SHIMADZU

WP 097

Development of Glycosaminoglycans Analysis Method for Dried blood spot as a Second-tier Test in Mucopolysaccharidoses Screening

Hironori Kobayashi[1,2]; Misa Tanaka[1]; Ryota Okazaki[2]; Tsubasa Oguni[2]; Miki Matsui[1]; Yoshitomo Notsu[2]; Shozo Yano[2]; Akihiro Kunisawa[3]; Michelle Zipse[4]; Takanari Hattori[3]; Jun Watanabe[3]; Shunji Tomatsu[5]; Takeshi Taketani[1]

1) Department of Pediatrics, Shimane University Faculty of Medicine, Shimane, Japan; 2) Laboratories Division, Shimane University Hospital, Shimane, Japan; 3)Shimadzu Corporation, Kyoto, Japan;

4) Shimadzu Scientific Instruments, Columbia, MD; 5)Nemours Children's Health, Delaware Valley, Wilmington, DE

1. Overview

The novel GAG analysis method based on enzyme digestion and LC/MS/MS related to mucopolysaccharidoses was developed and evaluated.

2. Introduction

Mucopolysaccharidoses (MPS) are a group of inborn errors of metabolism caused by the deficiency of lysosomal enzymes to metabolite glycosaminoglycans (GAGs), and undegraded GAGs accumulate systemically in the whole body. In the MPS newborn screening, the enzyme activity measurement is carried out; however, in this assay, the pseudodeficiency (PD) samples are detected, in which the enzyme activity level is low despite not being a patient, resulting in many false positive incidences. Until now, we have demonstrated the usefulness of GAG assay using dried blood spots (DBS) as the second-tier test of MPS I and II. Nevertheless, the conventional method has problems in stability, reproducibility, and cost. In this study, we have aimed to evaluate the newly innovated GAG detection method.

3. Methods

3-1. Sample Preparation

DBS samples were obtained from 100 healthy newborns and 3 MPS II patients. NeoLSD MSMS Kit (PerkinElmer, United States) for identifying MPS II was used as the reference enzyme activity assay to compare with the results of the GAGs assay. GAGs were extracted from 2 DBS punches (3 mm), and the samples were subjected to heating and disaccharification treatment and analyzed; standards (dermatan sulfate (DS), heparan sulfate (HS), keratan sulfate (KS) standard, chondroisin) and GAG-digesting enzymes (chondroitinase B, heparitinase, keratanase) were added. ΔDi-4S was used for an index of DS, ΔDiHS-0S and ΔDiHS-NS for an index of HS, mono-sulfated KS (Gal-GlcNAc(6S)) and di-sulfated KS (Gal(6S)-GlcNAc(6S)) for an index of KS, respectively, and those quantities were measured by LC-MS/MS analysis.

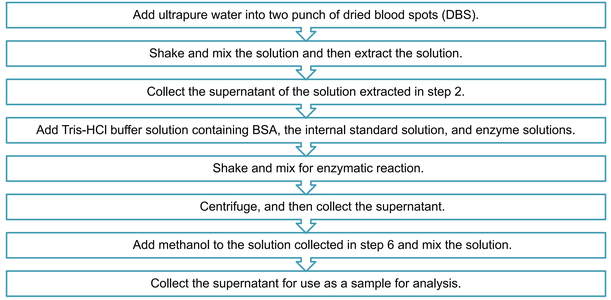

Figure 1. Workflow from analyte extraction from dried blood spot to LC/MS/MS analysis (GAGs assay).

3-2. Analytical Conditions

UHPLC conditions (Nexera™ X3 system)

Column:	HILIC Column (Amide)
Mobile phase:	A: 5 mmol/L Ammonium formate/water
	B: 5 mmol/L Ammonium formate/acetonitrile
Flow rate:	0.3 mL/min
LC Mode:	Gradient mode
Injection vol.:	3 μL
Column temp.:	40°C

MS conditions (LCMS™-8060)

Ionization:	ESI Negative , MRM mode		
DL temp.:	250 °C	HB temp.:	400 °C
Interface temp.:	300 °C	Nebulizing gas:	3.0 L/min
Drying gas:	10 L/min		
Heating gas:	10 L/min		

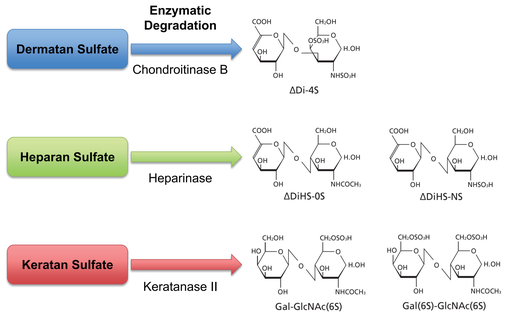

Figure 2. Glycosaminoglycans and Sulfated Disaccharides.

4. Results

4-1. Method development and evaluation

To develop the LC-MS/MS method with the enzyme digestion, MRM conditions for ΔDi-0S, ΔDi-4S, ΔDiHS-0S, ΔDiHS-NS, mono-sulfated KS, di-sulfated KS, and chondrosine were optimized. LC-MS/MS analysis was performed by Nexera™ X3 system coupled with an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). As a result of the optimization, the following MRM transitions were used; m/z 458.0 > 300.1 for ΔDi-4S, m/z 378.0 > 259.2 for ΔDiHS-0S, m/z 415.9 > 138.1 for ΔDiHS-NS, m/z 462.0 > 361.0 for mono-sulfated KS, m/z 270.7 > 220.1 for di-sulfated KS and m/z 354.0 > 193.1 for chondrosine.

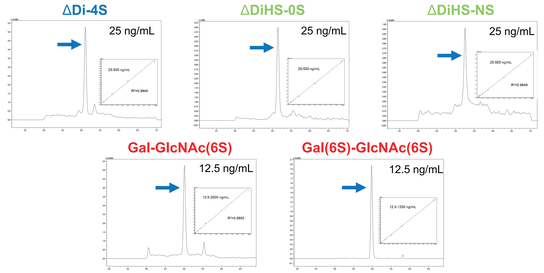

Figure 3. MRM chromatogram of standard solutions.

As shown in Figure 3, highly sensitive and high-resolution analysis was achieved for 5 disaccharides generated from GAGs. The results of the quantitative test and the reproducibility test are shown in Tables 1 and 2, respectively.
These results show that the developed method is suitable for analysis of GAGs.

Table 1. Quantification test results.

	Di-4S	DiHS-0S	DiHS-NS	KS I	KS II
LLOQ (ng/mL)	25	25	25	12.5	12.5
ULOQ (ng/mL)	500	500	500	2500	1250
R^2	0.9949	0.9992	0.9949	0.9950	0.9984

Table 2. Repeatability test results.

	Retention Time %RSD (n=6)				
	Di-4S	DiHS-0S	DiHS-NS	KS I	KS II
Upper limit concentration	0.098	0.099	0.096	0.105	0.050
Lower limit concentration	0.062	0.075	0.057	0.122	0.100
	Peak Area %RSD (n=6)				
	Di-4S	DiHS-0S	DiHS-NS	KS I	KS II
Upper limit concentration	2.19	3.21	3.55	2.54	3.47
Lower limit concentration	6.29	5.33	10.11	4.57	5.10

4-2. Application of novel GAG assay to DBS samples

DBS samples from healthy newborns and MPS II patients were measured using the developed GAG assay. Furthermore, the amount of enzyme activity was measured by enzyme activity assay for the same sample and compared with the GAG assay.

As shown in Table 3, good retention time and area repeatability were obtained even in the real sample.

Table 3. Repeatability test results for real samples.

	Retention Time %RSD (n=10)					
	Di-4S	DiHS-0S	DiHS-NS	DiHS-6S	KS I	KS II
Sample A	0.278	0.238	0.232	-	0.257	0.243
Sample B	0.178	0.188	0.191	0.286	0.142	0.146
Sample C	0.282	0.286	0.273	-	0.242	0.231
	Peak Area %RSD (n=10)					
	Di-4S	DiHS-0S	DiHS-NS	DiHS-6S	KS I	KS II
Sample A	15.73	6.96	18.16	-	2.66	23.38
Sample B	20.13	4.82	20.13	24.30	4.61	12.08
Sample C	15.29	5.45	10.86	-	2.66	14.86

The mean values of healthy newborns of ΔDiHS-0S and ΔDiHS-NS were 220.0 ng/ml and 118.7 ng/ml, respectively. On the other hand, ΔDiHS-0S and ΔDiHS-NS in samples of 3 MPS II patients were 505.06, 353.30, 642.10 ng/ml and 322.89, 353.30, 325.00 ng/ml, respectively. This result demonstrated that HS levels in the MPS II patient group were significantly elevated and that the GAG method made it possible to distinguish between healthy controls and MPS II patients. Therefore, the proposed GAG assay was practicable as the second-tier test of MPS. However, we could not test the PD samples (by enzyme activity assay), which were not available because of a small number of healthy control samples. Efficacy of the two-tier screening method to eliminate false-positive samples could not be proved.

Table 4. Quantitative results obtained by DBS sample analysis.

	ΔDiHS-0S Concentration	ΔDiHS-NS Concentration	Enzyme Activity amount
Healthy control Average	220.0 ng/ml	118.7 ng/ml	14.3259
MPS II Patients	505.06 ng/ml	322.89 ng/ml	0.039
	353.30 ng/ml	353.30 ng/ml	0.047
	642.10 ng/ml	325.00 ng/ml	0.110

5. Conclusions

- We have developed the glycosaminoglycan assays for mucopolysaccharidoses using LC-MS/MS.
- In the LC-MS/MS method based on enzyme digestion, we could analyze 5 disaccharides from GAGs with high sensitivity and high-resolution.
- DBS samples from healthy newborns and MPS II patients were measured, then we demonstrated it is possible to distinguish between healthy controls and MPS II patients.

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