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

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Conflict of Interest:
All the authors have contributed to this "Original Article" and have no conflict of interest with any other party.
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Compliance with Ethics
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Informed consent was obtained from the patients and/or their guardians through the physicians who recruited the patients with MPS at each local institute, and approved by the local Institutional Review Board (IRB).

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Tsutomu Shimada contributed to the concept of the project, the planning, execution of experiments (LC-MS/MS), data analysis, and reporting of the work described in the article.
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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 ten seconds (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 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 blood, between those calculated using conventional LC-MS/MS and HT-MS/MS, 2) that levels of HS in 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 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.

Keywords

automated high-throughput mass spectrometry; mucopolysaccharidoses; heparan sulfate; biomarker; newborn screening

1. Introduction

Mucopolysaccharidoses (MPS) are a group of lysosomal storage diseases caused by deficiency of the lysosomal enzymes required for 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 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 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 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.

Newborn screening (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 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 blood, urine, and/or dried blood spot (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 ten seconds, 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 (Absorption, Distribution, Metabolism and Excretion) 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 blood and dried blood spots (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, [DiHS-0S, 2-acetamido-2-deoxy-4-O-(4-deoxy-a-L-threo-hex-4-enopyranosyluronic acid)-D-glucose; DiHS-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 DiHS-0S (100 μg/ml), DiHS-NS (100 μg/ml), and IS (5 mg/ml) were prepared separately in ddH$_2$O. Standard working solutions of DiHS-0S and DiHS-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), 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 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 10K membrane filters (PALL corporation, NY, USA). The filter plates were placed on the receiver and centrifuged at 2,000 g for 15 min to remove free disaccharides. The membrane plates were transferred to a fresh receiver plate. Standards were added to unused wells of the filter plate. Then, 20 μl of IS solution (500 ng/ml), 70 μl of 50 mM Tris–hydrochloric acid buffer (pH 7.0), and 10 μl of heparitinase solution (2 mU per sample) were added to each filter well. The plate was incubated in a water bath at 37 °C for 15 hr and centrifuged at 2,000 g for 15 min. The receiver plate containing disaccharides was stored at –20 °C until analysis by HT-MS/MS or LC-MS/MS. disks of DBS samples (3 mm in diameter) were punched out and incubated in 0.1% bovine serum albumin for 15 min on Omega 10K membrane filters. After centrifuge at 2,000 g for 15 min, the mixture of IS, 50 mM Tris–hydrochloric acid buffer (pH 7.0), and heparitinase solution as described above was added to each filter well, and processed as per the blood samples.

2.2.2. Automated high-throughput mass spectrometry (HT-MS/MS) system (RapidFire)—Samples stored at –20 °C were allowed to thaw at ambient temperature. General operation of the HT-MS/MS has been described previously [35]. In brief, 10 μL of sample was aspirated directly from microtiter plates and loaded onto the RapidFire microscale solid-phase extraction cartridge D (graphitic carbon) to remove salts with HPLC-grade water in a 2.5-sec wash cycle. Analytes were then coeluted into the mass spectrometer in a 3.5-s elution cycle using 25% acetonitrile, 25% acetone containing 5 mM ammonium acetate. DiHS-0S, DiHS-NS and IS were measured using a Sciex API 4000 QQQ mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) in negative ESI mode

following multiple-reaction monitoring (MRM) transitions at 378.2/174.3, 416.0/137.7 and 354.3/193.1, respectively. Sample data were integrated using the RapidFire Integrator software (Agilent Technologies, Inc: Santa Clara, CA) resulting in individual AUC (area under the curve) values for each of the three analytes in each sample. Each sample was prepared and analyzed in duplicate on separate days. HS levels were measured and averaged.

2.2.3. LC-MS/MS—The chromatographic system consisted of the 1260 Infinity LC (Agilent Technologies, Palo Alto, CA, USA) and a Hypercarb column (2.0 mm i.d. 50 mm, 5 μm, Thermo Electron, USA). The 6460 Triple Quad mass spectrometer (Agilent Technologies) was operated in the negative ion detection mode. The procedure was described previously [18]. The MRM of each disaccharide was the same as used for HT-MS/MS analysis.

2.2.4. Method validation—Intra-day precision evaluated as coefficient of variation (CV) was determined by replicate analyses (n = 5) of three different control blood samples. Inter-day precision was determined by replicate analyses (n = 5) of three different control blood samples on three separate days.

The selectivity of the assay was investigated by processing and analyzing five independent samples by the method described above without enzymatic digestion. Calibration curves were constructed by plotting the peak area ratio of the analytes to IS against the concentration of the analytes. Each calibration curve consisted of seven calibration points. HS values obtained by HT-MS/MS were compared with those by LC-MS/MS.

2.2.5. Data analysis—The data were analyzed by ANOVA and Tukey's test. Analysis was performed using SPSS for Windows (version 22.0, IBM, Chicago, IL, USA).

3. Results

3.1. MRM of HT-MS/MS

Typical MRM of HT-MS/MS for DiHS-0S standards with serial dilutions and samples is shown in Fig. 1. Similar MRMs were obtained for IS and DiHS-NS. The HT-MS/MS system allowed us to measure 8 – 9 samples per min.

3.2. Precision

Results of intra- and inter-assay precision for DiHS-0S and DiHS-NS in control blood samples are as follows. The intra-assay precision values/coefficient of variation (CV) determined from the analysis of DiHS-0S and DiHS-NS for control blood samples are less than 14.7 and 14.9%, respectively. The inter-assay precision values/CVs for these disaccharides in control blood samples are less than 13.9 and 13.7%, respectively. These results demonstrate the reproducibility and precision of the method.

3.3. Correlation between HT-MS/MS and LC-MS/MS

All blood samples were also measured with the LC-MS/MS system that separates the disaccharides with a chromatographic system. There were strong correlations between HT-

MS/MS and LC-MS/MS in measurements of both ΔDiHS-0S ($r^2 = 0.9409$) and ΔDiHS-NS ($r^2 = 0.889$), and regression coefficients for ΔDiHS-0S and ΔDiHS-NS were 1.2 and 1.0, respectively (Fig. 2)

3.4. Measurements of GAGs in blood

3.4.1. Control—The levels of ΔDiHS-0S and ΔDiHS-NS were the highest in newborns and decreased until three years of age. After three years of age, the levels of these two HS remained stable (Fig. 3).

3.4.2. Mucopolysaccharidosis type II (MPS II)—Levels of blood ΔDiHS-0S and ΔDiHS-NS in untreated patients with a severe form of MPS II (n = 24) were significantly higher, compared with those in control groups (Table 1, Fig. 3). The increase in untreated patients with an attenuated form of MPS II (n = 9) did not reach statistical significance with the small sample size. The level of ΔDiHS-0S in untreated patients with a severe form of MPS II was higher than that in patients with an attenuated form. The level of ΔDiHS-NS was also elevated in the untreated patients with the severe form of MPS II compared to those with the attenuated form (Table 1).

The levels of ΔDiHS-0S in blood from HSCT-treated patients with a severe form of MPS II 8.9 years (average) after treatment were significantly reduced, compared with untreated patients (Table 1). Levels of ΔDiHS-0S in blood from these HSCT treated patients were also significantly lower than in blood from ERT-treated patients (Table 1). Seven patients with the severe form of MPS II provided blood specimens before and an average of two years after starting ERT. Levels of ΔDiHS-0S and ΔDiHS-NS were significantly decreased after ERT treatment (Fig. 4).

3.4.3. Mucopolysaccharidosis type III (MPS III)—Untreated patients with MPS IIIA and MPS IIIB (n = 6 and n = 11, respectively) had significant elevation of levels of ΔDiHS-0S and ΔDiHS-NS from blood compared with those in control groups (Table 2; Fig. 3). A 15 year-old MPS IIIB female, who had undergone successful HSCT at 1 year and 10 months, had blood levels of ΔDiHS-0S and ΔDiHS-NS in the normal range (Table 2). This patient could speak with limited words, recognize her parents, and walk by herself at the age of 15 years. Thus, her cognitive and physical function was much better, compared with that in untreated age-matched MPS IIIB patients, indicating that the biochemical correction by HSCT resulted in an improved clinical outcome.

3.4.4. Mucopolysaccharidosis type IV (MPS IV)—Blood HS level in untreated and ERT-treated patients with MPS IVA (n = 11 and n = 6, respectively) showed the same levels as normal controls. Untreated patients with MPS IVB also had the same range of HS compared with normal controls (Table 3).

3.5. Measurements of GAGs in newborn DBS

The levels of ΔDiHS-0S and ΔDiHS-NS extracted from DBS of newborns later diagnosed with MPS I and MPS III were significantly elevated compared to control newborn samples. The levels of ΔDiHS-0S and ΔDiHS-NS from DBS of a newborn later diagnosed with MPS

II were 3 and 1.5 times higher than in DBS from control newborns (Table 4). For all three forms of MPS, DiHS-0S distinguished patients from controls better than DiHS-NS.

4. Discussion

We have demonstrated 1) that the RapidFire platform is capable of detecting disaccharides of DiHS-0S and DiHS-NS in blood with the same reproducibility, sensitivity, and specificity as described for the LC-MS/MS method [7, 14–17, 19–22], suggesting that chromatographic separation of disaccharides is not essential for detection and identification of DiHS-0S and DiHS-NS, 2) that HS assays by HT-MS/MS can distinguish untreated patients with MPS II or III from healthy controls, showing the potential use of this technology as a diagnostic platform, 3) that patient groups treated by ERT or HSCT had reduced HS levels, suggesting that HS measured by HT-MS/MS can be used as a biomarker for monitoring therapeutic effect, 4) that the run time of six to eight seconds per sample provides 10–100 fold higher throughput when compared to traditional LC-MS/MS, indicating that HT-MS/MS may be useful for mass screening applications for MPS, and 5) that levels of HS in newborn DBS were elevated in all MPS I, II and III patients, suggesting that HT-MS/MS could be used in a NBS to identify these patients.

In MPS, the undegraded GAGs are stored in lysosomes, secreted into the circulation and excreted into the urine. In this study, blood HS was digested to disaccharides by heparitinase using the same method as described for the LC-MS/MS method [7, 14, 15]. Two subclasses of HS (DiHS-0S and DiHS-NS) were detected by HT-MS/MS simultaneously. The levels of DiHS-0S and DiHS-NS in blood samples from both control and patient subjects, determined by HT-MS/MS, correlated strongly with those determined by LC-MS/MS, both in this and previous studies [15, 17]. The calculated values of DiHS-0S and DiHS-NS using both methods were similar, indicating that LC-MS/MS and HT-MS/MS are comparable in sensitivity and specificity.

Overall, the levels of DiHS-0S and DiHS-NS obtained in blood of normal controls and MPS patients by HT-MS/MS was equivalent to those obtained by LC-MS/MS, indicating that the use of the HT-MS/MS platform did not affect the specificity and sensitivity of the assay. The HT-MS/MS system does not only acquire data much faster than traditional LC/MS/MS systems, but also provides data of a similar quality without using a time-consuming separation process.

MPS II results from a deficiency of iduronate-2-sulfatase, leading to accumulation of HS and DS. In this study DiHS-0S and DiHS-NS levels increased in untreated patients with a severe form of MPS II compared with those in the control groups, as described previously using the LC-MS/MS method [7, 15]. There was a significant increase in the level of DiHS-0S in patients with a severe form of this MPS compared to those with an attenuated form, and the level of DiHS-0S in the ERT- or HSCT-treated group was significantly lower compared with that in the untreated group. Furthermore, the level of DiHS-0S was significantly lower in blood from patients treated by HSCT than in blood from patients treated by ERT. Levels of DiHS-NS were also lower in blood from the HSCT treated patients but the difference was not statistically significant. Consistent with our results,

Tanaka et al reported that HSCT given during early stages of MPS II provides effectiveness toward brain and heart disorders with decrease of urinary GAGs level and that patients treated by ERT at a similar stage showed less reduction of urinary GAGs [36]. For ERT, the timing of blood draw after a round of therapy is likely to affect blood levels of GAGs, so more detailed studies are needed to determine whether HSCT or ERT is better at reducing GAGs. In future studies it will be of great interest to explore whether differences in DiHS-0S and/or DiHS-NS levels are associated with clinical severity and/or improvement of CNS involvement.

MPS IIIA and MPS IIIB result from deficiencies of enzymes heparan N-sulphatase and α-N-acetylglucosaminidase, respectively, leading to accumulation of HS. The levels of DiHS-0S and DiHS-NS in untreated patients with MPS IIIA or MPS IIIB were significantly raised compared with those in normal controls, as described previously using LC-MS/MS [7, 15]. The frequency of MPS IIIB is higher in a regional district in Japan, based upon the founder effect [37]. It is noteworthy that one Japanese MPS IIIB patient who received successful HSCT when under two years of age had DiHS-0S and DiHS-NS levels in the normal range. After over 13 years follow-up, the level of neurocognition in this patient is far better than in age-matched untreated patients, although neurological involvement still remains obvious. The finding that the primary storage material, HS, is within the normal range is consistent with the less severe clinical course in this patient, showing that both DiHS-0S and DiHS-NS should be good biomarkers for monitoring the clinical course and therapeutic efficacy in MPS III.

MPS IVA and MPS IVB are caused by the deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS) and β-galactosidase (β-Gal), respectively, and consequently KS but not HS should accumulate in blood. In this study we found that HS levels in patients with MPS IV were the same as the control group, consistent with the predicted result and previous reports [15, 17]. In the United States, nearly 4.2 million neonates undergo NBS annually [38]. There are no specific incidence studies of MPS in the USA, but we estimate that approximately 200 newborns are affected with MPS annually in USA [39–45](20–26). In this study, we show that the levels of DiHS-0S and DiHS-NS from DBS of newborn MPS I, II and III patients are higher than newborn control. De Ruijter et al also reported that the LC-MS/MS method could show elevation of DiHS-0S in the patients with these forms of MPS using newborn DBS samples [20]. Our results lead the possibility of applying HT-MS/MS to NBS for MPS, although further studies with more samples are required. The fact that HT-MS/MS can process the same samples with throughputs up to 10–100 folds faster than conventional LC-MS/MS makes this platform a more appropriate system, due to time-saving and potential cost reductions for NBS.

HS can be elevated in blood due to release from the endothelium during ischemia/reperfusion injury and severe inflammation [46, 47]. It can also be elevated in blood after virus- infection and septic shock [48, 49]. Thus, HT-MS/MS could be a novel high throughput system to identify biomarkers for not only MPS but also for these more common disorders. This manuscript has focused on assay of HS, but CS, DS and KS assays could also be developed for HT-MS/MS to screen for other forms of MPS and other diseases.

In conclusion, we have demonstrated a unique HT-MS/MS method for measurement of HS that can distinguish MPS patients from controls with a similar reproducibility, sensitivity and specificity as established by LC-MS/MS methods, and that the method can be applied to analysis of newborn DBS. The HT-MS/MS should provide both a rapid throughput and economic advantages for monitoring, diagnosis, and NBS for MPS and has the potential of being applied to other disorders.

Acknowledgments

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Highlight

- Automated high-throughput mass spectrometry (HT-MS/MS) is comparable with LC-MS/MS in sensitivity and specificity.

- Levels of HS in blood are significantly elevated in patients with MPS II and III.

- Levels of HS in patients with a severe form of MPS II are higher than that with an attenuated form.

- Reduction of blood HS level is seen in MPS II patients treated with ERT or HSCT.

- Levels of DiHS-0S and DiHS-NS in DBS from newborn MPS I, II and III patients are elevated compared to newborn controls.
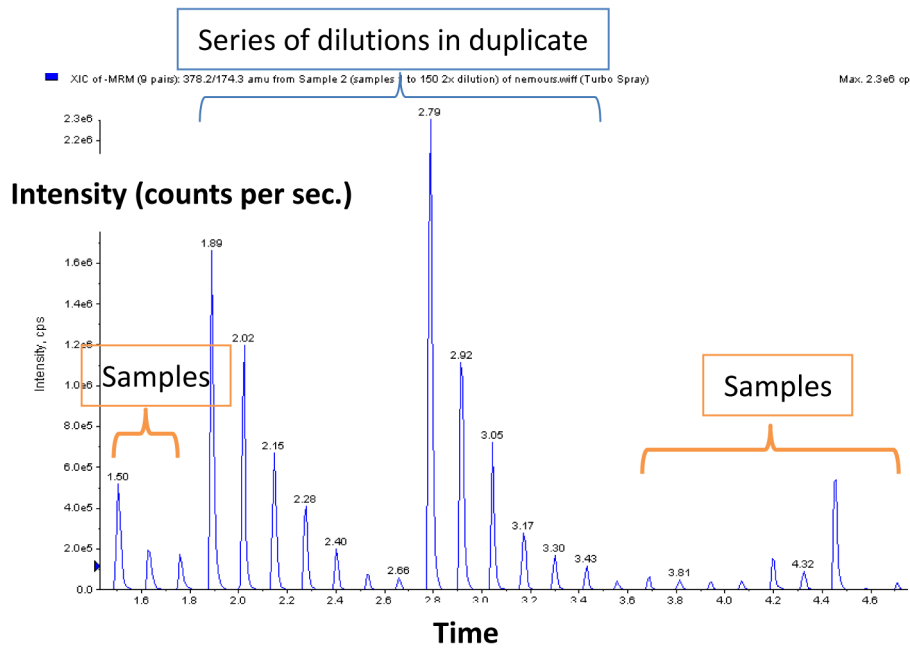
Figure 1.
MRMs of HT-MS/MS. Multiple injections of DiHS-0S (Q1; 378.2, Q3; 174.3) with a
series of dilutions (15.625, 31.25, 62.5, 125, 250, 500, and 1000 ng/ml) in duplicate show
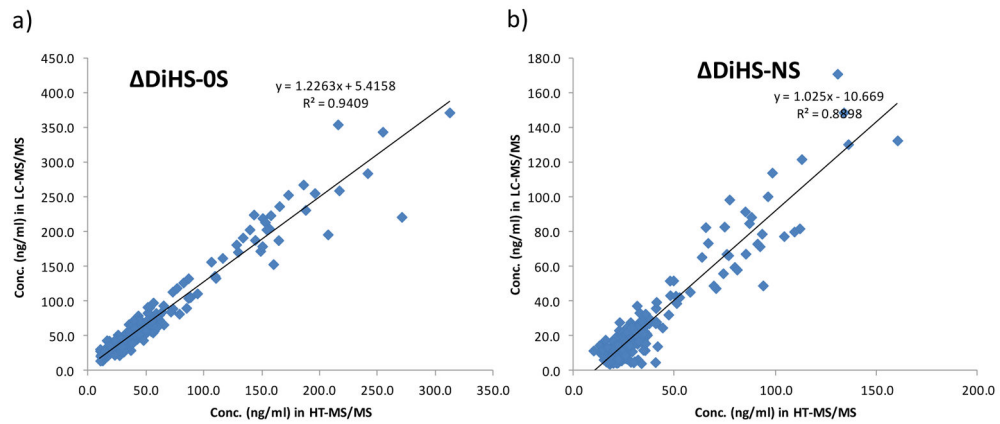seven gradient peaks per set of dilutions (surrounding samples also shown).

Figure 2.

Correlation in HS concentration between LC-MS/MS and HT-MS/MS.

a)   DiHS-0S; b)   DiHS-NS. Coefficient of determination is $r^2 = 0.9409$ and $r^2 = 0.889$ for

   DiHS-0S and b)   DiHS-NS, respectively; showing a strong correlation in   DiHS-0S and

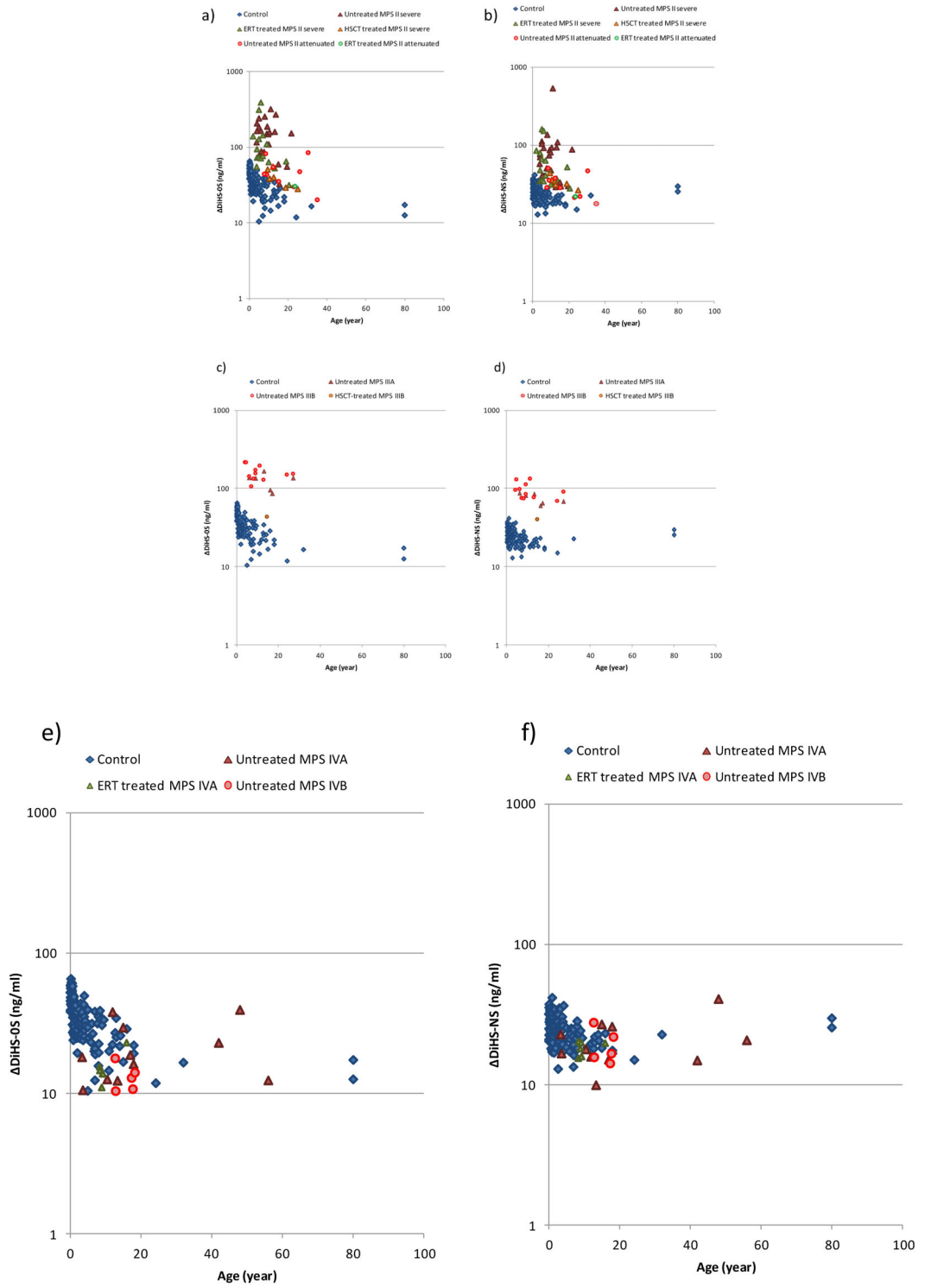   DiHS-NS between LC-MS/MS and HT-MS/MS in human blood samples (n = 228).

Figure 3.
HS levels in serum of patients with MPS and control subjects. a)   DiHS-0S in patients with MPS II, b)   DiHS-NS in patients with MPS II, c)   DiHS-0S in patients with MPS III, d)

DiHS-NS in patients with MPS III, e)   DiHS-0S in patients with MPS IV, and f)   DiHS-NS in patients with MPS IV.

Results of all specimens from patients and control subjects were plotted on a semilogarithmic scale with respect to age (years).
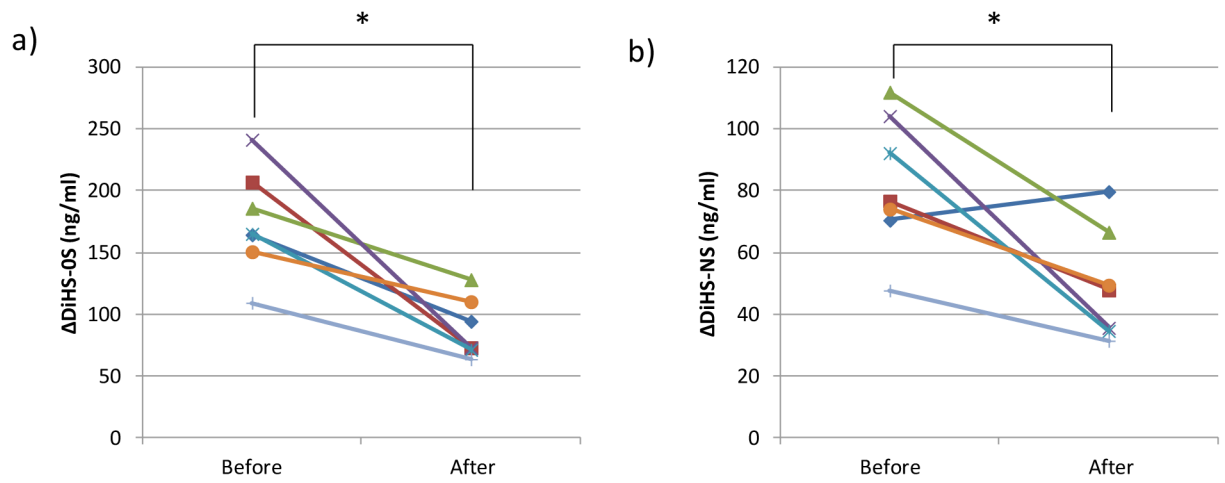
Figure 4.
HS levels in serum of seven patients with MPS II who underwent ERT for more than two years. a) DiHS-0S; b) DiHS-NS. Each line describes an individual patient. *: shows significant difference between pre-treatment and post-treatment.

Table 1

Levels of DiHS-0S and DiHS-NS in patients with MPS control subjects over 3 years of age.

	DiHS-0S (ng/ml)	DiHS-NS (ng/ml)
Control	27.5 ± 8.7	22.7 ± 4.8
Untreated MPS II (severe)	144.5 ± 77.9^a	89.4 ± 100.1^a
ERT-treated MPS II (severe)	116.1 ± 101.0^a	61.0 ± 41.4
HSCT-treated MPS II (severe)	$40.3 \pm 11.1^{b,c}$	34.1 ± 5.9
Untreated MPS II (attenuated)	49.2 ± 21.9^b	32.2 ± 11.5
ERT-treated MPS II (attenuated)	30.5	22

Number of each group is as follows; control (n = 59), untreated MPS II (severe; n = 24), ERT-treated MPS II (severe; n = 15), HSCT-treated MPS II (severe; n = 6), untreated MPS II (attenuated; n = 9), and ERT-treated MPS II (attenuated; n = 1).

[a] significantly different from the control at $p < 0.05$,

[b] significantly different from the untreated MPS II severe at $p < 0.05$,

[c] significantly different from the ERT-treated MPS II severe at $p < 0.05$

Table 2

Levels of DiHS-0S and DiHS-NS in patients with MPS III and control subjects over 3 years of age.

	DiHS-0S (ng/ml)	DiHS-NS (ng/ml)
Control	27.5 ± 8.7	22.7 ± 4.8
Untreated MPS IIIA	126.6 ± 30.0[a]	75.0 ± 11.5
Untreated MPS IIIB	161.3 ± 35.6[a]	95.0 ± 22.3[a]
HSCT treated MPS IIIB	43.6	40.5

Number of each group is as follows, Control (n = 59), untreated MPS IIIA (n = 6), untreated MPS IIIB (n = 11), and HSCT-treated MPS IIIB (n = 1)

[a] significantly different from the control at $p < 0.05$

Table 3

Levels of DiHS-0S and DiHS-NS in patients with MPS IV and control subjects over 3 years of age.

	ADiHS-0S (ng/ml)	DiHS-NS (ng/ml)
Control	27.5 ± 8.7	22.7 ± 4.8
Untreated MPS IVA severe	20.9 ± 10.3	20.6 ± 8.3
ERT treated MPS IVA severe	15.2 ± 4.0	18.2 ± 2.1
Untreated MPS IVB attenuated	13.1 ± 3.0	19.2 ± 5.5

Number of each group is as follows, Control (n = 59), untreated MPS IVA (n = 11), ERT-treated MPS IVA (n = 6), and untreated MPS IVB (n = 5)

Table 4

Levels of DiHS-0S and DiHS-NS in newborn DBS from control and patients with MPS.

(ng/spot)	DiHS-0S	DiHS-NS
Control	0.10 ± 0.02	0.11 ± 0.05
MPS I	0.49 ± 0.13 *	0.29 ± 0.11 *
MPS II	0.33	0.14
MPS III	0.50 ± 0.10 *	0.30 ± 0.13 *

Number of each group is as follows, Control (n = 22), MPS I (n = 6), MPS II (n = 1), and MPS III (n = 5)

* significantly different from the control at $p < 0.05$.