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Glycosaminoglycans detection methods: Applications of mass spectrometry

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Abstract

Glycosaminoglycans (GAGs) are long blocks of negatively charged polysaccharides. They are one of the major components of the extracellular matrix and play multiple roles in different tissues and organs. The accumulation of undegraded GAGs causes mucopolysaccharidoses (MPS). GAGs are associated with other pathological conditions such as osteoarthritis, inflammation, diabetes mellitus, spinal cord injury, and cancer. The need for further understanding of GAG functions and mechanisms of action boosted the development of qualitative and quantitative (alcian blue, toluidine blue, paper and thin layer chromatography, gas chromatography, high pressure liquid chromatography, capillary electrophoresis, 1,9-dimethylmethylene blue, enzyme linked-immunosorbent assay, mass spectrometry) techniques.

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Contributions to the project: Francyne Kubaski is the primary author for this review article and an expert in molecular biology. She has contributed to the concept and planning of the article, collection of previous articles and data, and reporting of the work described. Harumi Osago is the primary author for this review article and an expert in molecular biology. She has contributed to the planning, data analysis, and reporting of the work described.

Robert W. Mason PhD is a molecular biologist and chemist and has over 30 years of experience in chemistry. He has contributed to the concept, planning of the project, informed consent, analysis of data, and reporting of the work described.

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The availability of quantitative techniques has facilitated translational research on GAGs into the medical field for: 1) diagnosis, monitoring, and screening for MPS; 2) analysis of GAG synthetic and degradation pathways; and 3) determination of physiological and pathological roles of GAGs.

This review provides a history of development of GAG assays and insights about the use of tandem mass spectrometry and its applications for GAG analysis.

Keywords

Glycosaminoglycans; Mass spectrometry; Alcian blue; ELISA; Chromatography; mucopolysaccharidoses

1. Introduction

Glycosaminoglycans (GAGs) are negatively charged linear polysaccharides composed of repeating disaccharides with variable sulfation levels [1–5]. They are classified in five major groups according to the repeating subunit as: 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) (Fig.1) [1–6] (Table 1).

GAGs are one of the most important components of extracellular matrix (ECM) and are found in multiple tissues [7]. Polymeric GAGs are covalently bound through a linkage region to core proteins to produce proteoglycans (PGs) or remain as free polysaccharides [4,8,9,10]. PGs are associated with various physiological functions such as hydration and swelling pressure to the tissue to absorb compressional forces, regulation of collagen fibril formation, modification of the activity of transforming growth factor- β , and the major anionic site responsible for the charge selectivity in glomerular filtration. Sulfation patterns in the GAG chains play significant roles by allowing interactions, normally of an ionic nature, with growth factors. The core proteins are not just scaffolds for GAGs, they also contain domains that have particular biological activities [11]. Many PGs are multifunctional molecules that engage in different specific interactions simultaneously. Studies on GAGs and PGs have shown their importance in biological processes such as: cancer progression, angiogenesis, development, growth, microbial pathogenesis, cellular signaling (growth factors, cell surface receptors, cytokines, chemokines, enzymes, complement proteins), and anticoagulation [12–32]. One of the major clinical applications for GAG analysis is with the study of inherited metabolic disorders, particularly mucopolysaccharidoses (MPS). In MPSs, GAG degradation pathways are disrupted due to enzyme deficiency. Enzyme deficiency causes undegraded GAGs to accumulate in multiple tissues leading to organ dysfunction represented by a variety of clinical signs and symptoms such as skeletal dysplasia, short stature, mental retardation, heart valve disease, hearing loss, corneal clouding, hepatosplenomegaly, and umbilical and inguinal hernias. Untreated patients with the severe form die of respiratory failure, heart disease, and brain damage within the first two decades of life [33]. Establishment of GAG measurement facilitates diagnosis, prediction of clinical severity, prognosis, therapy monitoring (biomarker), and disease screening [34].

Several qualitative and quantitative methods (toluidine blue; alcian blue; paper and thin-layer chromatography, 1,9-dimethylmethylene blue; chromatography: gas and high-performance liquid chromatography - HPLC; capillary electrophoresis; enzyme-linked immunosorbent assay - ELISA; mass spectrometry MS) have been developed to determine the significance of many roles of GAGs in biological processes.

Dye-spectrometric methods including alcian blue [35] and dimethylmethylene blue (DMMB) [36–41] have been used to assay total urinary GAG. Thin-layer chromatography (TLC) has been used to separate specific GAGs, but this method has not been adapted to measure GAGs in blood or tissue extracts. Sensitivity and specificity of the dye-spectrometric and TLC methods are not sufficient to detect all types of MPS, especially MPS IV.

HPLC is a sensitive, reproducible, and accurate method to assay each specific GAG but cannot be applied to mass screening because the method is complex and time-consuming [42–44]. ELISA assays for CS, DS, KS, and HS in blood and urine have been established that are rapid and reproducible but expensive. Thus, establishment of a simple, accurate, reproducible, and cost-effective GAG assay method is urgently needed to apply to not only for clinical indications but also for basic research.

Tandem mass spectrometry (MS/MS) has more recently been developed to assay disaccharides derived from CS, DS, HS, and KS in blood, urine, tissues, and/or dried blood spots (DBS) [45–47]. The liquid chromatography (LC) MS/MS method not only shows sensitivity and specificity for detecting all subtypes of GAGs, but is also helpful in elucidating biological roles of GAGs and aiding diagnosis and therapeutic monitoring of MPS. The main limitation of LC processing is the long run time that limits its use in high-throughput screening. An automated high-throughput mass spectrometry (HT-MS/MS) system eliminates the chromatographic process allowing running time in 10 s, while maintaining the quality and accuracy of standard LC/MS/MS platforms.

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

2. GAG assay methods

2.1. Toluidine blue staining

Toluidine blue (TB), or tolonium chloride, is a thiazine that has acidophilic metachromatic properties. It was discovered by William H. Perkin in 1856 and is used to detect GAGs due to its high affinity for acidic tissues [48–49].

TB staining is based on metachromasia, a principle in which the dye reacts with electronegative groups in tissues to produce colors of different wavelengths according to the GAG concentration but does not alter the chemical structure of the GAGs [49–50]. The negatively charged sulfates in the GAGs neutralize the positive charge of toluidine blue, leading to dye aggregation by hydrophobic bonding and van der Waals interactions [51].

Detection methods using gel electrophoresis followed by staining with TB have been developed to detect low levels of GAGs in tissue extracts [52]. TB staining is widely used for pathohistology to detect GAG accumulation in tissues sectioned with 0.5 μm thickness providing the best resolution of storage materials (Fig. 2); however, TB method cannot be applied to quantitative analyses of GAGs since TB reacts with other unrelated negatively charged molecules.

2.2. Paper and thin layer chromatography

GAGs can be separated by paper and thin-layer chromatography [53]. Tissue extracted GAGs can be purified by paper chromatography in the presence of zinc (0.25 M zinc acetate solvent) at pH 4.0 and then precipitated with ethanol to remove impurities [54].

In 1969, Teller et al. developed a TLC method using formate buffer/isopropanol, 60:40, to separate GAGs in 5–6 h [55]. Lipiello et al. used ethanolic solutions (60, 50, 40, 30, 20%) of calcium salts (2.5 and 5% respectively) acidified with acetic acid to separate CS, DS, and KS [56]. Humbel et al. applied the same method to analyze these GAGs in urine samples. They used 5% calcium acetate in 10% ethanol to improve separation of DS [57]. TLC methods separate GAGs based on their size and sulfation levels, and are generally faster and have better sensitivity for small oligosaccharides with low net charge than gel separation methods (polyacrylamide gel electrophoresis-PAGE and fluorophore assisted carbohydrate electrophoresis-FACE) [58].

GAGs can be separated by chromatography on silicated glass paper [59] or filter papers [60]; however both require several elution steps. In 1966, Wusteman et al. reported separation of GAGs using thin layers of silica gel and detection with selective spray reagents (metachromatic spray-toluidine blue or azure A; orcinol-sulphuric acid; nitrous acid-indole; naphthoresorcinol spray; Morgan-Elson spray) [61]. In 1984, Säämänen et al. reported the use of scanning spectrophotometry at 232 nm to detect unsaturated GAGs digested with chondroitinase AC II separated by TLC on cellulose. Samples are scanned by a chromatogram spectrophotometer (Zeiss KM 3, Carl Zeiss) with 232 nm reflectance to measure the light intensity reflected from the plate [62].

Thus, TLC was developed to identify each specific GAG; however, overlapping retention factors (R_F) of different GAGs can lead to miss-identification of MPSs. For example, keratan sulfate (KS) that accumulates in MPS IV does not completely separate from C6S, making differential diagnosis of these forms of MPS difficult [63]. Sensitivity and specificity of the TLC methods are not sufficient to detect all types of MPS, especially MPS IV. Another disadvantage of the TLC method is that it is not applicable to blood or tissue extracts without prior protease, nuclease or hyaluronidase digestion. Thus, TLC methods are no longer widely used for GAG analyses.

2.3. Alcian blue

Alcian blue (AB) is a tetravalent cationic dye with a hydrophobic core that contains a copper ion. The dye interacts with high specificity to sulfated GAGs by ionic interactions and has been used to quantify GAGs [35]. It is also used in combination with silver to stain GAGs after separation by PAGE [58,64–66]. However, the use of AB is limited due to problems

with precipitation and interference due to interaction with other negatively charged molecules [67–68].

In 1964, Scott et al. demonstrated that AB forms insoluble complexes with acidic GAGs [69]. In 1973, Whiteman added $MgCl_2$ to prevent AB from binding to proteins in acidic solutions [70]. In 1973, Hata et al. reported the use of 0.1% AB in two-dimensional electrophoresis on cellulose acetate strips with 0.1 M pyridine/0.47 M formic acid buffer (pH 3) followed by 0.1 M barium acetate solution (pH 8) [71]. The sensitivity of the assay was improved by adding dimethyl sulphoxide to the assay solution [72].

The AB method can be limited by the presence of negatively charged molecules such as amino acids, which can interfere with the binding of AB to GAGs [73]. The AB staining is used to detect GAGs in combination with electrophoresis as well as tissue staining (Fig. 3). This method is not sensitive and specific enough to measure GAGs in blood or tissue extracts without prior protease, nuclease or hyaluronidase digestion and cannot distinguish specific GAGs by itself.

2.4. Dimethylmethylene blue

Dimethylmethylene blue (DMMB) is another cationic dye that binds to sulfated GAGs and results in an absorbance shift when it binds to GAGs [73]. Reference values for DMMB staining are dependent on age [36]. The DMMB test is one of the most used methodologies to quantify GAGs [74–79]. It takes advantage of the metachromatic properties of the dye, and it can be used in solution [80–81] or in solid-phase [82].

DMMB was first used as a histochemical stain in 1969 by Taylor et al. and as a colorimetric assay in tissues by Humbel et al. in 1974 [83,74]. The assay described by Humbel et al. was limited due to instability of the dye that led to precipitation of the GAG complexes [74]. In 1982, Farndale et al. overcame these limitations by substituting formate for the citrate/phosphate buffer used in the original assay [81].

Sabiston et al. developed an automated method [84], and Panin et al. adapted the method to use DMMB to measure GAGs in cetylpyridinium chloride precipitated urine [85]. Chandrasekhar et al. used DMMB to measure GAGs after chromatographic elution with guanidium chloride (GuHCl) [86] and Whitley et al. refined the method to measure GAGs in small amounts of sample [37].

Heparin should not be used as an anticoagulant because it interferes with the DMMB test, as do drugs that contain artificial coloring agents [37,87]. The test is also limited by purity of the dye because contaminating sulphur can cause false negatives [63]. The amount of protein present in the sample can also lead to false negative results, in which, high protein concentrations were seen with significantly decrease in GAG results [73]. In 1990, Goldberg et al. modified the DMMB protocol by addition of bovine serum albumin (BSA) and phosphate-buffered saline (PBS) to measure GAGs in chondrocyte culture media that contained high levels of protein [88]. de Jong et al. improved the method by increasing the pH (pH 8.8) so that proteins are mostly negatively charged and consequently do not bind to GAGs [78].

Orii's group had first demonstrated that preliminary DMMB test of 10,000 urine samples from 6-month-old infants and under 1-year-old MPS patients provided normal distribution of GAG levels and cut-off values. The same group performed successive MPS screening of around 130,000 urine from the infants from 1993 to 2000. These studies led to the first identification of two patients with MPS II [41]. Thus, DMMB method is still used for the screening of MPS as a feasible, reproducible and economical tool while the disadvantage is that this method cannot be applicable to blood and tissue specimens directly and cannot separate specific GAGs.

2.5. Capillary electrophoresis

The use of capillary electrophoresis (CE) for GAG analysis is beneficial due to high power of separation resolution and simplicity of analysis [97]. Separation is dependent on the amount of GAGs, size, purity, charges, and degree of sulfation [98–103]. In 1979, Cappelletti' group demonstrated that high-resolution electrophoresis of GAGs in tissue and AB staining can differentiate each type of MPS and normal control samples by 560 nm (Gelman Automatic Computing Densitometer ACD-18) followed for GAG analysis in urine by Orii's and Hopwood's groups (Fig. 4) [104–105]. The detection of GAGs separated by CE is based upon either direct UV detection [99], indirect UV detection [106–107], generation of metal complex-copper complexes [108–109], or mass spectrometry [110–112].

The use of electrophoresis method provides the advantage of simultaneous assays for several GAGs with high sensitivity and reproducibility. However, it only allows semi-quantitation and is only applicable to urine. Due to improvements in mass spectrometry and HPLC techniques, it is expected that CE will not be used for GAG analysis in the future.

2.6. Gas chromatography

Gas chromatography is based on vaporization of the compounds that are injected into a heated column with elution in an inert gas. In 1969, Kaplan D. suggested that MPS types could be classified according to the hexosamine content of urinary mucopolysaccharides (GAGs); however, it was extremely hard to identify the relative amount of DS and HS in samples by column chromatography [89]. This led to the use of gas-liquid chromatography (GLC) for GAG analysis in 1974 [90]. In this method, Murphy et al. analyzed GAGs in urine from MPS patients. The GAGs were hydrolyzed with hydrochloric acid to yield glucosamine and galactosamine. The sugars were then acetylated and separated by GLC. CS and DS yield galactosamine while HS yields glucosamine, allowing the discrimination of MPS III from MPS I and II [90].

In 1998, Toida et al. developed a quantitative and qualitative analysis of GAGs by gas chromatography-mass spectrometry (GC/MS). GAG samples were hydrolyzed by methanolysis, and the iduronic and glucuronic acids were derivatized to trimethylsilyl ethers prior to GC-MS [91]. The use of mass spectrometry (MS) coupled with GC allows separation and quantitation of sub-microgram quantities of the sugars.

2.7. High-performance liquid chromatography

High performance liquid chromatography (HPLC) is a separation method based on differential interaction of compounds with adsorbent materials in a column, leading to different elution times for specific compounds. It has been used to quantify GAGs after depolymerization of polysaccharides, followed by separation of resulting disaccharides, detected by UV absorbance or fluorescence [92–93]. In 1984, Kodama et al. quantified disaccharides of CS (Di-0S, Di-4S, Di-6S, Di-diS) by digesting GAGs with chondroitinase and measuring fluorescence of the disaccharides after modification with 2-aminopyridine and separation by HPLC [94]. In 1986, they developed an HPLC method for differential diagnosis of MPS [95].

HPLC is a sensitive, reproducible, and accurate method to assay each specific GAG; however, it cannot be applied to mass screening because the method is complex and time-consuming [43–44,96]. Thus, HPLC protocols are combined with mass spectrometry (MS) to identify and quantify eluted disaccharides with or without modification (see Section 2.9).

2.8. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a technique based on the binding of an antigen to an antibody that is linked to an enzyme and detection by hydrolysis of a substrate to the linked enzymes [113]. There are different types of ELISA: direct [114], indirect [115], sandwich [116], competitive [117].

ELISA assays developed for GAGs can measure levels of KS [118–120], HS [121–122], C4S [123], C6S [123], DS [123], and hyaluronic acid [124]. ELISA protocols have also been developed to detect GAGs in cells and on cell-surfaces [125–126]. Measurement of GAGs by sandwich ELISA is still commonly used, particularly in clinical settings. The advantages of its use are: feasibility, sensitivity, reproducibility and quantitation requiring only a simple ELISA plate reader. Disadvantages are cost since no current assay can detect several GAGs simultaneously requiring multiple assays.

2.9. Mass spectrometry

Mass spectrometry (MS) is a technique that measures compounds based on their mass-to-charge ratio (m/Q , m/q , m/Z , or m/z) [127–129]. Different ionization sources can be used, e.g. electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI), fast atom bombardment (FAB), chemical ionization (CI), matrix-assisted laser desorption/ionization (MALDI) [130–135].

The principles that led to mass spectrometry were discovered over 100 years ago by the Nobel laureate Sir John Thomson who discovered the electron and was the first to demonstrate separation of isotopes of a stable element [129]. Mass spectrometry has now become one of the most useful analytical techniques due to its specificity, accuracy and sensitivity [127–128,136] and is considered one of the most successful and useful techniques applied for newborn screening (NBS) [137].

GAG analyses have been performed in different types of mass analyzers: time-of-flight (TOF) [138], ion trap (IT) [139–140], Fourier transform ion cyclotron resonance (FTICR) [141], and triple quadrupole (QQQ) [45–47].

MS analysis can be full-spectrum to detect all intact ions in mixtures of unknown compounds, ion monitoring to measure levels of known intact ions, or multiple reaction monitoring (MRM) to measure different intact/product ion pairs that can distinguish compounds that have ions of identical mass/charge ratio but have different fragments [145–147].

Mass spectrometry methods are superior in accuracy, speed, sensitivity, and specificity to other detection methods. There are many protocols described using MS/MS for GAG quantification: e.g. sulfated GAGs in multiple cell lines [4], urinary GAGs [46–47], mono and disaccharides in tissue extracts [45], plasma/serum or urinary GAGs [144–151], GAGs in articular cartilage and yellow ligament [152], and GAGs from dried blood spots [153].

In 2001, Oguma et al. developed an ESI mass spectrometry protocol for quantification of HS and KS from serum and plasma, and in 2007 improved this method by including analysis of DS and adapting the method to measure GAGs in dried blood spots (DBS) [45–47,146–147] (Fig. 5). Polysaccharides were digested with heparitinase, keratanase, and chondroitinase B to release HS, KS and DS, respectively, and the unmodified disaccharides were then detected by LC/ESI/MS/MS [45–47,146–147]. In 2014, Osago et al. described a more complete method for one-shot analysis of disaccharides derived from all four classes of GAGs using LC/ESI/MS/MS (2014) [152] (Fig. 6). This protocol enabled identification and quantitation of 23 different disaccharides (8 CS/DS, 1 hyaluronic acid, 12 HS, and 2 KS) including di- and tri-sulfated species. Applying the method for analysis of disaccharides obtained by enzymatic digestion (chondroitinase ABC, hyaluronidase, heparitinase, and keratanase) of articular cartilage GAGs, they showed the characteristic composition of GAGs in the cartilage. In this method, disaccharides that have the same molecular mass but different structures (isomers) are separated stereospecifically on a porous graphitized carbon column and then identified with MRM transitions having the same Q1 but different Q3 specific to each disaccharide. Thus, the method distinguishes isomers in the different classes as well as in the same class with sulfate(s) at different positions, such as CS-2S, CS-4S, CS-6S, HS-2S, and HS-6S. This method increases the number of disaccharides in different classes that can be measured in a single analysis [152].

Lawrence et al. [154] published a protocol for detection of the non-reducing ends of GAGs [154]. In MPS diseases, lack of a specific enzyme leads to accumulation of polysaccharides with a specific sugar with a non-reducing end. After digestion of extracted polysaccharides with bacterial enzymes, non-reducing sugars are labeled by reductive amination with isotopic aniline, and the modified sugars quantified by LC/MS/MS [154]. This method can clearly distinguish 8 different forms of MPS from unaffected controls. This sophisticated derivatization method has not yet been adapted for higher throughput methods needed for routine laboratory use or NBS, but shows promise as a method to identify very selective biomarkers.

An acid-catalyzed chemical process (methanolysis) has been also developed by using a single reagent (methanolic hydrochloric acid) aiming at the analysis of individual GAGs by LC/MS/MS. This procedure was described in 2011 [155] and yields, among other oligosaccharides, desulfated and derivatized disaccharides. Specific disaccharides related to DS and HS were selected, optimized and quantified by MS/MS after chromatographic separation. This versatile procedure has been adapted for the analysis of GAGs from various samples, including urine [155–157], cerebrospinal fluid (CSF) [158], and animal tissues [159]. The same procedure has been used to analyze CS and KS [160]. This method has been used for high-risk screening, diagnosis, and longitudinal evaluation of patients under therapy.

One of the limitations of the LC separation techniques needed to quantify individual GAGs is that the length of time needed for separation of each sample is not compatible with high volume newborn screening programs. MS/MS can be associated with high throughput (HT) platforms to overcome this limitation. Shimada et al. [153] published a study comparing analysis of HS using an automated high-throughput mass spectrometry (HT/MS/MS) with analysis using a conventional LC/MS/MS system [153]. The HT platform used was RapidFire (Agilent Technologies) in which samples are adsorbed onto a matrix for concentration and desalting prior to injection into MS/MS with no chromatographic separation, allowing samples to be analyzed every 10 s.

As an alternative to measuring GAGs, MS has also been used to measure levels of specific enzymes that have reduced activity in MPS. In 2001, Chamolet et al. developed strategies for enzyme assay from re-hydrated dried blood spots (DBS) [161–167], allowing the use of DBS for enzyme assay by LC/MSMS for NBS in many disorders including Gaucher, Niemann-Pick A/B, Pompe, Fabry, Krabbe, Hurler syndrome (MPS I), Maroteaux-Lamy syndrome (MPS VI), and Morquio syndrome type A (MPS IVA) [168–174].

3. Applications of GAG assays

GAGs are widely distributed and associated with physiological and pathological roles depending upon specific GAG as described above. Therefore, establishment of accurate, rapid, sensitive, and specific measurements of specific GAGs has been urgently required. MS/MS based GAG assays are applied to not only diagnosis and therapeutic efficacy for MPS but also other disorders such as: mucopolysaccharidoses [120–122], cancer [175–176], osteoarthritis [177], rheumatoid arthritis [178], diabetes [179–180], infectious diseases [181], and spinal cord injury [182] where GAG(s) are down or up regulated.

4. Conclusions

Several methods have been developed for GAG quantification, but most of the earlier developed methods require large amounts of samples and provide limited information about specific GAGs [4,182]. The development of mass spectrometry detection methods allows a fast, sensitive, accurate measurement for GAGs analysis (Figs. 5, 6).

The fastest method, HT/MS/MS, cannot distinguish all isomers of individual disaccharides, but it has a similar sensitivity and reproducibility as conventional LC/MS/MS and thus may

be more appropriate for NBS programs to measure elevation of GAGs in MPS. Individual enzyme deficiencies that lead to elevation of GAGs could then be determined in a second screen for individual enzyme activities.

Overall, MS/MS assay contributes greatly to broad fields associated with primary or secondary metabolic pathway of GAGs.

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Class	Abbreviation	Structure	R ₁	R ₂	R ₃	M.W.
CS/DS	ΔCS-0S	ΔUA(β1-3)GalNAc	H	H	H	379.3
	ΔCS-2S		SO ₃ H	H	H	459.4
	ΔCS-4S		H	SO ₃ H	H	459.4
	ΔCS-6S		H	H	SO ₃ H	459.4
	ΔCS-2S4S		SO ₃ H	SO ₃ H	H	539.4
	ΔCS-2S6S		SO ₃ H	H	SO ₃ H	539.4
	ΔCS-4S6S		H	SO ₃ H	SO ₃ H	539.4
	ΔCS-1S		SO ₃ H	SO ₃ H	SO ₃ H	619.5
HA	ΔHA	ΔUA(β1-3)GlcNAc	-	-	-	379.3
HS	ΔHS-0SNH	ΔUA(α1-4)GlcN	H	H	H	337.3
	ΔHS-0S		H	H	Ac	379.3
	ΔHS-2SNH		SO ₃ H	H	H	417.3
	ΔHS-6SNH		H	SO ₃ H	H	417.3
	ΔHS-NS		H	H	SO ₃ H	417.3
	ΔHS-2S		SO ₃ H	H	Ac	459.4
	ΔHS-6S		H	SO ₃ H	Ac	459.4
	ΔHS-2S6S		SO ₃ H	SO ₃ H	Ac	539.5
	ΔHS-2SNS		SO ₃ H	H	SO ₃ H	497.4
	ΔHS-6SNS		H	SO ₃ H	SO ₃ H	497.4
	ΔHS-2S6SNH		SO ₃ H	SO ₃ H	H	497.4
	ΔHS-1S		SO ₃ H	SO ₃ H	SO ₃ H	577.5
KS	KS-6S	Gal(β1-4)GlcNAc	H	SO ₃ H	-	463.4
	KS-6S6S		SO ₃ H	SO ₃ H	-	543.4

Fig. 1.

Structure of glycosaminoglycans. CS: chondroitin sulfate, DS: dermatan sulfate, HA: hyaluronic acid, HS: heparan sulfate, KS: keratan sulfate, UA: uronic acid, GlcA: glucuronic acid, Δ: unsaturated. *Reproduced with permission from [ref. 152].*

Fig. 2.
TB staining in growth plate of wild-type (left) and MPS VII (right) mice (12 weeks old).
Chondrocytes in a wild-type mouse are stained while chondrocytes in MPS VII is ballooned and vacuolated.

Fig. 3.

AB staining for trachea with a 23-year-old MPS IVA patient and AB staining for electrophoretic urinary GAG from MPS IVA patients. Chondrocytes and their extracellular matrix as well as tracheal glands were stained with deep blue (left). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Electrophoresis on a mono-dimensional run (left) and densitography (right) of urinary GAGs from MPS patients. Urinary GAGs were extracted by cetylpyridinium chloride (VPC) method and were separated by the electrophoresis. A mono-dimensional electrophoresis of urinary specimens from MPS I, II, III, IVA, VI, and VII patients and healthy control shows clear separation of specific GAGs (DS1, HS, DS2, C4S, C6S, and KS) (left). A healthy control sample yields C4S, C6S, and HS. Samples from MPS I and II patients provide more DS and HS. Samples from MPS III patients show a strong band of HS and there is no difference of the HS band between subclasses of MPS III. An MPS IVA sample provides a characteristic KS band while an MPS VI sample yields a thick DS band. Each separate GAG band is semi-quantified by Densitometer (right), and each type of MPS provides a unique pattern of densitography apart from the normal control pattern.

Fig. 5. Multiple reaction monitoring (MRM) of DBS samples (control $\times$ MPS II patient). Chromatograms for disaccharides of chondrosine (IS), heparan sulfate (HS), mono-sulfated KS, di-sulfated KS. Equipment: 6460 Triple Quad MS/MS with 1260 infinity LC (Agilent Technologies). DBS: dried blood spot; IS: internal standard.

Table 1

History of glycosaminoglycan assay.

Year	Description	First.author(s)	Ref.
1856	Discovery of toluidine blue	Perkin W.H.	[48]
1897	Development of mass spectrometry	Thomson J.J.	[129]
1956	Use of alcian blue for staining of acidic carbohydrates	Mowry et al.	[184]
1956	Use of alcian blue for mucopolysaccharidoses	Runoe et al.	[185]
1969	Development of GAG thin layer chromatography (TLC)	Teller et al.	[55]
1971	Spot test azure A dye staining of GAGs	Berman ER	[187]
1974	Analysis of GAGs with DMB	Humbel et al.	[74]
1974	Separation of urinary GAGs through gas chromatography	Murphy et al.	[90]
1978	GAG purification with paper chromatography	Sato et al.	[54]
1983	Separation of GAGs using paper thin chromatography	Rajendra V.	[53]
1985	HPLC for heparin	Rice et al.	[92]
1993	ELISA for C4S/C6S/DS	Shibutani et al.	[123]
1994	ELISA for KS	Møller et al.	[118]
1996	GAGs through capillary electrophoresis	Linhardt et al.	[97]
1997	ELISA for HS	Najjam et al.	[121]
2001	Single GAG measurement by LC-MS/MS	Oguma et al.	[45–47]
2007	Multiple GAG assay in plasma/serum or urine	Oguma et al.	[146–147]
2010	KS levels in plasma of MPS patients	Tomatsu et al.	[188]
2011	Methanolysis reaction for GAGs	Auray-Blais et al.; Zhang et al.	[155,158]
2012	ELISA for hyaluronic acid	Yang et al.	[124]
2012	HPLC-MS/MS for HS	Ruijter et al.	[186]
2012	Use of non-reducing ends of GAGs as biomarkers	Lawrence et al.	[154]
2013	Newborn screening for MPS measuring GAGs with LC-MS/MS	Tomatsu et al.	[150]
2014	Spectrometry for GAGs in cell lines	Kiselova et al.	[4]
2014	Methanolysis for animal tissue GAGs	Trim et al.	[159]
2014	Measurement of GAGs in articular cartilage and yellow ligament	Osago et al.	[152]
2014	Reversed-phase HPLC/MS for GAGs extracts	Zhu et al.	[145]
2014	Automated high-throughput mass spectrometry for HS	Shimada et al.	[153]

TLC: thin layer chromatography; GAGs: glycosaminoglycans; DMB: dimethylmethylene blue; HPLC: high performance liquid chromatography; C4S: chondroitin-4-sulfate; C6S: chondroitin-6-sulfate; DS: dermatan sulfate; ELISA: enzyme-linked immunosorbent assay; KS: keratan sulfate; HS: heparan sulfate; LC/MS/MS: liquid chromatography tandem mass spectrometry; CSF: cerebrospinal fluid.