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# Evaluation of a rapid LC-MS/MS method to measure simultaneously IDUA and IDS enzymes activities in dried blood spots

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## 1. Overview

A novel and rapid LC-MS/MS method was developed to simultaneously measure the activities of lysosomal enzymes for newborn screening

## 2. Introduction

Mucopolysaccharidoses (MPSs) is a group in lysosomal storage disorders (LSDs) caused by a deficiency of lysosomal hydrolases responsible for the catabolism of glycosaminoglycans (GAGs). Some techniques such as fluorometric and mass spectrometric assays have been developed to measure these enzyme activities for the purpose of newborn screening. The use of mass spectrometric techniques has exhibited advantages over the other techniques in the ability to multiplex several enzymes in one assay. In this study, we report a novel method using tandem mass spectrometry that is capable of simultaneous measurement of the activities of the MPS enzymes including IDUA (MPS I) and IDS (MPS II) in a short time scale. In this presentation, the developed method is detailed.

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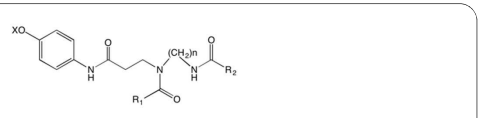
## 3. Methods

Assay buffer, cocktail of 2 enzyme substrates, internal standards and quality control (QC) dried-blood sample (DBS) were purchased from PerkinElmer Inc. A disc (3 mm) was punched from each DBS sample and placed into a 96-well plate. Assay cocktail was added to each well, and the whole plate was shaken at 37ºC for 16 hours. Thereafter, quenching of the enzyme assay was followed by liquid-liquid extraction for purification.



| Compound | X | R1 | R2 | n |
|---|---|---|---|---|
| MPS-I Substrate | α-Iduronosyl | Methyl | Phenyl | 6 |
| MPS-I Product | H | Methyl | Phenyl | 6 |
| MPS-II-Substrate | α-Iduronosyl-2-sulfate | n-Butyl | Phenyl | 6 |
| MPS-II Product | α-Iduronosy | n-Butyl | Phenyl | 6 |

Figure 1 2 substrates for 2 enzymes; IS are deuterated forms of enzyme products

On-column analysis was performed using a LC-MS/MS system consisting of UHPLC with a triple quadrupole mass spectrometer (NexeraTM with LCMS-8050, Shimadzu Corporation, Kyoto, Japan). The mobile phases used were (A) 0.1 % formic acid in water and (B) 0.1 % formic acid in acetonitrile. LC-MS/MS with electrospray ionization was operated in multiple-reaction-monitoring (MRM) mode.

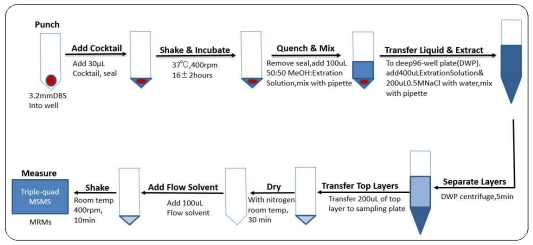



Figure 2 2-plex NeoLSD reagent workflow

### Analytical Conditions

**HPLC conditions (Nexera MP system)**

**On Column Analysis**

Mobile phase A : Water + 0.1 % formic acid
Mobile phase B : Acetonitrile + 0.1 % formic acid
Flow rate : 0.4 mL/min

- 6 minutes method
Injection volume : 1 μL
Column : Phenomenex Kinetex XB-C18 150 mm x 2.1 mm, 1.7 μm
Time program : 0 min. B 30% > 0.5 min. B 30 % > 3.5 min. B 100 % > 5.0 min. B 100 % > 5.01 min. B 30 %> 6.0 min. B 30 %

- 2 minutes method
Injection volume : 1 μL
Column : Phenomenex Kinetex XB-C18 30 mm x 2.1 mm, 1.7 μm
Time program : 0 min. B 30% > 0.1 min. B 30 % > 0.7 min. B 100 % > 1.3 min. B 100 % > 1.31 min. B 30 %> 2 min. B 30 %

**MS conditions (LCMS-8050)**

Ionization ESI, Positive MRM mode

Table 1 MRM Transitions

| Compound | Precursor ion *m/z* | Product ion *m/z* |
|---|---|---|
| MPSI-IS | 431.3 | 322.3 |
| MPSI-P* | 426.3 | 317.3 |
| MPSII-IS | 649.4 | 364.4 |
| MPSII-P | 644.4 | 359.4 |

* P means product

## 4. Results

This presentation is the result of the project of AMED (Japan Agency for Medical Research and Development). In this study, we developed a method for simultaneous analysis of two enzyme activities (2-plex) as MPS-I, MPS-II typical in Japan using LC-MS/MS. The method requires up to 6 minutes per sample on this LCMS-8050 with an Nexera MP HPLC system. In the flow injection method, MRM product peaks derived from in-source breakdown of substrates are observed, especially IDS (Iduronate-2-sulfatase) for MPS-II. As UHPLC led to the full separation of enzymatic product and substrate peaks, this in-source breakdown was of no concern. Although this method requires 6 minutes cycle now, the method has the potential to be shortened as Liu Y et al. reported [Liu Y et al., Clin Chem. 2017, 63(6):1118-1126]. We evaluated the possibility to shorten the analysis cycle to 2 minutes to make it more suitable for newborn screening.

### 4-1. On-column analysis

In each MRM chromatogram of products, an interfering peak from each of substrate at different retention time was observed (red arrow in Figure 3). Those peaks were considered as the substrates caused by in-source decay from the each product.
In flow injection analysis (FIA), the signal is detected even without enzyme activity since substrate peaks could not be chromatographically separated from enzymatic reaction products.
On column analysis showed that the product and IS were detected at the same retention time and products were separated from each substrate for MPS-I and MPS-II. The results from the control dried-spot (Low- activity) with 6 minutes and 2 minutes method are shown in Figure 3.

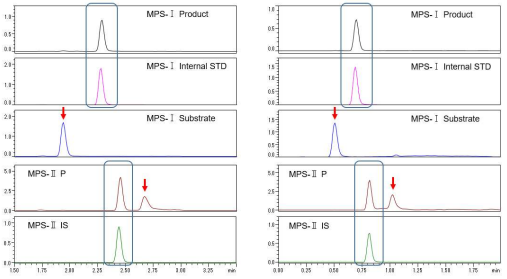
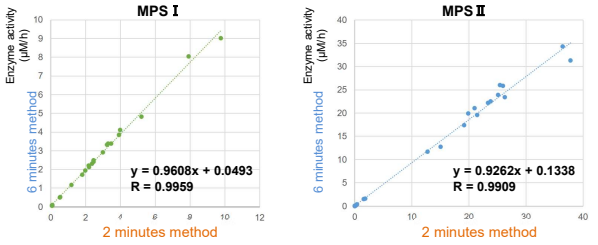
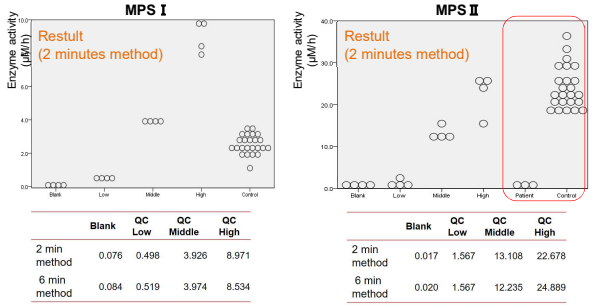



Figure 3 MRM chromatograms of each target compound with on-column analysis. Left: Low- activity QC, 6 minutes method; Right: Low-activity QC, 2 minutes method

### 4-2. Results of enzyme activity evaluationId sample

We evaluated 6 min and 2 min methods for simultaneous analysis of two enzyme activities (2-plex) of MPS-I and MPS-II. The correlation coefficients between these methods showed good correlation with R = 0.9959 for MPS Ⅰ and R = 0.9909 for MPS Ⅱ, and this rapid 2 min method was considered particularly useful for newborn screening. In addition, the samples from MPS Ⅱ affected patients showed a significantly lower enzyme activity than the healthy newborns and could be clearly distinguished (Figure. 4).




| | Blank | QC Low | QC Middle | QC High |
|---|---|---|---|---|
| 2 min method | 0.076 | 0.498 | 3.926 | 8.971 |
| 6 min method | 0.084 | 0.519 | 3.974 | 8.534 |

| | Blank | QC Low | QC Middle | QC High |
|---|---|---|---|---|
| 2 min method | 0.017 | 1.567 | 13.108 | 22.678 |
| 6 min method | 0.020 | 1.567 | 12.235 | 24.889 |

Figure 4 Comparison of enzyme activity results between 6 minutes and 2 minutes methods

## 5. Conclusions

- ✓ Measurement of multi enzyme activities in DBS by the on column method (multi-plex assay) was developed, and the analysis time was further reduced to 2 minutes.
- ✓ The 2 minutes method could clearly distinguish enzyme activity between MPS Ⅱ patients and healthy newborns.