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# Advances in glycosaminoglycan detection

**Shaukat A. Khan**[1], **Robert W. Mason**[1,2], **Hironori Kobayashi**[3], **Seiji Yamaguchi**[3], **Shunji Tomatsu**[1,3,4,5,6,*]

[1]Nemours/Alfred I. duPont Hospital for Children, Wilmington, DE

[2]Department of Biological Sciences, University of Delaware, Newark, DE

[3]Department of Pediatrics, Shimane University, Shimane, Japan

[4]Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan

[5]Department of Pediatrics, Thomas Jefferson University, Philadelphia, PA

[6]Department of Pediatrics, Shimane University, Shimane, Japan

## Abstract

**Background—**Glycosaminoglycans (GAGs) are negatively charged long linear (highly sulfated) polysaccharides consisting of repeating disaccharide units that are expressed on the surfaces of all nucleated cells. The expression of GAGs is required for embryogenesis, regulation of cell growth and proliferation, maintenance of tissue hydration, and interactions of the cells via receptors. Mucopolysaccharidoses (MPS) are caused by deficiency of specific lysosomal enzymes that result in the accumulation of GAGs in multiple tissues leading to organ dysfunction. Therefore, GAGs are important biomarkers for MPS. Without any treatment, patients with severe forms of MPS die within the first two decades of life.

**Scope of review—**Accurate measurement of GAGs is important to understand the diagnosis and pathogenesis of MPS and to monitor therapeutic efficacy before, during, and after treatment of the disease. This review covers various qualitative and quantitative methods for measurement of GAGs, including dye specific, thin layer chromatography (TLC), capillary electrophoresis, high-performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), gas chromatography, ELISA, and automated high-throughput mass spectrometry.

**Major conclusion—**There are several methods for GAG detection however, specific GAG detection in the various biological systems requires rapid, sensitive, specific, and cost-effective methods such as LC-MS/MS.

**General significance—**This review will describe different methods for GAG detection and analysis, including their advantages and limitation.

[*]**Address for correspondence:** Shunji Tomatsu MD PhD, Nemours/Alfred I. duPont Hospital for Children, Address: DuPont Experimental Station, Bldg. E400. #5205, 200 Powder Mill Rd. Wilmington, DE 19803, Office Tel: 302-298-7336, stomatsu@nemours.org.

**Keywords**

glycosaminoglycans; chondroitin sulfate; dermatan sulfate; heparan sulfate; keratan sulfate; tandem mass spectrometry (MS/MS)

## 2. Introduction

Glycosaminoglycans (GAGs) are linear, negatively charged polysaccharides classified into five families, based on their structure: chondroitin sulfate (CS) (glucuronic acid and N-acetylgalactosamine), dermatan sulfate (DS) (iduronic acid or glucuronic acid and N-acetylgalactosamine), heparan sulfate (HS) (iduronic acid or glucuronic acid and N-acetylglucosamine), keratan sulfate (KS) (galactose and N-acetylglucosamine), and hyaluronan (HA) (glucuronic acid and N-acetylglucosamine) [1, 2].

GAGs are important components of the extracellular matrix (ECM) and are found in multiple tissues [3]. Polymeric GAGs are covalently bound through a linkage region to core proteins to produce proteoglycans (PGs) or remain as free polysaccharides [4–7]. Studies on GAGs and PGs have shown their importance in biological roles, including cancer progression, angiogenesis, development, growth, microbial pathogenesis, cellular signaling (growth factors, cell surface receptors, cytokines, chemokines, enzymes, complement proteins), and anticoagulation [8–21]. GAGs are important biomarkers for inherited metabolic disorders particularly mucopolysaccharidoses (MPS). MPS are caused by the defeciency of specific lysosomal enzymes that lead to the accumulation of GAGs in multiple tissues and successive multiple organ dysfunctions. Without any treatment, patients with severe forms of MPS die within the first two decades of life [22]. Thus, it is important to establish GAG measurements, which will facilitate diagnosis, predict clinical severity and prognosis, enable therapeutic monitoring (biomarker), and provide disease screening for MPS [23].

This review manuscript focuses on the history of GAG assay development with qualitative and quantitative methods with a more detailed discussion on current applications of mass spectrometry for GAG analysis.

## 3. Methods for GAG analysis

There are several methods for measurement of GAG that have been reported in the literature, which are summarized below.

### 3.1. Dye-specific method

Dye specific methods include various stainings; toluidine blue, alcian blue, and dimethylmethylene blue, which detect intact polymer GAGs.

Toluidine blue (TB) is a simple staining technique, discovered by William Henry Perkin in 1856 [24]. Several applications of this method have been reported for the detection of GAGs in urine or other biological tissue or fluid [25–29]. TB is not suitable for quantitative

analysis of GAG because of nonspecific binding with negatively charged molecules in the biological matrix.

Alcian blue (AB) has also been used for the detection of GAGs in biological fluid and urine [30, 31]. Although AB dye interacts with negatively charged sulfated GAGs, it cannot distinguish between specific GAG. There are several reports where GAG detection was performed in urine by AB in combination with electrophoresis [25, 29, 32–34]. AB staining is not applicable to measure GAGs in blood and tissue due to a lack of sensitivity and specificity.

Dimethylmethylene blue (DMMB) has also been used to detect GAGs in biological fluids. Taylor and Jeffree first described 1,9 dimethylmethylene blue as a sensitive histochemical stain for the induction of metachromasia [35]. The DMMB dye was later developed as a colorimetric assay [36], modified by Farndale et al. [37], and adapted by Panin et al. for urinary GAGs [30]. The method was modified by Whitley et al. and utilized as a direct assay for urinary GAGs [38, 39]. DMMB was used by Stone to measure 600 urine samples with one false negative sample (Morquio A) [40]. Later reports, however, indicate that the false negative data using this method is almost 15% for all MPS patients and an even higher percentage for MPS IV patients [41–43]. The purity of dye also impacts on GAG detection and may contribute to false negative data [44].

### 3.2. Paper and thin layer chromatography

Paper and thin-layer chromatography (TLC) uses the same principle using either paper (cellulose) or glass coated with a thin layer of silica, which is used as a stationary phase, and various solvents are used as mobile phase. Caster and Dorstewitz identified known mammalian mucopolysaccharides, including hyaluronic acid, heparin, heparin monosulfate, and chondroitin sulfates A, B, and C with some certainty but KS with less confidence [45]. The method is useful only as a tentative examination of intact GAGs with a limited type of specimen mixture. Stao and Gyorkey used paper chromatography to purify GAGs from tissue extracts using zinc acetate as a solvent system [46]. Lippiello and Mankin [47] used TLC with a different acidic solution of calcium acetate and ethanol and were able to separate CS, DS, and KS. Teller and Ziemann developed the TLC method and separated urinary GAGs from different MPS [48]. There are several reports [47–50] regarding the use of TLC for GAG analysis, mainly in the urine. The main limitation of TLC is that the retention factors of different GAGs overlap, thus leading to wrong identification of MPS, especially MPS IV. These methods are inadequate for the determination of GAGs from blood and tissue extracts without prior treatments. Lack of sensitivity and specificity also limit these methods for the identification of GAGs.

### 3.3. Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) is also known as liquid secondary ion mass spectrometry (LSI-MS). FAB-MS uses a beam of neutral atoms such as argon or xenon bearing high kinetic energy for the ionization of in volatile ionic or nonionic compounds present either in the solid state or as a solution in a glycerol matrix.

Linhardt et al. reported an analysis of larger, heparin-derived oligosaccharides and a variety of disaccharides obtained from different GAGs by FAB-MS, demonstrating the versatility of this technique [51]. Negative ion FAB-MS has been used in the analysis of monosulfated disaccharides. Lamb et al. analyzed the isomers of CS, DS, and HS with differences in sulfate position and linkage position [52]. The full-scan mass spectra are useful in differentiating isomers when the sulfate group resides on different saccharide units. Li et al. also used negative ion FAB-MS to characterize sulfated unsaturated disaccharides from heparin and heparan sulfate [53]. Analysis of GAGs using FAB-MS has been reported by several other investigators [54–60]. The major limitation of this method is that it is not readily compatible with HPLC; thus, isomers cannot be resolved.

### 3.4. Matrix-assisted laser desorption/ionization (MALDI)

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) method was first developed for protein analysis [61]. It was subsequently applied to the detection of complex substances such as peptides [62], oligonucleotides [63], and glycolipids [64], as well as larger molecules such as synthetic polymers [65], glycoproteins, and polysaccharides [66] without prior derivatization. Other investigators also have been successfully analyzed underivatized oligosaccharides by MALDI-MS with detection limits down to the low pmo1 range [67–70]. Tissotet et al. used strategies to analyze highly sulfated heparins by MALDI-TOF massspectrometry [71]. However, losses of sulfate group equivalents were reported by this method [72]. Bultel et al. minimized the extent of sulfate losses by adding sodium to the matrix solution [73]. Infrared MALDI is considered softer than UV-MALDI and has been used in the analysis of HS and CS disaccharides [74]. The results showed that disaccharides were detected as sodium adducts in negative mode with minimal losses of sulfate equivalents. Sisu et al. developed a method for CS and DS using MALDI-MS [75]. Since CS and DS constitute a subgroup of sulfated GAGs for which the degree of sulfation varies between species and tissues, Kisolova et al. [6] developed a new strategy applying MALDI-TOF MS in positive ion mode for semi-qualitative and quantitative analysis of CS/DS-derived disaccharide units. Hsieh et al. also developed nanodiamond-based affinity purification and detection of sulfated GAGs using MALDI-TOF claiming specific, sensitive, and potentially useful for clinical diagnosis of MPS [76]. This method is, however, incompatible with chromatographic systems and, therefore, unable to resolve isomers, which limit its usefulness in the clinical setting.

### 3.5. Gas chromatography

Gas chromatography (GC) is a simple, multifaceted, highly sensitive, and rapidly applied technique for the separation of molecules. Nakamura and Tamura used this technique to analyze mono and disaccharides in human blood and urine after oral administration [77]. Gordon and Haust reported urinary GAGs in 23 MPS patients [78]. Urinary GAGs analysis has been reported in normal [79, 80] and MPS patients by several researched using gas chromatography [81–83]. GC, coupled with mass spectrometry, has been used for the analysis of iduronic and glucuronic acid [84]. Traditional GC/MS uses “hard” ionization technique such as electron ionization (EI), which fragments the molecules in an extensive way. When GC is coupled with API/MS, it preserves molecular ion from fragmentation [85].

### 3.6. High-performance liquid chromatography (HPLC)

Toyoda et al. developed fluorometric detection of heparin and heparan sulfate using simple and reverse-phase ion-pair chromatography [86, 87]. Studelska et al. reported quantification of glucosamine- and galactosamine containing GAGs after the reversed-phase separation of their fluorescent isoindole derivatives, including heparin, HS, KS, CS, DS, and hyaluronan [88]. A similar method has been used by several investigators to identify oligosaccharides [88–91]. This method is sensitive and very specific and, therefore, has been used to identify various MPS patients by analyzing intact urinary GAGs [92]. An example of HPLC analysis of HA, CS/DS GAG-disaccharides using HPLC is presented in Figure1.

### 3.7. HPLC with ion-exchange chromatography

Ion exchange chromatography is also applied for intact GAG detection. Radhakrishnamurthy et al. have quantified uronic acid in chondroitin sulfates and dermatan sulfate mixtures by Dowex 1 $Cl^-$ column fractionation of GAG from aortas of different animal species [94]. Heparin, HS, CS, DS, and KS were analyzed using reverse-phase ion-pairing HPLC and ion-exchange HPLC with suppressed conductivity detection. The results were compared with those obtained by strong anion-exchange HPLC using UV detection [95].

Campo et al. used weak anion-exchange Ecteola-cellulose to detect GAGs in plasma and serum using HPLC [96]. This method is not frequently used since it is tedious and time-consuming.

### 3.8. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent (ELISA) assay is a commonly used analytical biochemistry assay. Tomatsu et al. developed ELISA to measure KS and HS in blood and urine samples of MPS and ML patients [23, 41, 97, 98]. Several other investigators used ELISA to measure KS and HS in MPS patients [99–102]. Other GAGs, including CS, DS, and hyaluronan, were also identified by this method [103–107]. Although the ELISA method is a qualitative and quantitative method for the measurement of intact specific GAGs, it cannot be used to identify multiple GAGs simultaneously.

### 3.9. Capillary electrophoresis

Capillary electrophoresis (CE) is a highly sensitive chromatographic method for intact GAGs and GAG-derived disaccharide analysis. This method has high resolving power, separation efficiency, and short analysis time. Chang et al. demonstrated 2-aminoacridone derivatized GAG disaccharides by CE-laser-induced fluorescence and stated that this technique is 100 times more sensitive than traditional UV detection at 232 nm [108]. Hopwood and Harrison have used CE to detect urinary GAGs from control and MPS patients [109]. Cappelletti et al. separated all animal GAGs using CE [110]. Several reports describe CE combined with mass spectrometry for simultaneous separation and determination of GAGs [111–113]. An example of capillary electrophoresis analysis of heparin (HP) and HS, GAG-disaccharides, is presented in Figure 2.

### 3.10. HPLC with ESI mass spectrometry

Electrospray ionization (ESI) is a soft ionization method that is generally used in combination with liquid chromatography and is most suitable for mass spectrometric (MS) analysis of glycosaminoglycans [114]. The spray conditions can be modified to suppress the loss of sulfo groups and enhance the abundance of certain types of ions, such as, for example, sodium cationized or protonated species and ions with specific charge states [115]. Oguma et al. developed methods to analyze GAGs (HS, KS, and DS) from serum/plasma using ESI mass spectrometry [116–120]. The method uses the lyase enzymes of chondroitinase B, keratinase II, and heparitinase, which digest polysaccharides to DS, KS, and HS disaccharides. The produced disaccharides were measured by LC/ESI/MS/MS. A similar enzymatic digestion method was used by Osago et al. [121], which was able to measure not only all classes of GAGs (CS/DS, KS, HS, and hyaluronic acid) but also isomeric disaccharide compounds. Numerous reports have described the measurement of GAGs in blood/plasma and urine samples from normal control and MPS patients by using enzyme-mediated digestion [3, 23, 122–128]. Detection of non-reducing end of GAGs was developed by Lawrence et al. [129, 130]. In this method, the lack of lysosomal enzyme results in the accumulation of characteristic non-reducing terminal carbohydrate structures. GAGs are enzymatically depolymerized, releasing unique mono-, di-, or trisaccharides from the non-reducing ends of the chains, which are subsequently labeled by reductive amination with heavy isotope-labeled aniline. The modified sugars are quantified by LC/MS/MS. This method is broadly applicable to all MPS disorders utilizing tissue, blood, and urine. However, this method cannot detect MPS IV.

Identification of GAGs (CS, DS, HS, and KS) from the urine of MPS patients by a chemical degradation by methanolysis followed by LC/MS/MS of the liberated disaccharides was developed by Auray-Blais et al. [131–133]. The method has been adapted for the identification and quantification of GAGs from urine [134, 135], cerebrospinal fluid (CSF), serum [136, 137], and animal tissues [138]. Recently, Trim et al. applied butanolysis followed by LC/MS/MS to quantify heparan sulfate in urine from MPS patients. (MPS I, II, III and VI) [139]. The sensitivity of this method is very high when optimized for HS.. The sensitivity of the new technique is illustrated by the quantification of HS in 5 μL urine samples from MPS patients and healthy controls. HS was quantifiable in all samples, including controls. Disaccharide reaction products were further characterized using exact mass MS/MS. However, this method is unable to identify subclasses of GAGs. Most recently, Forni et al. reported simultaneously quantification of urinary HS and DS from MPS (I, II, III, VI, and VII) patients using butanolysis derivatization followed by LC/MS/MS [140].

### 3.11. HPLC with atmospheric-pressure chemical ionization mass spectrometry

Atmospheric pressure chemical ionization (APCI) is a soft ionization method similar to chemical ionization used for polar and relatively less polar thermally stable compounds with molecular weight less than 1500 Da. McEwen et al. have described for the analysis of solids, liquids, and biological tissues by APCI LC/MS [141]. The method can be used in combination with either gas chromatography or liquid chromatography and is qualitative and quantitative analysis of mono and disaccharides. Ricochon et al. have reported the

quantification of a large pool of mono and disaccharides in complex matrices by APCI mass spectrometry (APCI-MS) [142]. Zhou et al. have reported the determination of glucosamine in human plasma using HPLC-APCI-MS/MS [143].

### 3.12. Automated flow-injection mass spectrometry

Automated high-throughput mass spectrometry (HT-MS/MS) using flow-injection is a very rapid method for GAG analysis compared with standard LC-MS/MS. The HT-MS/MS system excludes chromatographic separation; thereby allowing sample-to-sample cycle times to be processed faster than LC-MS/MS. This method was originally developed for high-throughput newborn screening for inborn errors of metabolism by analysis of acylcarnitines and amino acids in DBS [144]. The complete automation of the sample introduction has been allowed the analysis of up to 200 samples in one injection sequence, at a rate of one sample every 3 min, with excellent separation.

Moreover, the innovative HT-MS/MS system (RapidFire; Agilent Technologies) enables the sample to be aspirated to a matrix for concentration and desalting, followed by direct injection into MS/MS without chromatographic separation. Each sample is processed within 10 seconds. In 2014, Shimada et al. have reported that this HT-MS/MS system is applied to assay HS in blood samples from control and patients with MPS II, III, or IV and in DBS from newborn controls and patients with MPS I, II, or III [145]. The study concluded that the innovative HT-MS/MS system provides 10–100 fold higher throughput than LC-MS/MS-based methods with similar sensitivity and specificity in an HS assay and suitable for diagnosis, monitoring, and newborn screening of MPS. Tomatsu et al. reported HS and KS analysis by HT-MS/MS, compared with LC/MS/MS and found a moderate correlation in serum HS and KS measurements of control subjects between LC-MS/MS and HT-MS/MS indicating that each assay is comparable [3].

The HT-MS/MS (RapidFire) system has been validated as suitable for many drug discoveries [146–150] and Absorption, Distribution, Metabolism, and Excretion (ADME) based applications [151–154]. The method could be valuable in newborn screening of MPS, whereby over one million of samples might be screened for GAG on an annual basis. The main drawback of this method is that disaccharides with identical molecular weights cannot be distinguished.

## 4. Advantages and limitations of methods for GAG analysis

Dye specific methods are simple and have been widely used for quantitative analysis of urinary GAGs but are unable to quantify specific GAG in blood and DBS, resulting in false negative data. Similarly, TLC is a very simple method used to identify specific GAGs in urine but not in blood and tissue extracts without prior treatment. Overlapping various GAGs affected by the retention factors results in misidentification. In contrast, HPLC is a very sensitive, highly reproducible, and efficient method for separation and detection of GAGs and other compounds within 10–30 min. and therefore produces quicker, and sometimes more accurate, results.

Co-elution of two compounds with similar structure and polarities is a limitation of this method. Capillary electrophoresis (CE) is also a highly sensitive method for GAGs and GAG-derived disaccharide analysis. Due to high resolving power, separation efficiency, and short analysis time, it is used by several investigators, as mentioned above. Another method for GAG analysis is ELISA, which is a sensitive and specific method; however, due to the high cost and inability to identify multiple GAGs simultaneously, this method is not widely used. LC/MS/MS is a highly specific and sensitive technique, which measures analytes in complex mixtures either in positive ion mode and/or negative ion mode. There are several protocols that have been developed in the past decades as described above. A variety of analyte separation techniques like capillary electrophoresis, gas chromatography, and HPLC are united with mass spectroscopy for simultaneous separation and determination of analytes called CE-MS, GC–MS, and HPLC-MS, respectively [111, 112, 133, 155, 156] which makes its application even wider. Although mass spectrometry has many advantages and its wider application makes it the best tool in the modern period however, it is very expensive, and not every lab can afford it.

Various LC-MS/MS-based GAG analysis methods, which are in clinical practice, their advantages and limitations have been summarized in table 1.

## 5. Conclusion

Although several procedures have been established to measure GAGs, dye specific and TLC methods are not used these days due to their limitation to detect all GAGs. Mass spectrometry is now widely used for measuring all species of GAGs in a biological systems including blood, urine, DBS, and tissue for prognosis, diagnosis, monitoring, and screening purposes. Due to itswide range of applications, LC-MS/MS is considered as the best analytical tool for basic or clinical research in the biomedical field and the method of choice for GAG analysis.

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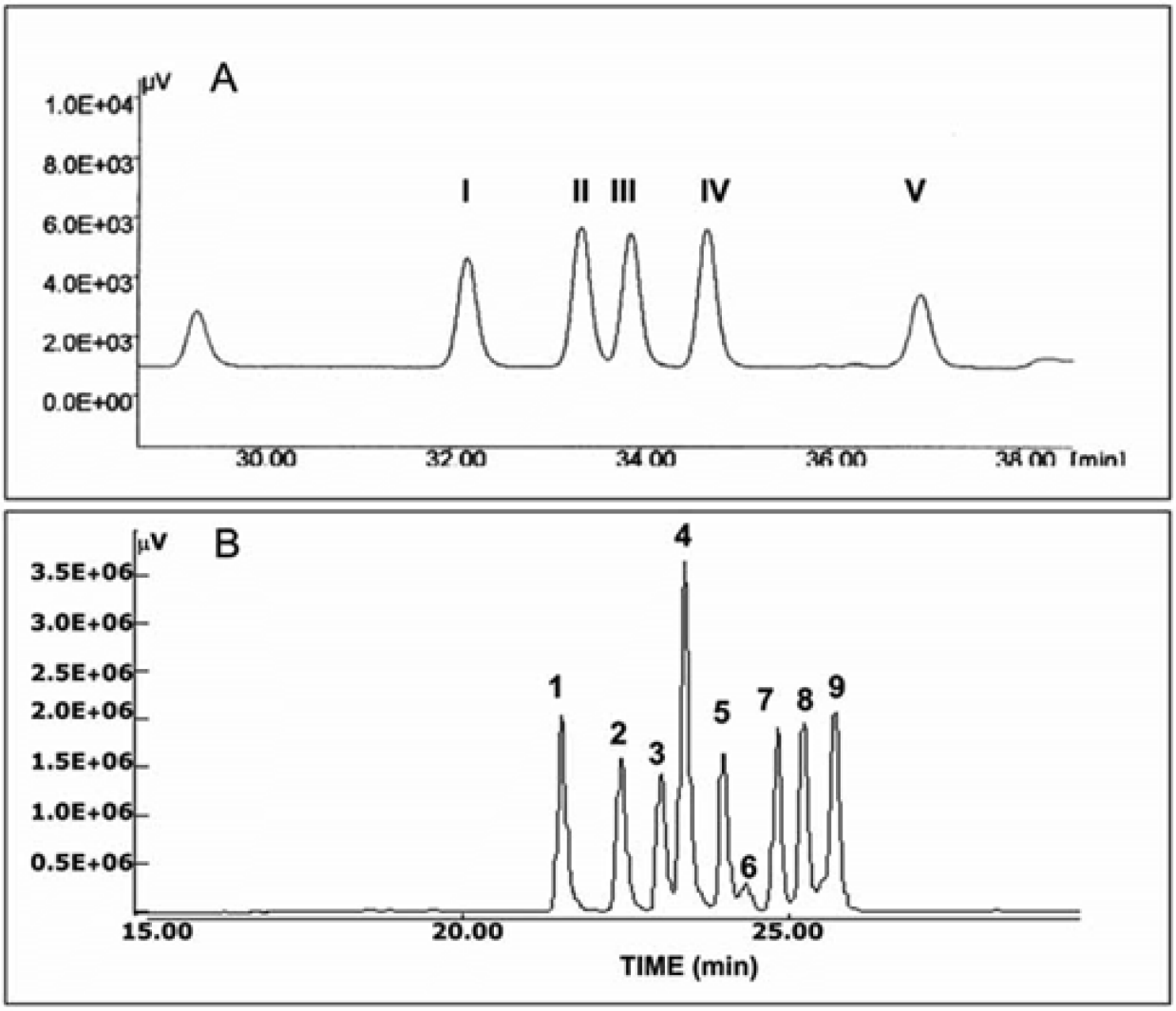


**Figure 1.**

Adapted from Karousou et al. [93]. HPLC analysis of HA, CS/DS GAG-disaccharides. The standard mixture of HA and CS/DS-disaccharide was derivatized with AMAC and analyzed with HPLC using a reversed-phase column (C-18, 4.6 × 150 mm). Products were detected with a fluorophore detector (ex = 442 nm and em = 520 nm). Panel A: separation of a mixture of commercial standard mono-sulfated-disaccharide from CS/DS and HA; I: di-mono4S; II: dimono6S; III: di-0 HA; IV: 0 CS; V: GalNAc. Panel B: separation of a mixture of commercial standard-disaccharide from HA and CS/DS GAGs. Peaks corresponds to: 1 di-tri(2,4,6)S; 2 di-di(2,4)S, 3 di-di(4,6)S, 4 di-di(2,6)S, 5 di-mono4S, 6 di-mono2S, 7 di-mono6S, 8 di-0S HA, 9 di-0S CS.

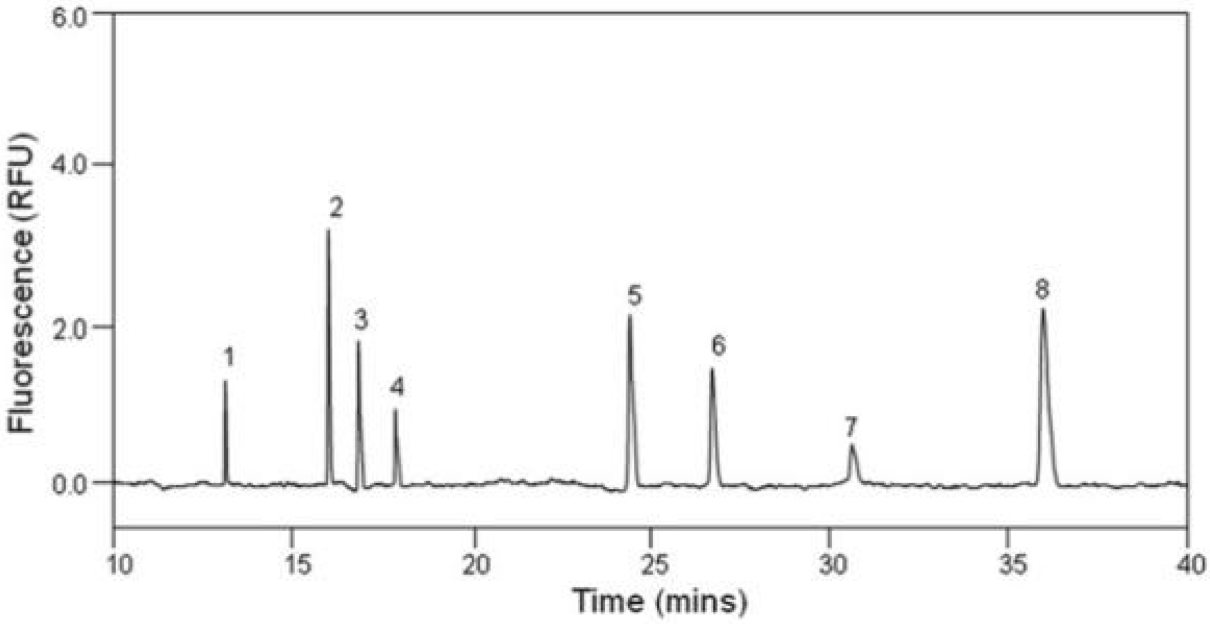


**Figure 2.**

Adapted from Chang et al. [108]. Electrophoregram of eight HP/HS Δ-disaccharides. The analysis was performed at 25°C, pressure injection of 50 mbar × 10 s, using 50 mM phosphate buffer, pH 3.5, under 30 kV with reversed polarity.

**Table 1.**

LC-MS/MS-based GAG analysis methods and their advantages and limitations.

| LC/MS/MS Methods | GAGs | Sample | Advantages | Disadvantage | Reference |
|---|---|---|---|---|---|
| Enzymatic degradation and LC/ESI/MS/MS | CS, DS, HS, KS | Plasma, serum, urine, DBS, tissue extracts, and CSF | Simultaneous identification of all GAGs Identification of subclasses of CS, HS, and KS Quantification of sulfation level | Variation in the ionization efficiency of different disaccharides. | [116–119, 123, 126, 157, 158] |
| Methanolysis and LC/MS/MS | CS, DS, HS, and KS | Urine, blood, CSF, and tissues | Simultaneous identification of all GAGs. Simple steps for preparing samples and reagents that are easily available. No use of an expensive enzyme. | Not applicable for blood KS. No detection of subclass of CS, HS, and KS | [131, 132, 136–138, 159] |
| Butanolysis and LC/MS/MS | DS, HS | Urine | Simultaneous identification of DS, HS. Simple steps for preparing samples and reagents that are easily available. No use of an expensive enzyme. | Not applicable in blood. No detection of subclass of CS, HS, and KS | [139, 140] |
| Atmospheric pressure chemical ionization mass spectrometry | Glucosamine Purified disaccharides from CS | Plasma | The main usage of APCI is for polar and relatively less polar, thermally stable compounds with molecular weight less than 1500 Da. Useful in drug discovery. | Not wide application in biological fluid for GAG detection. | [58, 143, 160] |
| MALDI-TOF massspectrometry | Heparin, CS, DS | Tissue | Creates singly charged species, greatly simplifies the interpretation of the data. Applicability to high-throughput experiments, robustness, relatively high tolerance to salts and other contaminants. | The low extent of desorption induced by the laser for samples containing a large number of acidic groups, together with the facile loss of sulfate moieties in the MALDI source has been major limitations of this technology for GAG analysis. | [6, 71, 75] |
| Fast atom bombardment mass spectrometry | Heparin, CS, DS, HS, hyaluronic acid, and KS | Commercially obtained disaccharides | The procedure is simple and rapid. Useful for high mass and thermally labile compounds. Relatively tolerant of variation in sampling. Good for a large variety of compounds | Analyte must be soluble in the matrix. Bad for multiple charged (>2 charges) compounds. Low sensitivity. Not compatible with HPLC. Cannot distinguish between isomers. | [51, 52] |
| Isotope Labeling mass spectrometry | CS, HS, and KS | CHO cells, and mice tissue | The technique is adaptable to all types of GAGs | Several different isotopes used, including reductive amination, biotin with single deuterium$^{13}$C incorporation, etc. Procedures are tedious, time-consuming and have limited applications. | [130, 161–163] |
| Non-reducing end of GAG and mass spectrometry | HS, DS | Blood, urine, tissue | All MPS can be distinguished based on specific non-reducing end GAGs. | This method is not applicable to identify MPS IV based on non-reducing end KS. | [129] |
| Automated flow injection mass spectrometry (high throughput MS/MS; RapidFire) | HS, KS | Blood, DBS | Within 10 seconds per sample compared with LC/MS/MS. Much higher throughput than LC-MS/MS-based methods with similar sensitivity. | Isomeric disaccharides cannot be distinguished. | [3, 145] |