Structure Analysis

### Molecular Weight Analysis of GAGs

Polyacrylamide gel electrophoresis (PAGE) analysis can be conveniently applied to analyze the molecular weight of sulfated GAGs. Gels on which GAGs have been





**FIGURE 3.2** (a) Gradient polyacrylamide gel electrophoresis (PAGE) analysis with Alcian Blue staining of glycosaminoglycan samples before and after treatment with heparin lyases. Lane 1 is intact porcine intestinal heparan sulfate (HS); lane 2 is porcine intestinal HS after treatment with heparin lyase 1, 2, and 3; lane 3 is a hexasulfated tetrasaccharide standard derived from heparin (HP) indicated by the arrow; lane 4 is a mixture of HP-derived oligosaccharide standards enzymatically prepared from bovine lung HP—the numbers indicate their degree of polymerization (i.e., 4 is a tetrasaccharide); lane 5 is intact porcine HP; lane 6 is porcine intestinal HP after heparin lyase 1 treatment; lane 7 is human liver HS; lane 8 is human liver HS after heparin lyase 3 treatment. (b) A plot of log molecular weight of bovine lung HP-derived oligosaccharide standards as a function of migration distance of each oligosaccharide from which the average molecular weight of HP and HS can be calculated. (See color plate 6.)

fractionated can be visualized with Alcian Blue with or without silver staining and the bands can be scanned and digitized. The average MW of a GAG is then calculated based on a mixture of HP-derived oligosaccharide standards prepared through the partial enzymatic depolymerization of HP. PAGE analysis of HS purified from human placenta is shown in Figure 3.2 [30]. The polydispersity of GAGs is observed as a broad smear in PAGE and a numerical value for the dispersity can be calculated.

Gel permeation chromatography (GPC), which separates molecule solely on the basis of differences in molecular size has been used for the MW analysis of GAGs. Dextran, dextran sulfates, or GAGs of different MWs can be used as standards in a GPC column to calibrate the MW of GAGs. Refractive index detection is typically used in this method [52].

### Disaccharide Analysis

Disaccharide analysis is one of the most important ways to characterize a GAG, which consists of repeating disaccharide units composed of different monosaccharide residues, linkages, and sulfation patterns. Disaccharide analysis typically follows the complete enzymatic digestion of GAGs by corresponding lyases. There are several techniques used to measure the resulting disaccharide composition.

Capillary electrophoresis (CE) is often used in disaccharide analysis because of its high resolving power and sensitivity. The methods for CE disaccharide analysis are presented in Table 3.3 [53–76]. CE can be used with UV, fluorescence, or MS for detection of analyte.

Table 3.3 Capillary electrophoresis methods and operating condition used for analysis of glycosaminoglycan disaccharides

Analytes	Operating conditions (capillary type, operating buffer, voltage, wavelength)
<b>High pH, normal polarity</b>	
HA- and CS-derived- $\Delta$ -disaccharides [53]	UFS, 10 mM borate-50 mM boric acid (pH 8.8), 10 kV, 232 nm
HA- and CS-derived- $\Delta$ -disaccharides [54]	UFS, 100 mM borate-25 mM sodium tetraborate (pH 9.0), 15 kV, 214 nm (PMP derivatives)
<b>High pH + additives, normal polarity</b>	
HA-derived- $\Delta$ -di-, tetra- and hexasaccharides; CS-derived- $\Delta$ -disaccharides [55]	UFS, alkaline borate (pH 9.0)+TBA 17 kV, 232 nm
HA-derived- $\Delta$ -disaccharides, saturated oligosaccharides and CS-derived- $\Delta$ -disaccharides [56]	UFS, (1) 40 mM Pi-10 mM borate (pH 9.0) + 40 mM SDS, 15 kV, (2) 200 mM Pi (pH 3.0) - 15 kV, 232 nm for $\Delta$ -di- and 200 nm for oligosaccharides
CS-derived- $\Delta$ -disaccharides [57]	UFS, 40 mM Pi-10 mM borate (pH 9.0) + 40 mM SDS, 15 kV, 232 nm
CS-derived- $\Delta$ -disaccharides [58]	UFS, 18 mM borate-30 mM Pi (pH 7.0) + 50 mM CTAB, - 20 kV, 232 nm
DS- and HS-derived- $\Delta$ -disaccharides [59]	UFS, 50 mM borate 10 mM boric acid + 50-100 mM triethylamine (pH 8.8-10.4), 30 kV, 214 nm
HS-derived- $\Delta$ -disaccharides and tetra-, hexa- or higher oligosaccharides [60]	UFS, 10 mM borate (pH 8.81) + 50 mM SDS, 20 kV, 232 nm
Acetylated heparin-derived trisulfated disaccharides [61]	UFS, 10 mM borate (pH 8.81) + 50 mM SDS, 20 kV, 232 nm or 206 nm
HS-derived- $\Delta$ -disaccharides [62]	UFS, 10 mM borate (pH 8.5) + 50 mM SDS, 20 kV, 231 nm
Coated capillary	
CS-derived- $\Delta$ -disaccharides [63]	Polyether-coated fused silica, 50 mM Pi (pH 5.0) + 0.1 mM spermine - 20 kV, LIF (325/425) (ANDSA derivatives)
<b>Low pH, reversed polarity</b>	
CS- and HS-derived- $\Delta$ -disaccharides, saturated and - $\Delta$ -oligosaccharides [64]	UFS, 25 mM Pi (pH 3.0), - 15 kV, 254 nm (AMAC derivatives)
CS-, HS-, and heparin-derived- $\Delta$ -disaccharides [65]	UFS, 20 mM Pi (pH 3.48), - 8 kV, 232 nm
HA- and CS-derived- $\Delta$ -disaccharides [66]	UFS, 15 mM Pi (pH 3.00), - 20 kV, 232 nm
CS-derived- $\Delta$ -di- and oligosaccharides [67,68]	UFS, 15 mM Pi (pH 3.0), - 20 kV, LIF (AMAC derivatives)
HP-derived- $\Delta$ -di- and oligosaccharides [69]	UFS, 20 mM Pi (pH 3.48) - 8 kV, 232 nm
HS-/heparin-derived- $\Delta$ -disaccharides [70]	UFS, 15 mM Pi (pH 3.5) - 20 kV, 232 nm
HP-derived- $\Delta$ -di-and oligosaccharides [71]	UFS, 20 mM Pi (pH 3.48) - 18 kV, 232 nm
HS-derived- $\Delta$ -disaccharides [72]	UFS, 20 mM Pi (pH 3.5) - 15 kV, 232 nm
HP-derived- $\Delta$ -disaccharides [73]	UFS, 60 mM formic acid (pH 3.40), - 15 kV (+ pressure gradient), 231 nm
Microemulsion electrokinetic capillary chromatography	
CS-derived- $\Delta$ -disaccharides [74]	UFS, microemulsion buffer, 30 kV, 260 nm

<sup>a</sup> $\Delta$  is a disaccharide or oligosaccharide having a  $\Delta$ 4,5-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid ( $\Delta$ UAp) residue at its non-reducing end as a result of the action of a polysaccharide lyase.

Strong ion-exchange (SAX) high-performance liquid chromatography (HPLC) has also been used for disaccharide analysis [77,78]. This method is also widely used in oligosaccharide mapping. SAX-HPLC relies on UV detection at 232 nm and thus has rather low sensitivity, limiting its utility for microanalysis of GAGs prepared from small tissue or cell culture samples.

Reversed phase (RP)-HPLC is another method to analyze disaccharide composition. This method often utilizes fluorescence derivatization of the disaccharides with 2-aminoacridone or some other fluorescent tag [79]. RP-HPLC has been used for the disaccharide analysis of both HS and CS/DS obtained from biological samples. The pre-analysis derivatization of disaccharides, without sample clean-up, is followed by RP-HPLC separation and can detect as little as approximately 100 pg (approximately  $10^{-13}$  mol) of each disaccharide present in the mixture, thereby requiring >10 ng of total GAG for analysis.

Reversed-phase ion-pairing (RPIP)-HPLC is also widely used recently to analyze disaccharide composition. Different conditions are listed in Table 3.4 [80–95]. This analysis is generally performed with post-column fluorescent derivatization of disaccharides. The detection sensitivity of this method is picomolar per each disaccharide. Unfortunately, post-column derivatization requires a custom built, temperature-controlled, post-column reactor with two additional HPLC pumps. Recently, in-line electrospray ionization (ESI) mass spectrometry (MS) has been used in place of fluorescence detection to obtain comparable analytical sensitivity while simplifying this method. RPIP-HPLC with ESI-MS detection has also been used for oligosaccharide mapping.

ESI-MS and ESI-MS-MS and multidimensional mass spectrometry ( $MS^n$ ) can be relied on for the quantification of the isomeric disaccharides of GAGs without the use of chromatography or prior separation. The compositional analysis of disaccharide constituents of HP/HS can be achieved from a full-scan  $MS^1$  spectra using an internal standard and a calculated response factor for each disaccharide [96]. Diagnostic product ions from  $MS^n$  spectra of isomeric disaccharides can provide quantitative analysis of the relative amounts of each of the isomers in mixtures. This protocol was validated using several quality control samples and showed satisfactory accuracy and precision. Using this quantitative analysis procedure, percentages of disaccharide compositions for heparins from porcine and bovine intestinal mucosa and heparan sulfate from bovine kidney were determined.

CS/DS and KS disaccharides were also identified using a combination of electrospray ionization MS and tandem MS [97,98].

Reverse phase ion-pairing (RPIP) HPLC-MS is the most recent method to analyze disaccharide qualitatively and quantitatively. RPIP is the primary method to separate disaccharides and MS is used to detect and confirm the structure of each disaccharide. Selected methods are listed in Table 3.4. The extracted ion chromatogram (EIC) of the HPLC-MS analysis of 8 HP/HS disaccharides and the MS of each disaccharide is shown in Figure 3.3 [90]. The composition is obtained by integration of the peak area of the EIC and the structure of the disaccharides present in this mixture is unambiguously established from the MS. Based on the improvement in

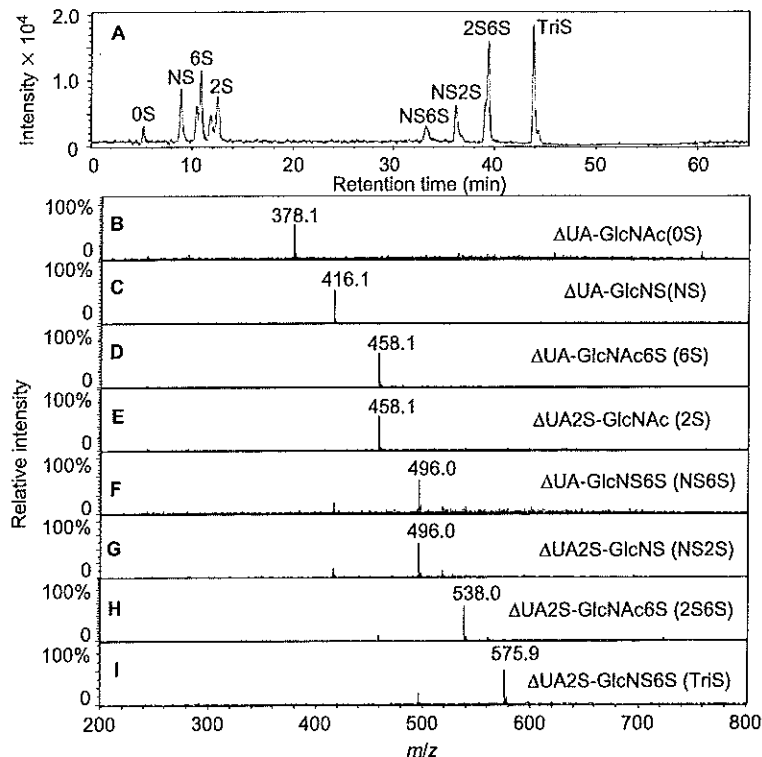
**Table 3.4 Ion-pairing reverse-phase HPLC methods and conditions for disaccharide analysis and oligosaccharide mapping**

Column	Ion-pairing reagent	Sample	Detector
Regular C18 column [80]	Tetrabutylammonium hydroxide	HA derived oligosaccharides	Post-column derivatization by 2-cyanoacetamide monitored at 270 nm
Supelcosil LC18 column [81]	Tetrabutylammonium	Twelve HP/HS disaccharides	232 nm
Cosmosil packed ODS column [82]	Tetrabutylammonium phosphate	PNP labeled HP/HS disaccharides	390 nm
Regular C18 column [83]	Triethylammonium, dibutylammonium, tributylammonium, tripropylammonium, tetrapropylammonium, and tetrabutylammonium acetate	HP/HS disaccharides, oligosaccharides, heparosan oligosaccharides	232 nm and MS
Regular C18 column [84,85]	Tributylammonium acetate	HP-derived oligosaccharides	232 nm and MS
TSKgel Super-Octyl column [86–88]	Tetrabutylammonium hydrogensulfate	HP/HS disaccharides CS/DS disaccharides	Post-column derivatization by 2-cyanoacetamide monitored at 346 nm of excitation and 410 nm of emission with a fluorescence detection
XTerra MS C18 column [89]	Tripropyl ammonium acetate and butyl ammonium acetate	HP oligosaccharides	232 nm and MS
Agilent capillary C18 column [90,91]	Tributylammonium acetate	HP/HS disaccharides CS/DS disaccharides	MS
Agilent capillary C18 column [92,93]	Tributylammonium acetate	HA, heparosan and CS oligosaccharides	MS
Regular C18 column [94]	Tributylammonium acetate	Non-sulfated CS oligosaccharides	MS
Acquity UPLC BEH C18 column [95]	Tributylammonium acetate	HP/HS disaccharides	232 nm and MS

separation method and application of an internal standard, the HP/HS disaccharide composition was quantified [95]. The method offers the advantage of rapid analysis with minimum sample consumption and without the need for sample preparation or further purification. This fast and reliable method is suitable for structural characterization and quantification of pharmaceutical HP preparations and samples of HP/HS isolated from a variety of biological sources.

### Oligosaccharide Mapping and Analysis

Oligosaccharide mapping techniques have been applied to GAGs to understand the structural features responsible for their activity differences. Oligosaccharide mapping

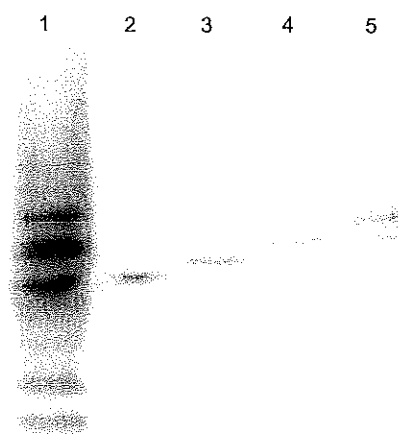


**FIGURE 3.3** Heparin (HP)/heparan sulfate (HS) disaccharide analysis by liquid chromatography/mass spectroscopy (LC-MS). (A) Extracted ion chromatography; (B–I) mass spectra of each disaccharide in negative mode. (See color plate 7.)

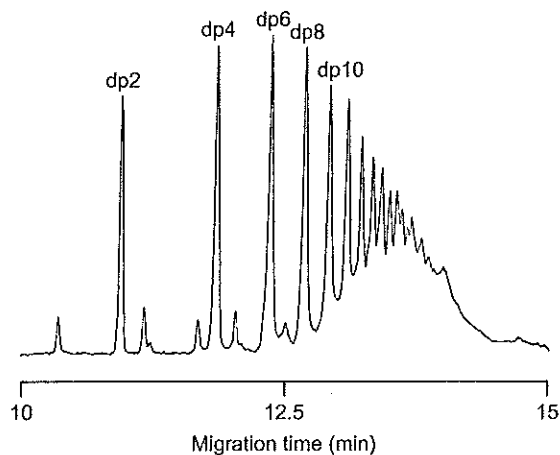
of GAGs represents an approach comparable to the peptide mapping of proteins. Oligosaccharide mapping can provide an estimate of molecular weight by detection of oligosaccharides arising from chain ends and gives information on the presence and distribution of discrete oligosaccharide sequences within a GAG [99]. Oligosaccharide mapping is a valuable method for the analysis of fine structure and to better understand the sequence of complex GAGs. Oligosaccharide mapping is also useful for making rapid assessments of the molecular distinctions between GAGs from different tissues and species [100–102]. The method involves specific enzymatic or chemical (i.e., through the use of nitrous acid) scission of polysaccharide chains followed by high-resolution separation of the degradation products by gradient PAGE (Figure 3.4) [103], GPC, CE (Figure 3.5) [103], SAX-HPLC (Figure 3.6a) [104], and other chromatographic methods. The molecular structural information of purified oligosaccharides (such as mass, degree of sulfation, and sequence) can be elucidated using ESI-MS (Figure 3.6b) [104]. Preparative separations can provide sufficient pure oligosaccharide for detailed structural analysis using nuclear magnetic resonance (NMR) spectroscopy (Figure 3.6c) [104].

### Sequence Analysis

Tandem MS has been recently applied to GAG [105]. Successful ionization and detection of GAG-derived oligosaccharides by tandem MS, including HA [106–109], KS



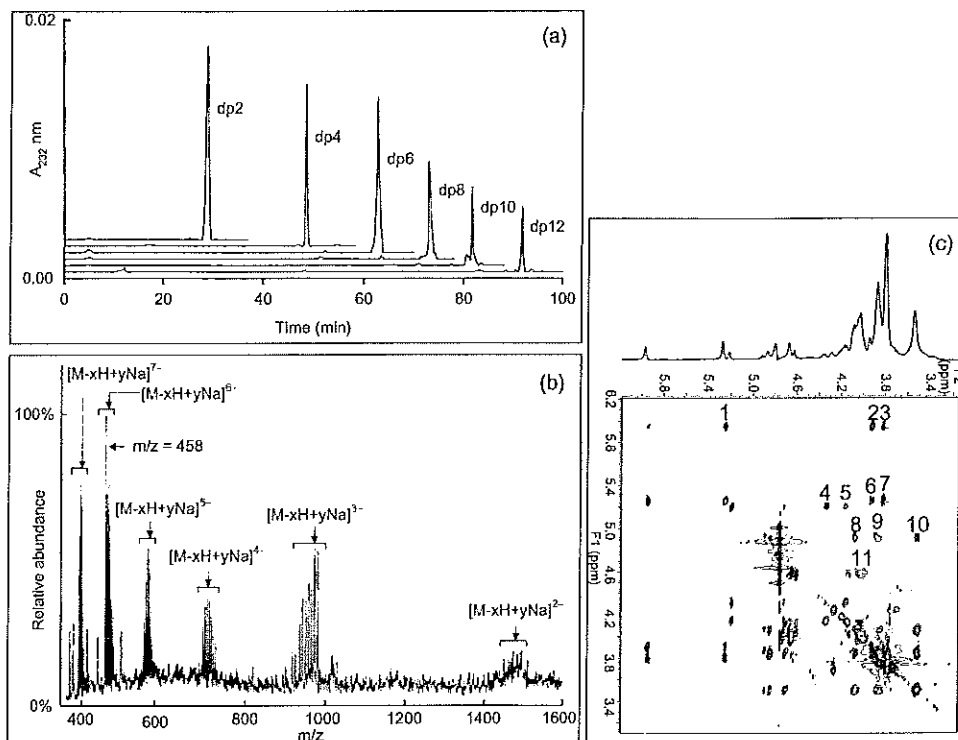
**FIGURE 3.4** Polyacrylamide gel electrophoresis (PAGE) analysis of acharan sulfate (AS)-derived oligosaccharides visualized with Alcian Blue. Lane 1: Oligosaccharide standards derived from bovine lung heparin; Lane 2: AS oligosaccharide dp10; Lane 3: AS oligosaccharide dp12; Lane 4: AS oligosaccharide dp14; Lane 5: AS oligosaccharide dp16.



**FIGURE 3.5** Capillary electrophoresis (CE) analysis of acharan sulfate after 40% digestion with heparin lyase II. Absorbance at 230 nm is plotted as a function of migration time. The peaks corresponding to the disaccharide (2mer) through octasaccharide were confirmed using authentic standards that had been previously prepared and characterized in our laboratory. The peaks of deca-saccharide (10 mer) and higher oligosaccharides (not labeled) were also separated with high resolution.

[110–112], CS [113–116], DS [104], and HP/HS [96,117–118] oligosaccharides have been reported.

Since there is no sequence heterogeneity in HA, the structure is confirmed by chain length. Fragment ions can be useful for determining the terminal sugars in the HA oligosaccharides. Fragment ions show difference of  $m/z=174.8$  ( $\text{GlcA}_p\text{-H}_2\text{O}$ ) or  $m/z=201.8$  ( $\text{GlcN}_p\text{Ac-H}_2\text{O}$ ). Glycosidic cleavage fragment ions were also observed in MS/MS and tandem MS of KS, CS/DS and HS/HP oligosaccharides, which confirmed



**FIGURE 3.6** (a) Analytical strong ion-exchange (SAX) high-performance liquid chromatography (HPLC) analysis of individual dermatan sulfate-derived oligosaccharides. (b) Electrospray ionization mass spectrometry (MS-ESI) analysis of dermatan sulfate dodecasaccharide. Six ion clusters are marked with charges ranging from  $-7$  to  $-2$ . The number of protons lost ( $x$ ) ranged from 1 to 12 and the number of sodium atoms added ( $y$ ) ranged from 0 to 9. (c) Two-dimensional  $^1\text{H}$ -NMR TOCSY spectrum of dermatan sulfate dodecasaccharide is presented. The cross-peaks are assigned as: 1,  $\Delta\text{UA}_p$  H1/H4; 2,  $\Delta\text{UA}_p$  H3/H4; 3,  $\Delta\text{UA}_p$  H2/H4; 4,  $\alpha$ -GalpNAc H1/H2; 5,  $\alpha$ -GalpNAc H1/H3,H6; 6,  $\Delta\text{UA}_p$  H1/H3; 7,  $\Delta\text{UA}_p$  H1/H3; 8, IdoAp H1/H4; 9, IdoAp H1/H3; 10, IdoAp H1/H2; and 11,  $\beta$ -GalpNAc H1/H2,H3.

the residues and composition. The cross-ring cleavage fragment ions in tandem MS can distinguish GAG oligosaccharides with different linkage and sulfo patterns.

Most recently, electron detachment dissociation (EDD) has been applied to analyze the sequence of HS oligosaccharides, including substituent position and epimerized residues of HS oligosaccharides [119,120]. Compared to collisionally activated dissociation (CAD) and infrared multiphoton dissociation (IRMPD) MS, EDD provides improved cross-ring fragmentation important for determining the pattern of sulfation, acetylation, and hexuronic acid stereochemistry ( $\text{GlcA}_p$  and  $\text{IdoA}_p$ ) on a GAG oligosaccharide. The MS/MS spectra on  $[\text{M}-2\text{H}]^-$  precursor ion of  $\Delta\text{UA}_p$ - $\text{GlcN}_p$ - $\text{S-IdoA}_p$ - $\text{GlcN}_p$ - $\text{Ac6S}$  in different dissociation are shown in Figure 3.7. Also this technology was applied to sequence DS oligosaccharides. The exact sequence of DS tetrasaccharides was assigned by the detail fragmentation in EDD-MS/MS [121,122]. Based on the extensive fragmentation observed in EDD, these complicated sulfated oligosaccharides can be sequenced unambiguously.

Electrospray ionization Fourier transform-ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) was first applied to identify several major components

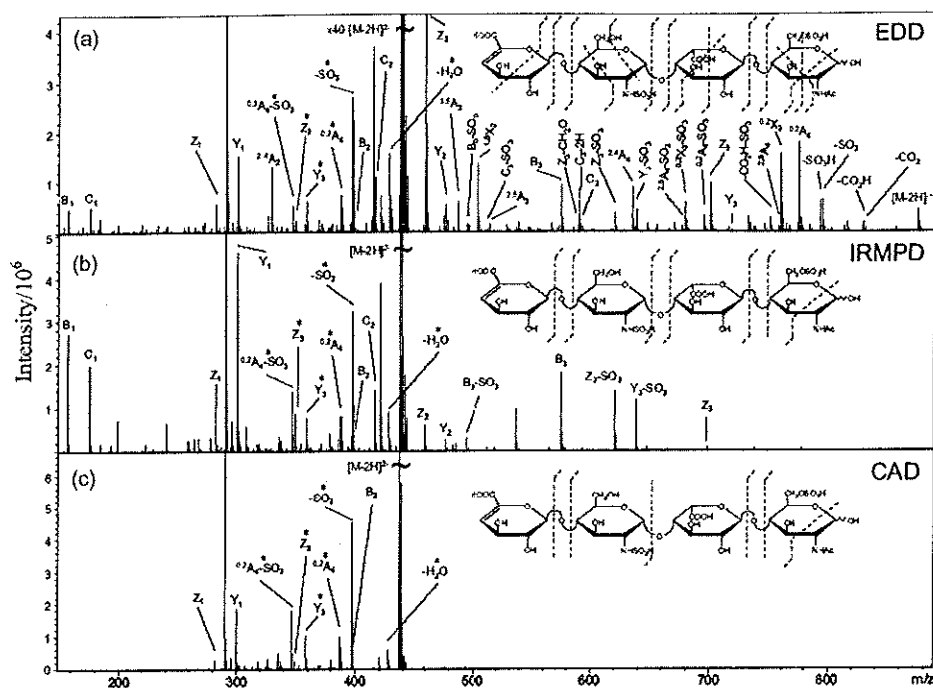


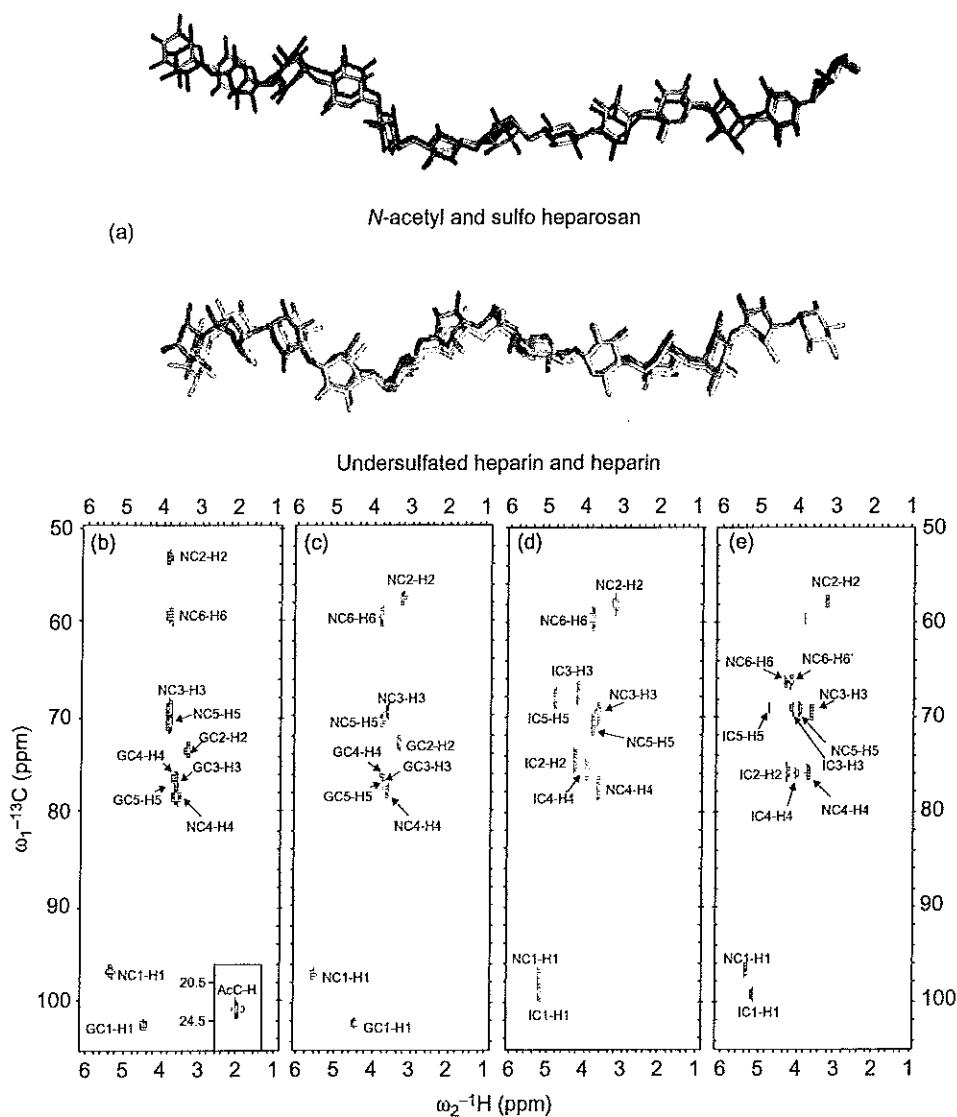
FIGURE 3.7 Tandem mass spectra of the  $[M-2H]^{2-}$  precursor ion of heparan sulfate tetrasaccharide, obtained by using (a) electron detachment dissociation (EDD), (b) infrared multiphoton dissociation (IRMPD), and (c) collisionally activated dissociation (CAD). Doubly-charged product ions are indicated with an asterisk.

in intact GAG chain mixture [123]. Bikunin is a serine protease inhibitor found in human amniotic fluid, plasma, and urine. Bikunin is posttranslationally modified with a CS chain, O-linked to a serine residue of the core protein. The CS chain of bikunin plays an important role in the physiological and pathological functions of this PG. While no PG or GAG has yet been sequenced, bikunin, the least complex PG, offered a compelling target. ESI-FTICR-MS permitted the identification of several major components in the GAG mixture having molecular masses in a range of 5505–7102 Da. This is the first report of a mass spectrum of an intact GAG component of a PG. FTICR-MS analysis of a size-uniform fraction of bikunin GAG mixture obtained by preparative polyacrylamide gel electrophoresis, allowed the determination of chain length and number of sulfo groups in the intact GAGs.

### Conformational Analysis

Nuclear magnetic resonance (NMR) is useful to characterize intact GAG chains. The composition of a chemoenzymatically synthesized HS GAG was characterized by  $^1\text{H-NMR}$  [124]. The sequence, linkage, and conformation were elucidated. The three-dimensional structure of GAGs can also be simulated based on these NMR data (Figure 3.8) [124,125].





**FIGURE 3.8** High-field NMR spectra and three-dimensional structures of chemo-enzymatically synthesized heparin (HP) and its precursors; (a) Three-dimensional structures of chemo-enzymatically synthesized HP and its precursors; (b–e) heteronuclear multiple quantum coherence of chemo-enzymatically synthesized HP and its precursors. (See color plate B.)

## Conclusion

The progress of structure analysis of GAGs relies on highly efficient isolation/purification techniques, and high-sensitivity, information-rich analytical instruments. With modern MS techniques, structural information, such as molecular weight, monosaccharide composition, number and position of sulfo groups, composition of disaccharide blocks, and sequence of highly charged sulfated carbohydrates can be obtained. High sensitivity of MS is now available for the microanalysis of carbohydrates derived from biological samples.

The fragility of sulfo groups during ionization steps and tandem MS dissociation steps makes the MS analysis challenging. Optimization of MS conditions reduces undesired fragmentation. Tandem MS has the potential to become a robust method to completely sequence complex oligosaccharides. Mechanistic studies of oligosaccharide behavior in MS and tandem MS will help advance methods development. Accumulation of MS data of various oligosaccharides will be useful to establish a library of sulfated oligosaccharides sequences. The progress of structure analysis of GAGs should dramatically improve our understanding of their biological functions and help in the development of structure–activity relationships for these important biopolymers.

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