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Novel heparan sulfate assay by using automated high-throughput mass spectrometry: Application to monitoring and screening for mucopolysaccharidoses

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ABSTRACT

Mucopolysaccharidoses (MPS) are caused by deficiency of one of a group of specific lysosomal enzymes, resulting in excessive accumulation of glycosaminoglycans (GAGs). We previously developed GAG assay methods using liquid chromatography tandem mass spectrometry (LC–MS/MS); however, it takes 4–5 min per sample for analysis. For the large numbers of samples in a screening program, a more rapid process is desirable. The automated high-throughput mass spectrometry (HT-MS/MS) system (RapidFire) integrates a solid phase extraction robot to concentrate and desalt samples prior to direction into the MS/MS without chromatographic separation; thereby allowing each sample to be processed within 10 s (enabling screening of more than one million samples per year). The aim of this study was to develop a higher throughput system to assay heparan sulfate (HS) using HT-MS/MS, and to compare its reproducibility, sensitivity and specificity with conventional LC–MS/MS.

HS levels were measured in the blood (plasma and serum) from control subjects and patients with MPS II, III, or IV and in dried blood spots (DBS) from newborn controls and patients with MPS I, II, or III. Results obtained from HT-MS/MS showed 1) that there was a strong correlation of levels of disaccharides derived from HS in the blood, between those calculated using conventional LC–MS/MS and HT-MS/MS, 2) that levels of HS in the blood were significantly elevated in patients with MPS II and III, but not in MPS IVA, 3) that the level of HS in patients with a severe form of MPS II was higher than that in an attenuated form, 4) that reduction of blood HS level was observed in MPS II patients treated with enzyme replacement therapy or hematopoietic stem cell transplantation, and 5) that levels of HS in newborn DBS were elevated in patients with MPS I, II or III, compared to those of control newborns.

In conclusion, HT-MS/MS provides much higher throughput than LC–MS/MS-based methods with similar sensitivity and specificity in an HS assay, indicating that HT-MS/MS may be feasible for diagnosis, monitoring, and newborn screening of MPS.

1. Introduction

Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases caused by deficiency of the lysosomal enzymes required for the degradation of glycosaminoglycans (GAGs) such as chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS), and hyaluronan. There are 11 known enzyme deficiencies, resulting in seven distinct forms of MPS with a collective incidence of more than 1 in 25,000 live births. Accumulation of GAGs causes progressive damage of multiple tissues including the brain, lung, heart, liver, kidney, and bone. Most clinical signs and symptoms for MPS patients do not appear immediately after birth; however, the subsequent onset of clinical signs and symptoms progresses with age. Although the symptoms and severity of MPS vary with individual patient and subtype of

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MPS, the average life span in patients with a severe form is one to two decades if untreated.

Currently, enzyme replacement therapy (ERT), hematopoietic stem cell transplantation (HSCT), substrate reduction therapy (SRT), and gene therapy are clinically in use or are being investigated in clinical trials for some types of MPS patient. Starting these treatments at birth or at a very early stage will provide the most significant impact on the clinical course of the disease [1]. To provide better quality of life for the patients, early diagnosis and treatment are required. Since unique clinical features in most MPS patients are not apparent until they are around two years old and general physicians have little knowledge of MPS, most patients are misdiagnosed or undiagnosed until irreversible damage to the brain and/or bones has started. Thus, a novel, accurate, sensitive, economical and rapid diagnostic method applicable to newborn screening (NBS) for MPS is critical to improving therapeutic efficacy.

Currently, conventional screening methods for MPS are dye-spectrometric methods such as dimethylmethylene blue (DMB) [2–4] and Alcian Blue [5,6] which measure total GAGs from urine samples. When urine assays provide a positive result, the definitive diagnosis is determined by measuring the enzyme activities in lymphocytes or fibroblasts. However, these current methods cannot be applied to blood and/or tissue extracts without prior protease, nuclease or hyaluronidase treatment [7]. Total GAG concentration in urine does not reflect the severity of the neurological or skeletal signs and symptoms [4] and substantial overlap of the total urine GAG level between age-matched controls and MPS IV patients is observed [4], resulting in misdiagnosis of many patients. Thin layer chromatography, which is a semi-quantitative method for each GAG, is used to allow preliminary classification of patients among subgroups to reduce the number of enzyme assays necessary for final diagnosis [8,9]. There are several other methods for measuring specific GAGs in the blood, including ELISA [10,11] and HPLC [12,13]. However, ELISA kits are not commercially available for all types of GAGs and they cannot measure subtype of HS or KS. HPLC is not suitable for mass screening since the assay is time-consuming with low sensitivity.

NBS is recognized as an essential, preventive public health program for early identification of diseases in newborns that can affect their long-term health. A suitable method for NBS must be inexpensive and should be performed using a dried blood spot (DBS) from a Guthrie card. At present there is no reliable NBS to cover all types of MPSs. We and others have developed new methods to assay CS, DS, HS, and KS simultaneously in the blood, urine, and/or DBS samples by using liquid chromatography tandem mass spectrometry (LC–MS/MS) [7,14–23]. The LC–MS/MS method not only shows sensitivity and specificity for detecting all subtypes of MPS, but also monitors therapeutic efficacy in MPS patients and animal models [1,16,22,24,25]; however, since LC processing is time consuming, the main drawback of this method is throughput. This factor may limit its utility for assaying large numbers of samples.

The automated high-throughput mass spectrometry (HT-MS/MS) system (RapidFire; Agilent Technologies) eliminates the chromatographic process, enabling sample-to-sample cycle times to be reduced to seconds, thereby removing the bottleneck of throughput while maintaining the quality and reliability of standard LC–MS/MS read-outs. The sample is aspirated to a matrix for concentration and desalting, followed by direct injection into MS/MS without chromatographic separation. Each sample is processed within 10 s, yielding much faster throughput than traditional LC–MS/MS based methods. A single 384 well plate can be read in less than 40 min, indicating that this HT-MS/MS system can analyze over one million samples annually. Moreover, HT-MS/MS has been shown to provide sensitivity and specificity equivalent to a scintillation proximity assay [26], and an LC–MS/MS assay for some applications [19,27]. The speed and efficiency of HT-MS/MS can allow for experiments that would otherwise be deemed untenable under normal circumstances. The HT-MS/MS system has been validated as suitable for many drug discovery programs [26,28–34], and ADME (*A*bsorption, *D*istribution, *M*etabolism and *E*xcretion) based applications [27]. We recently reported that HT-MS/MS could measure HS levels in control human blood much faster than LC–MS/MS, and showed a strong correlation with levels measured by LC–MS/MS [19].

In this study we have evaluated a higher throughput system to assay HS levels in the blood and DBS from newborn patients with MPS by using HT-MS/MS, and have compared its reproducibility, sensitivity, and specificity with conventional LC–MS/MS.

2. Materials and methods

2.1. Materials

2.1.1. Standards and enzymes

To digest polymer HS to disaccharides, the enzyme heparitinase was obtained from Seikagaku Corporation (Tokyo, Japan). Chondrosine was used as an internal standard (IS), and unsaturated disaccharides, [ΔDiHS-0S, 2-acetamido-2-deoxy-4-O-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-D-glucose; ΔDiHS-NS, 2-deoxy-2-sulfamino-4-O-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-D-glucose] were used for generation of standard curves. Stock solutions of ΔDiHS-0S (100 μg/ml), ΔDiHS-NS (100 μg/ml), and IS (5 mg/ml) were prepared separately in ddH_2O. Standard working solutions of ΔDiHS-0S and ΔDiHS-NS, (15.625, 31.25, 62.5, 125, 250, 500, and 1000 ng/ml), each mixed with IS solution (5 μg/ml), were prepared.

2.1.2. Blood and DBS samples

We collected 95 blood (plasma or serum) samples from MPS patients as follows: 55 MPS II patients (age 2.0–35.0 years, mean 11.4 years; 45 severe, 10 attenuated), 18 MPS III patients (age 4.0–27.0 years, mean 12.5 years; 6 IIIA, 12 IIIB), and 22 MPS IV patients (age 3.4–56.0 years, mean 17.2 years; 17 IVA, 5 IVB). We obtained the samples from patients with MPS II who received ERT (16) or HSCT (9), and one patient with MPS IIIB who received HSCT. All patients were diagnosed as having below 5% of normal enzymatic activity. We also collected 133 blood (plasma or serum) samples from normal controls. The age of control samples ranged between 0 and 80 years (mean 5.2 years). In previous LC–MS/MS experiments, we confirmed that specificity and sensitivity of each GAG value were comparable using plasma or serum [7,14–17,19]. Diagnosis for MPS patients was confirmed by enzyme assay at each institute providing samples. The clinical severity in patients with MPS II was assessed by the presence (severe) or absence (attenuated) of central nervous system (CNS) involvement. Clinical phenotypes in all patients with MPS III and MPS IVA were defined as severe, and all patients with MPS IVB were defined as attenuated in terms of growth compared to MPS IVA patients.

We also collected DBS samples from 22 anonymous control newborns and 12 newborns with MPS (6 MPS I, 1 MPS II, and 5 MPS III). These MPS patients had been diagnosed enzymatically and clinically, and DBS samples obtained at birth were provided by the newborn screening center after diagnosis. These control and patient samples were assayed in a blinded manner.

Written informed consent was obtained for each patient prior to initiation of the study, and the study was approved by the institutional review board of each institute providing samples for this study.

2.2. Methods

2.2.1. Sample preparation

Blood specimens and standards were prepared as described previously [18,19]. First, 10 μl of each serum or plasma sample and 90 μl of 50 mM Tris–hydrochloric acid buffer (pH 7.0) were placed in wells of AcroPrep™ Advance 96-Well Filter Plates that have Ultrafiltration Omega 10 K membrane filters (PALL corporation, NY, USA). The filter plates were placed on the receiver and centrifuged at 2000 *g* for