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Newborn screening and diagnosis of mucopolysaccharidoses

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Abstract

Mucopolysaccharidoses (MPS) are caused by deficiency of lysosomal enzyme activities needed to degrade glycosaminoglycans (GAGs), which are long unbranched polysaccharides consisting of repeating disaccharides. GAGs include: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. Their catabolism may be blocked singly or in combination depending on the specific enzyme deficiency. There are 11 known enzyme deficiencies, resulting in seven distinct forms of MPS with a collective incidence of higher than 1 in 25,000 live births. Accumulation of undegraded metabolites in lysosomes gives rise to distinct clinical syndromes. Generally, the clinical conditions progress if untreated, leading to developmental delay, systemic skeletal deformities, and early death. MPS disorders are potentially treatable with enzyme replacement therapy or hematopoietic stem cell transplantation. For maximum benefit of available therapies, early detection and intervention are critical. We recently developed a novel high-throughput multiplex method to assay DS, HS, and KS simultaneously in blood samples by using high performance liquid chromatography/tandem mass spectrometry for MPS. The overall performance metrics of HS and DS values on MPS I, II, and VII patients vs. healthy controls at newborns were as follows using a given set of cut-off values: sensitivity, 100%; specificity, 98.5–99.4%; positive predictive value, 54.5–75%; false positive rate, 0.62–1.54%; and false negative rate, 0%. These findings show that the combined measurements of these three GAGs are sensitive and specific for detecting all types of MPS with acceptable false

negative/positive rates. In addition, this method will also be used for monitoring therapeutic efficacy. We review the history of GAG assay and application to diagnosis for MPS.

Keywords

Mucopolysaccharidoses; Tandem mass spectrometry; Newborn screening; Glycosaminoglycans; Monitoring

1. Introduction

Mucopolysaccharidoses (MPS) are caused by deficiency of lysosomal enzyme activities needed to degrade glycosaminoglycans (GAGs), which are long unbranched polysaccharides consisting of repeating disaccharides [1]. GAGs include: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. Their catabolism is inhibited singly or in combination depending on the specific enzyme deficiency. Lysosomal accumulation of GAG molecules results in cell, tissue, and organ dysfunction. In MPS, the undegraded or partially degraded GAGs are stored in lysosomes and/or secreted into the blood stream and subsequently excreted in urine. There are 11 known enzyme deficiencies that give rise to seven distinct MPS.

The MPS share many clinical features, although to variable degrees. Most MPS patients are asymptomatic at birth, with subsequent onset of clinical signs and symptoms. These clinical features include a chronic and progressive disease course, multiple organ system involvement, organomegaly, dysostosis multiplex, and abnormal facies. Hearing, vision, and cardiopulmonary functions are affected. Profound neurological impairment is characteristic of MPS I (Hurler syndrome), the severe form of MPS II (Hunter syndrome), and all subtypes of MPS III (Sanfilippo syndrome). MPS IV (Morquio syndrome) and MPS VI (Maroteaux–Lamy syndrome) have characteristic bone lesions without central nervous system (CNS) involvement. There is clinical similarity between different enzyme deficiencies and, conversely, a wide spectrum of clinical severity within any one enzyme deficiency [1]. In all cases, except for a few mild cases, the disease is ultimately fatal with an average life expectancy of one to two decades if untreated. MPS can be found worldwide. Although the overall incidence of MPS is estimated as higher than 1:25,000 live births, the incidence of a particular type of MPS varies (Table 1) [2–8]. Scott et al. (2013) showed that in a pilot study of 110,000 dried blood spot (DBS), the incidence of MPS I is 1 in 35,700 [9].

We estimate that approximately 200 newborns affected with MPS are born annually in the United States.

The stepwise degradation of GAGs requires four exoglycosidases, five sulfatases, and one nonhydrolytic transferase. One endoglycosidase also participates in the degradation [10]. Understanding the degradation of GAGs is relevant to establishing new screening methods and monitoring therapies. The backbone of the CS chain consists of repetitive disaccharide units containing D-glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc) residues, whereas DS is a stereo-isomeric variant of CS with varying proportions of L-iduronic acid (IdoA) in place of GlcA. CS and DS are involved in cell adhesion and neuritogenesis [11].

The HS-GAG chains are linear polysaccharides composed of alternating *N*-acetylated or *N*-sulfated glucosamine units (*N*-acetylglucosamine, GlcNAc, or *N*-sulfoglucosamine, GlcNS) and uronic acids (GlcA or IdoA). HS is involved in diverse biologic functions including activation of growth factors [11]. The backbone of the KS chain consists of repetitive disaccharide units containing lactosamine, which is composed of galactose (Gal) and GlcNAc residues. KS is involved in cell motility, embryo implantation, wound healing, corneal transparency, extracellular matrix of cartilage, and neuronal regeneration [12].

Therapies for MPS have been developed experimentally and clinically. These include hematopoietic stem cell transplantation (HSCT), enzyme replacement therapy (ERT), gene therapy, and substrate reduction therapy (SRT), all of which lead to the partial restoration of the enzyme activity or inhibition of GAG synthesis. HSCT is not entirely effective and has a relatively high mortality rate, primarily from graft versus host disease [13,14]. Treating MPS with ERT relies on the cellular uptake of the enzyme by receptor-mediated endocytosis. ERT has been quite successful in animal models, leading to application in human patients [15–19]. ERT is FDA approved for use in patients with MPS I [20], MPS II [21,22], and MPS VI [23–26]. Patients treated with ERT demonstrate clinical improvement of somatic manifestations and quality of life. Trials for MPS IVA and other types of MPS are under way. While ERT holds much promise for the treatment of MPS, current experience with MPS animal models indicates that it is unlikely that therapeutic amounts of enzyme: 1) cross the blood brain barrier to correct the CNS pathology and 2) reach the bone cells to correct the skeletal pathology. Experimental gene therapies and SRT are under way in vivo [27–29].

The most commonly used methods for diagnosis of MPS are dye-spectrometric methods such as dimethylmethylene blue (DMB) [30–32] and alcian blue [33–35] on urine samples. The direct DMB method was automated for detection of MPS diseases [32]. However, these methods cannot be applied to blood specimens without prior protease, nuclease, or hyaluronidase digestion and are impractical for screening newborn urine since collection is difficult and storage of large volumes of urine samples is costly. These spectrometric assays are also not adequate for screening: 1) proteins in the blood specimen block binding of the dyes to GAG, 2) the staining dyes are prone to decomposition leading to a low signal to noise ratio, and 3) the dye methods cannot predict MPS subtypes. HPLC is a sensitive and specific method but not appropriate for mass screening since it is time-consuming and expensive [36–40]. Although sandwich ELISA methods to measure specific GAGs such as HS and KS have been developed to detect MPS [41–43], a method to detect DS is not available.

A suitable method for newborn screening (NBS) must be inexpensive and should be performed on DBS from a Guthrie card. Two other potential methods have been proposed [44]. One is an immune-capture method for detecting each deficient lysosomal protein from patients with MPS I, MPS II, MPS IIIA, and MPS VI [45,46], and the other one is a direct method, assaying individual enzyme activities for MPS I, MPS II, MPS IIIB, MPS IVA, MPS VI, and MPS VII patients [47–55]. These approaches, which rely on individual antibodies or enzyme activities for first-tier screening, are still being developed to detect all types of MPS. We have developed multiplex assays for three MPS-associated metabolites that make NBS possible. Since cost-performance is an important key to applicability, highly

efficient and inexpensive screening methodologies must be formulated and developed. Otherwise, the cost of screening for each type of MPS would be unacceptable, as the incidence ranges from approximately 1:100,000 births to 1:2,000,000 births. However, screening for MPS as a group with a combined incidence of higher than 1:25,000 births would be comparable with other existing screening programs for genetic disorders such as phenylketonuria (1:14,000) and galactosemia (1:50,000). Therefore, strategies that enable the simultaneous detection of a group of MPS by LC–MS/MS method are feasible for NBS [1]. Most NBS methods measure elevated substrates in organic acidemias, urea cycle disorders, and cystic fibrosis (CF). A two-tier strategy is universally used for CF [59–62].

The method described here for MPS has another advantage in that it can be used to monitor therapeutic effect [63,64] and to predict clinical severity.

The goal of this review is to describe the potential of tandem mass spectrometry assay of GAGs for NBS and diagnosis of MPS.

2. Historical review for GAG assay

2.1. Total urine GAG assay

The measurement of total GAG excretion in urine is widely used as a biomarker for MPS [65–67]. Urine GAG analysis is a useful initial screening test for MPS and may be also helpful for monitoring treatment efficacy. Measurement of total urine GAGs can be performed quantitatively and qualitatively. Both are recommended as part of the evaluation of patients for MPS.

In 1971, Berman et al. proposed a spot test for MPS (termed the Ames MPS paper spot) based on Azure A dye staining of GAGs in urine spotted on paper [68]. In 1985, Huang et al. measured the uronic acid content of the precipitated GAGs from MPS patients and age-matched normal controls by using spectrophotometric analysis of samples treated with cetylpyridinium chloride (CPC) [69]. In healthy individuals, uronic acid/creatinine ratios decreased until age 20 years old and then remained low. Most patients with MPS IV had higher uronic acid/creatinine ratios than age-matched controls. This same group also suggested that the Ames MPS paper spot test was a potential approach to screen MPS patients from a high risk group with suspected clinical features [70].

At the same timing, 1,9-dimethylmethylene blue (DMB) has been used to quantitatively detect GAGs in urine from older children [71,72]. The DMB method also measures the total amount of GAG in urine. In 1989, Whitley et al. showed that this testing could be used as a potential screen for any MPS disorder [73–75] as the test measures levels of all GAG species on the urine spot. In 1991, JG de Jong et al. proposed that the DMB method is more reliable than the Ames MPS paper spot [76].

In 2000, Orii's group reported that two MPS II patients were diagnosed by screening urine spot samples from approximately 130,000 six-month-old infants in a Japanese population using the DMB method [77, personal communication from T. Orii]. This was the first practical demonstration that GAG measurements can be used to screen for MPS. The limitations of the method are 1) some substances that respond to DMB are found in a diaper,

leading to around 3% of a false positive result and GAG/creatinine ratio is not stable until 4 days old, suggesting that we can apply this method to the urine samples from the 5th day newborns [77,78].

Other dye-based procedures such as the toluidine blue spot test [79] and the alcian blue spot test [80] have been evaluated for qualitative screening. Overall, spot tests suffer from a high incidence of false negative results [80] and cannot be applied to blood spot.

Elevated urinary GAG is normally specific for an MPS disorder; however, many patients have borderline or only slightly elevated urinary GAGs, especially those with MPS IV, resulting in false negatives in a screen [41,42,73,75,81,82]. Total urine GAG levels are also unlikely to reflect the clinical severity or predict the specific clinical signs and symptoms that might arise.

Thus, a traditional method for urine GAG is still used for a screening, when the subject is suspected as a clinically high risk group; however, it is not suitable for a general mass screening of newborns. The lack of adequate sensitivity, specificity, and reliability of these screening tests underscores the need for improved methods of GAG detection for MPS.

2.2. Assay for specific GAGs

Several methods have been described for detecting and monitoring specific GAGs in MPS.

2.2.1. Chromatography—In 1968, Orie described the measurement of urine GAG in normal male children by using CPC fractionation and column chromatography on Dowex 1X2, showing the compositions of each specific GAG in urine [83].

In 1980, Kimura et al. described the chemical structure of DS in the urine of a patient with MPS II through the analysis of disaccharide units. Urine was digested by chondroitinase ABC and the resultant disaccharides were separated on a Dowex 1 column [84]. In 1984, the same group fractionated urinary heparan sulfates (“HS”) from two siblings with MPS IIIB by chromatography on Dowex 1 and Sephadex G-50 columns [85].

In 1985, Hopwood and Elliott used Dowex 1 column separation to show that urine from MPS IIID, IVA, and VI patients contained elevated levels of sulphated *N*-acetylhexosamines compared with urine from normal individuals [86]. In 1995, K Murata et al. digested heterogeneous sulfated HS isomers with specific HS-lyases, heparitinase I, and heparinase, and separated the resultant unsaturated disaccharides (Di-SHS) by HPLC. The pattern of

Di-SHS obtained from that urine of a patient with MPS I indicated accumulation of unique urinary HS isomers in this MPS [87].

Enzymatic and chemical analyses of the structures of HS in the urine by patients with MPS III and II revealed that their non-reducing ends differ from each other and reflect the enzyme deficiency of the syndromes [88]. In addition to providing a new tool for the differential diagnosis of the MPS, these results bring new insights into the specificity of the heparitinases from *Flavobacterium heparinum*.

In 1998, Byers et al. used a combination of anion-exchange chromatography and 30–40% gradient polyacrylamide gel electrophoresis (gradient-PAGE) to purify and characterize urinary GAG from various MPS [65]. The urinary GAG from the different MPS displayed distinct patterns on gradient-PAGE, and further confirmation of MPS types and subtypes was demonstrated by an electrophoretic shift in the banding pattern after digestion with the appropriate MPS enzyme. They reported that each MPS disorder accumulates a unique spectrum of GAGs, with a non-reducing terminus that is specific for the deficient enzyme that causes each particular MPS disorder. The absolute correlation of the non-reducing terminal structure with a particular MPS and the availability of recombinant lysosomal enzymes provide the means for a rapid and accurate diagnosis of individual MPS.

Thus, qualitative urine-based testing methods can separate the various GAG species and thereby provide a starting path for differential diagnosis. GAGs are first isolated from the urine and then separated by thin layer chromatography or electrophoresis [69,70,89–92]. A GAG specific stain is used to visualize the GAGs and the relative position on the gel or plate is used to identify GAG types [93]. KS, the specific diagnostic GAG in patients with MPS IVA, can be difficult to separate and visualize by these methods and result in false negatives. Numerous methods, including electrophoresis and spectrophotometry, have been evaluated for the quantitative and/or qualitative analysis of urinary GAGs. However, all these methodologies are laborious, time-intensive, and non-specific [80].

2.2.2. ELISA—In 1988, the measurements of blood KS were accomplished using an inhibition ELISA by Thonar et al. [94]. His group measured blood KS level in healthy controls, suggesting that blood KS is age-dependent. In 2004, Tomatsu et al. developed a sandwich ELISA method for KS measurements, showing that KS levels in blood and urine of MPS IVA patients are markedly elevated and that clinical severity correlates with the levels of blood and urine KS [41,42]. In 2005, Tomatsu et al. demonstrated that blood KS is elevated in other types of MPS, suggesting the potential of use of blood KS for screening and monitoring other types of MPS patients in addition to MPS IV. In the same year, Tomatsu et al. developed a sandwich ELISA method for HS measurement and showed that blood and urine HS levels are elevated in MPS I, II, III, VI, and VII patients and correlate with clinical severity [43]. The results of these studies indicate that blood and urine KS and HS measurements could be used as a biomarker for assessing clinical severity at an early stage, and to monitor therapeutic effects [95]. ELISAs to measure KS, HS, or DS are not available commercially.

2.2.3. Tandem mass spectrometry (MS/MS)—In 2003, Ramsay et al. developed a method for the derivatization and quantification of sulfated *N*-acetylhexosamine-containing mono- and disaccharides from patient samples by electrospray ionization tandem mass spectrometry (ESI-MS/MS) [96]. Urine from most MPS types had significant increases in mono- and di-sulfated *N*-acetylhexosamines and mono-sulfated *N*-acetylhexosamine-uronic acid disaccharides. Analysis of plasma and DBS on filter paper collected from MPS patients showed elevations of total mono-sulfated *N*-acetylhexosamines but less than that seen in urine. However, direct quantification of GAGs by mass spectrometry is complex because of

their molecular heterogeneity, resulting in difficulty of application to mass screening and practical use for diagnosis of the patients.

The major GAGs that accumulate in MPS patients are copolymers that contain analogous repeating units (glucurono-, or idurono-sulfo-*N*-acetyl-glucosamine or galactosamine) [57]. These polymers are amenable to chemical and enzymatic degradation (methanolysis and hydrolysis) to produce simple molecules containing the repeating subunits. The sulfate groups are rapidly cleaved and the remaining acidic polysaccharides are hydrolyzed or methanolized at certain glycosidic linkages and dissociate to stable disaccharides as end products.

In 2007, Oguma and Tomatsu developed a highly sensitive, specific, accurate, and inexpensive strategy in which DS, HS, and KS disaccharides are produced from GAGs in blood and urine samples by hydrolysis and analyzed simultaneously by LC–MS/MS (Fig. 1) [56–58, 96–98]. Other groups have subsequently developed similar methods [99–100]. A significant advantage of this approach is that not only individual but multiple disaccharides are measured from a single sample, and that all types of MPS can be detected in a first-tier screening, with specific types confirmed in a second-tier screening. Blood, urine, or other biological samples are purified by filtration and GAGs digested with chondroitinase B, heparitinase, and keratanase II to yield disaccharides of DS, HS, and KS. After digestion, the samples are injected into the LC–MS/MS and results are compared with control samples (Fig. 2).

The LC–MS/MS method not only shows sensitivity and specificity for detecting all subtypes of MPS but also is able to monitor therapeutic efficacy in MPS patients and animal models [15,63,98–103]. This new method has an advantage of being both GAG-specific and quantitative.

In 2011, Auray-Blais et al. described that methanolysis can be used to prepare HS and DS for LC–MS/MS analysis from GAGs in urine of MPS patients [104,105]. Methanolysis has not yet been used to measure KS.

In 2012, Lawrence et al. showed that another new method with the enzyme digestion can detect DS and HS by analysis of non-reducing ends of urinary GAGs by LC–MS/MS. Currently, this method does not provide a measure of non-reducing ends for KS [106]. Overall, establishment of the method to measure the disaccharides rather than heterogeneous oligosaccharides make it feasible to interpret individual GAG values, leading to rapid and accurate diagnosis, prognosis, and monitoring therapies for MPS.

3. Update of LC–MS/MS method with enzyme digestion

3.1. Equipment

At present, the following LC–MS/MS instruments have been confirmed to provide good resolution for disaccharide analysis by multiple laboratories; Alliance 2795XE HPLC system/Quattro micro tandem quadrupole (Waters Corp, Milford, MA, USA); Ultra performance liquid chromatography (UPLC) Acquity system/Xevo TQ-S (Waters Corp, Milford, MA, USA); HP1100 system (Agilent Technologies, Palo Alto, CA, USA)/

API-4000 or API-5000 (AB Sciex, Foster City, CA, USA); Acquity HPLC system/Quattro Premier XE (Waters Corp, Milford, MA, USA); and 1260 infinity LC/6460 Triple Quad (Agilent Technologies, Palo Alto, CA, USA) [56–58,95–98,9–101,103–106, personal communication from Dr. K. Kida].

3.2. Standards

DiHS-6S (HS); DiHS-NS (HS); DiHS-OS (HS); Di-4S (DS); mono-sulfated KS — Gal β 1-4GlcNAc(6S); di-sulfated KS — Gal(6S) β 1-4GlcNAc(6S); and KS I (bovine cornea) digested with keratanase II to yield Gal β 1-4GlcNAc(6S), Gal(6S) β 1-4GlcNAc(6S), have all been used to produce standard curves for each specific GAG (Fig. 3). A common internal standard is chondrosine. The KS I standard is the most difficult to quantify. We assessed efficiency of KS I digestion by keratanase II using gel permeation chromatography. We found that 60% of KS I was digested with keratanase II and that the ratio of Gal(6S) β 1-4GlcNAc(6S) to Gal β 1-4GlcNAc(6S) was 42:58 (data not shown).

3.3. Disaccharide determination derived from GAGs

3.3.1. Recoveries—Extraction efficiencies of KS-derived and DS/HS-derived disaccharides from control samples are over 87% and almost 100%, respectively. These methods have been applied successfully to extract KS- and DS/HS-derived disaccharides from human serum [56,57].

3.3.2. Chromatography and selectivity—Chromatograms of extracts obtained from a control serum sample consist of Gal β 1-4GlcNAc(6S), Gal(6S) β 1-4GlcNAc(6S), DiHS-OS, DiHS-NS, and Di-4S. All disaccharides are eluted in less than 5 min (see Figs. 3, 4) [56,57].

3.3.3. Calibration curves—Calibration parameters of disaccharides derived from DS, HS, and KS and in human serum were assessed. Calibration curves for Gal β 1-4GlcNAc(6S), Gal(6S) β 1-4GlcNAc(6S), DiHS-OS, DiHS-NS, and Di-4S obtained on five separate days are linear over the concentration ranges of 0.14 to 7.1 μ g/ml, 0.06 to 2.9 μ g/ml, 10 to 1000 ng/ml, 5 to 500 ng/ml, and 10 to 1000 ng/ml, respectively. The correlation coefficients of determination (r) are not less than 0.99 [56,57].

3.3.4. Precision—The results of intra- and inter-assay precision for DiHS-OS, DiHS-NS, and Di-4S in control serum are as follows. The disaccharide (Di-4S) is detected by the same ions (m/z 462/97) as those of DiHS-6S. Thus, the total concentration of disaccharides expressed as Di-4S includes both Di-4S and DiHS-6S. The intra-assay precision values/coefficient of variation (CV) determined from analysis of DiHS-OS, DiHS-NS, and Di-4S for control serum are less than 6.7, 7.9, and 15.8%, respectively. The inter-assay precision values/CVs for these disaccharides in control serum are less than 5.2, 6.8, and 14.8%, respectively. The intra-assay precision values/CVs for Gal β 1-4GlcNAc(6S) and Gal(6S) β 1-4GlcNAc(6S) in control serum were less than 8.2 and 5.3% and inter-assay precision values/CVs were less than 6.6 and 5.7%. These results demonstrate the reproducibility and accuracy of the method [56,57].

3.4. Analysis of plasma and serum samples in MPS patients—Each type of MPS has a different pattern of elevated GAG levels in serum or plasma. We described concentrations of DS-, HS-, and KS-derived disaccharides and their composition ratios in human control and MPS patients [56–58].

3.4.1. Diagnosed MPS patients—Plasma samples were digested by heparitinase and chondroitinase B to produce disaccharides of DS and HS. Digested samples were assayed by LC–MS/MS. For the DS and HS assay, 120 samples from patients (MPS I, 22; MPS II, 27; MPS IIIA, 5; MPS IIIB, 4; MPS IIIC, 2; MPS IVA, 42; MPS VI, 4; MPS VII, 6) (age; 0–66 years) and 112 control samples (age; 0–62 years) were analyzed. The results were as follows.

- All MPS I, II, IIIA, IIIB, IIIC, and VI patients had significant elevations of plasma DS + HS (ave. 3024; 2763; 2610; 2933; 1605; 2700 ng/ml, respectively; min. 829 ng/ml, max. 23,800 ng/ml), compared with controls (ave. 448 ng/ml, min. 107 ng/ml, max. 776 ng/ml, $p < 0.0001$) (Figs. 4, 5). Specificity and sensitivity are 100%, if the cut-off value is 800 ng/ml between control and MPS samples [58].
- All MPS VI patients have significant elevation of plasma DS (ave. 2225 ng/ml, min. 1100 ng/ml; max. 4400 ng/ml), compared with controls (ave. 253 ng/ml, min. <1 ng/ml; max. 610 ng/ml; $p < 0.0001$) [58].
- Forty-four blood specimens from MPS IVA patients (ages 2–65 years; mean 14.3 years) were analyzed to confirm that the KS concentration is a suitable marker for early diagnosis and longitudinal assessment of disease severity. Blood specimens were obtained from patients categorized phenotypically as severe ($n = 33$), and attenuated ($n = 11$). Plasma samples were digested by keratanase II to produce disaccharides of KS [95,96].

Digested samples were assayed by LC–MS/MS. Plasma KS levels varied with age and clinical severity. Plasma KS levels in healthy controls ($n = 90$) in 0–5 years showed 1.843 ± 0.0634 $\mu\text{g/ml}$ while plasma KS levels in MPS IVA patients ($n = 25$) showed 7.445 ± 3.042 $\mu\text{g/ml}$ ($p < 0.0001$). There was no overlap in blood KS value between the age-matched MPS IVA patients and healthy controls (see Fig. 6), although there was no case of newborn MPS IVA available. By contrast, the predictive value of KS in older patients was poor, with levels indistinguishable from controls (Fig. 6). This clearly demonstrates that test should be performed at an early stage.

Thus, we can not only screen for a group of MPS but also infer MPS subtypes from specific patterns of elevation of each GAG, leading to simplification of the second-tier enzyme assays designed as confirmatory diagnostic tests to identify the deficient enzyme. These findings suggest that measurement of DS, HS, and KS levels by LC–MS/MS can be applied to screening for MPS I, II, III, IV, VI, and VII patients.

3.4.2. Monitoring therapies

3.4.2.1. MPS VII mice treated by ERT: We treated MPS VII mice with 12 weekly infusions of human β -glucuronidase (GUS), modified GUS, or vehicle control, and monitored DS (Di-4S) and HS (DiHS-0S plus DiHS-NS) levels.

Serum DS was elevated in untreated mutant mice over levels found in heterozygous control mice. Serum DS concentrations in untreated mice increased with age and reached over 7000 ng/ml by age 7 months. Serum HS concentrations in untreated mutant mice increased with age and reached around 4000 ng/ml by age 7 months. Serum HS in untreated mutant mice was significantly elevated at age 3 months compared with heterozygous control mice that had a relatively constant low level of HS over 7 months.

After 12 weekly infusions, all forms of GUS lowered serum DS and HS levels markedly. The concentration of serum DS was lower in mice treated with enzyme tagged with a short peptide consisting of acidic amino acids (D6- or D8-GUS) than the untagged enzyme (GUS). The same trend was seen for serum HS concentrations. The final serum HS in native GUS treated mice was higher than HS levels in tagged-GUS treated mice. Thus, this LC–MS/MS method shows efficacy of treatment with different enzyme formulations confirming the utility of this method for measuring biomarkers associated with MPS [63,107].

3.4.2.2. MPS IVA mouse treated by ERT: Mouse serum KS is not measurable by standard methods due to limited KS synthesis in rodents. The sensitivity of LC–MS/MS makes it feasible to assay serum KS in mice [15]. We treated MPS IVA mice with recombinant N-acetylgalactosamine-6-sulfate sulfatase (GALNS) and a sulfatase-modifier-factor 1 modified form of GALNS, for 12 weekly infusions. Serum KS levels were significantly reduced by 3 weeks compared with untreated mice and continued to decline over the 12 week duration of treatment. After 12 weeks of infusion, the average levels of serum KS were similar to those of normal mice [15].

3.5. Analysis of DBS samples in newborn controls and MPS patients

3.5.1. Newborn screening of MPS and its significance—NBS is recognized internationally as an essential, preventive public health program for early identification of disorders in newborns that can affect their long-term health. Early detection, diagnosis, and treatment of certain genetic, metabolic, or infectious congenital disorders can lead to significant reductions in disease severity, associated disabilities, and death. Currently, NBS represents the largest source of samples subjected to biochemical genetic testing worldwide. In the United States, the panel of NBS disorders varies from state to state, and decisions for adding or deleting tests involve many complex social, ethical, and political issues. Usually, disorders selected for NBS are tied to issues such as disorder prevalence, sensitivity, treatment availability, outcome, and overall cost effectiveness. Presently, NBS programs utilizing improvements in MS/MS screening procedures, screen for almost 50 disorders at birth and the number will continue to increase.

In the United States, NBS is performed for nearly 4.2 million neonates every year [108]. Almost 200 of these neonates are likely to be affected with MPS. Early detection of MPS

would enable genetic counseling of the parents and early treatment. At present NBS for MPS I has been conducted by the enzyme assay method and three patients were found [9]; however, NBS for other types of MPS has not started yet. Thus, a presumptive diagnosis of most MPS patients is made much later when children present with clinical signs and symptoms. Establishing standard methodologies to screen MPS at an early clinical stage should improve the clinical course significantly. Early detection allows medical intervention, which may ultimately slow progression of the disease and reduce morbidity and mortality [109]. There are several important factors supporting a decision to include MPS into NBS programs:

- Number of patients: Incidence of MPS collectively is higher than 1 out of 25,000 births and annually nearly 200 newborns are expected to be diagnosed with MPS in the United States.
- Clinical course: The onset of severe forms of these diseases occurs prior to one year of age, while the diagnosis is typically delayed until between two and four years of age for most types of MPS. Delay in diagnosis may lead to irreversible damage to bone and brain, resulting to early death.
- Availability of therapies: ERT, HSCT, and/or SRT treatments are available for several types of MPS. Clinical and experimental studies on MPS patients and animal models show that early diagnosis and early treatment provide better outcomes for these disorders.
- Natural history: The natural history of most types of MPS is under investigation and our understanding of the clinical features and their progression is evolving. Efficient and accurate NBS will prevent birth defects and will provide the affected infants a better quality of life.

Thus, we propose here a novel NBS system for MPS screened by LC–MS/MS.

To confirm effectiveness of simultaneous DS, HS, and KS assay by LC–MS/MS on DBS, we have analyzed DBS samples from newborn normal controls and newborns with MPS.

3.5.2. Normal healthy newborns—We measured DiHS-0S, DiHS-NS, Di-4S, and KS extracted from DBS of healthy normal newborns. We classified DBS samples into 6 groups by birth weight (<1500 g, 1500–1999 g, 2000–2499 g, 2500–2999 g, 3000–3499 g, >3500 g). Each group was comprised of 10 samples. We did not observe any significant difference in overall GAG composition by birth weight (Fig. 7).

3.5.3. Newborn MPS—When the elevation of GAGs is first detectable in human MPS patients is unknown. To assess whether our LC–MS/MS method can distinguish MPS newborns from the normal control newborns, we compared DS and HS levels in DBS samples from six neonates that were subsequently determined to have MPS (four MPS I, one MPS II, and one MPS VII) and control newborns (n = 326) in a double blind manner. All six cases (Table 2) had marked elevations of DS and HS (above 1500 ng/ml) compared with the values of normal control newborns (mean, 553 ± 146 ng/ml). DS and HS levels in another DBS sample from a one year-old infant with MPS I (Case 3) remained high since this infant

went untreated. This infant was a fraternal twin and DS and HS levels were clearly distinguishable between the unaffected and affected fraternal twin (800 ng/ml vs. 2660 ng/ml).

The overall performance metrics of DiHS-0S (HS), DiHS-NS (HS), and Di-4S (DS) values were as follows using a given set of cut-off values (Table 3, Fig. 8): sensitivity, 100%; specificity, 98.5–99.4%; positive predictive value, 54.5–75%; false positive rate, 0.62–1.54%; and false negative rate, 0% in any of these biomarkers. In 2012, Paracchini et al. reported the results of NBS for CF by assaying the immunoreactive trypsinogen level with the cut off of >99th centile. The results showed that among a total of 717,172 newborns screened, 7354 newborns were found positive to NBS. After the second screening by sweat test, 7234 newborns (98.4%: false positive) were negative or not confirmed, while 120 newborns (1.6%) were confirmed as a patient with CF [61]. These results support our hypothesis that using the LC–MS/MS method to measure GAGs can be applied to NBS for MPS.

Subsequently, Ruijter et al. assayed DS and HS levels from newborn DBS of 11 MPS I, 1 MPS II, and 6 MPS III patients, with phenotypes ranging from severe to relatively attenuated forms. The levels of DS and HS derived disaccharides in these DBS were compared with levels in DBS of newborn MPS I and MPS III heterozygotes and controls. The levels of DS and HS derived disaccharides were clearly elevated in all newborn DBS of MPS I, II, and III patients when compared with controls. In contrast, DBS of MPS I and III heterozygotes showed similar disaccharide levels when compared with control DBS [100].

Potential advantages of this current method are: i) inclusion of all types of MPS for the first-tier screening; ii) prediction of MPS type with elevation of specific GAGs such as DS, HS, and KS or in combination and, iii) monitoring efficacy of therapies.

We are conducting a pilot study of NBS for MPS patients, supported by NIH (1R01HD065767-01), using this LC–MS/MS method. Fig. 9 describes the two-tier strategy of NBS for MPS. GAG assay is the first tier to define a high risk group of MPS. Subsequently, enzyme assays are performed on blood spots from this high risk group in a second tier to confirm diagnosis. Enzyme assays will be conducted by using synthetic substrates as described elsewhere [47–55].

3.6. Limitations of current LC–MS/MS method

A limitation of the current method is that there is a possibility that we may obtain false negative results in samples from patients with very mild phenotypes, although treatment of such patients in the newborn period would not typically be necessary. Another major limitation of this method could be the chromatographic time (4 min) of LC, still limited throughput for mass screening. We may need additional improvements in the method such as use of a RapidFire system (Agilent Technologies) for NBS in practice, in which every sample can be assayed within 10 s.

4. Conclusion

In conclusion, MS/MS methodology for the analysis of disaccharides of GAG has become popular and should be available in diagnostic and clinical settings soon. With increased demand for MS/MS assays and the wider availability of instrumentation, MS/MS methodologies are likely to have a large impact in screening, diagnosis, and monitoring of MPS in the future.

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Abbreviations

CS	chondroitin sulfate
CF	cystic fibrosis
CNS	central nervous system
CPC	cetylpyridinium chloride
DBS	dried blood spot
DMB	dimethylmethylene blue
DS	dermatan sulfate
ERT	enzyme replacement therapy
ESI-MS/MS	electrospray ionization tandem mass spectrometry
GAG	glycosaminoglycans
GALNS	N-acetylgalactosamine-6-sulfate sulfatase
GUS	β -glucuronidase
HPLC	high performance liquid chromatography
HS	heparan sulfate
HSCT	hematopoietic stem cell transplantation
KS	keratan sulfate
LC-MS/MS	high performance liquid chromatography tandem mass spectrometry
MPS	mucopolysaccharidoses
NBS	newborn screening

SRT

substrate reduction therapy

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Fig. 1.
Production of disaccharides digested by the enzymes.

Fig. 2.
Sample preparation and apparatus with automated procedure.

Fig. 3.

Chromatograms for KS-I (bovine cornea), Di-4S (DS), DiHS-NS, DiHS-OS, and chondrosine (IS) disaccharides by using Hypercarb (2.0 mm $\times$ 50 mm, 5 μ m; Thermo Electron Corp.) with a solvent gradient of 0% to 100% 5 mM ammonium acetate (pH 10):acetonitrile. Polymer KS was separated with mono-sulfated KS [Gal β 1-4GlcNAc(6S)] and di-sulfated KS [Gal(6S) β 1-4GlcNAc(6S)] after digestion by keratanase II. Equipment: 6460 Triple Quad MS/MS with 1260 infinity LC (Agilent Technologies). IS: internal standard.

Fig. 4.

Chromatograms of extracts from control (left) and MPS II (right) blood spots. Equipment; HP1100 system/API-4000. The arrows show the peaks of each disaccharide. KS has two peaks: mono-sulfated KS [Gal β 1-4GlcNAc(6S)] (a major peak) and di-sulfated KS [Gal(6S) β 1-4GlcNAc(6S)].

Fig. 5.
Total DS and HS levels in blood of MPS patients. Equipment; HP1100 system/API-4000.

Plasma KS concentration in MPS IVA

Fig. 6.

Age-dependent KS in MPS IVA patients. White square; controls, red rhombus; MPS IVA patients with a severe form, green circle; MPS IVA patients with an attenuated form. Equipment; HP1100 system/API-4000.

Fig. 7.

Distribution of each GAG level in DBS of normal newborns by birth weight. Average of DiHS-0S, DiHS-NS, and Di-4S was 347 ng/ml (range: 282–502), 85 ng/ml (range: 64–159), and 96 ng/ml (range: 57–163), respectively. The average of KS was 1185 ng/ml (range: 684–1511). Yellow: DiHS-0S, green: DiHS-NS, pink: Di-4S, blue line: KS. Equipment; HP1100 system/API-4000.

Fig. 8. Frequency distribution of A) DiHS-NS, B) Di-4S, and C) DiHS-0S for DBS from 326 controls to 6 MPS cases. Values for 6 cases are marked with arrows. Bars represent controls with frequency. Equipment; HP1100 system/API-4000.

Fig. 9.
Proposed two-tier strategy of newborn screening for mucopolysaccharidoses.

Table 1

Mucopolysaccharidoses and GAG storage [1–9].

Disorder	Deficient	Incidence	Primary storage material
MPS I	α -L-Iduronidase	1/35,700	DS, HS
MPS II	Iduronate-2-sulfatase	1/93,000–200,000	DS, HS
MPS IIIA	Heparan-N-sulfatase	1/5,3000–370,000	HS
MPS IIIB	α -N-acetylglucosaminidase		HS
MPS IIIC	Acetyl-CoA: α -glucosaminide acetyltransferase		HS
MPS IIID	N-acetylglucosamine 6-sulfatase		HS
MPS IVA	N-acetylgalactosamine-6-sulfate sulfatase	1/7,6000–1,428,000	C6S, KS
MPS IVB	β -Galactosidase		KS
MPS VI	N-acetylgalactosamine-4-sulfatase	1/207,000–2,000,000	C4S, DS
MPS VII	β -D-Glucuronidase	1/345,000–2,100,000	C4, 6S, DS, HS,

Table 2

HS and DS levels from dried blood spot in six MPS newborns and controls (ng/ml).

Case	DiHS-0S	; DiHS-NS	Di-4S	Total HS/DS
Case 1 (MPS I)	277	158	1090	1525
Case 2 (MPS I)	285	180	1200	1665
Case 3 (MPS I)	280	110	2266	2656
Case 4 (MPS I)	4503	6700	67,222	78,425
Case 5 (MPS VII)	408	406	3417	4231
Case 6 (MPS II)	240	137	836	1210
Control (n = 326)				
Mean $\pm$ SD	117 $\pm$ 23	67 $\pm$ 28	370 $\pm$ 109	553 $\pm$ 146

Table 3

Sensitivity and specificity.

	DiHS-0S	DiHS-NS	Di-4S
Cut off point (ng/spot)	>2.5	>1.1	>8.0
Sensitivity (%)	100	100	100
Specificity (%)	98.5	98.5	99.7
Positive predictive value	54.5	54.5	85.7
False positive rate	1.5	1.5	0.3
False negative rate	0	0	0