SHIMADZU
Excellence in Science

Application News

No.C139

Liquid Chromatography Mass Spectrometry

Measurement of Enzymatic Activities in Dried Blood Spots with On-Line Solid Phase Extraction-LC/MS/MS System

Lysosomes are a type of intracellular organelle that uses a variety of hydrolytic enzymes to digest waste matter. To measure the enzymatic activity of lysosomes, methods using artificial fluorescent dyes and tandem mass spectrometry are used. Of these methods, tandem mass spectrometry offers the advantage of being able to measure multiple enzymatic activities at the same time.
In this example, a protocol developed at the Meyer Children's Hospital, Mass Spectrometry, Clinical Chemistry and Pharmacology Laboratory (Florence, Italy) was used to measure the enzymatic activity in dried blood spots (DBS) using an online solid phase extraction (SPE) - liquid chromatograph - tandem mass spectrometer (LCMS-8050) system. Because using this system results in samples being cleaned up during SPE, samples can be inserted directly for measurement after enzymatic reaction, without any pretreatment processes.

Sample Preparation and Analytical Conditions

Three enzymes were targeted, alpha-iduronidase (IDUA), acid alpha-glucosidase (GAA), and alpha-galactosidase A (GLA). DBS was used as sample. 3.2 mm diameter disks were punched from the DBSs and placed in a 96-well plate. Then a reaction solution containing respective enzyme substrates and internal standard substance (Genzyme) was added to each well and incubated for 22 hours at 37 ˚C. A flowchart of the preparation process is shown in Fig. 1.
Samples were analyzed using online SPE-LC and LCMS-8050 system. Respective reaction products were measured as the target compounds based on multiple reaction monitoring (MRM) using an internal standard substance. The structures of the target enzymatic reaction products and the internal standards are shown in Fig. 2. MRM transitions are listed in Table 1 and the LC and MS conditions in Table 2.

Online Solid Phase Extraction-Tandem Mass Spectrometer System

The online SPE-LC/MS/MS system configuration is shown in Fig. 3. When the enzymatic reaction was complete, the sample was injected directly and measured. The trapping and cleanup procedure was centered on a Perfusion column POROS® R1 and separation chromatography was performed through a Shim-pack XR-ODS. The two operations are articulated through the following steps. Upon the injection, the sample is cleaned through the Perfusion column with an aqueous solution (solvent A) and delivered by pump A at 1.2 mL/min for 1 min. With the activation of the valve, the Perfusion column is connected in line with the ODS column and both are flowed by 300 µL/min of organic solvent (solvent B). With the switching-back of the valve, occurring at 3 min, the Perfusion column is re-equilibrated with a solvent A and delivered by pump A at 1.2 mL/min for 2.2 min. Using this system eliminates the need for desalting and purification processes.

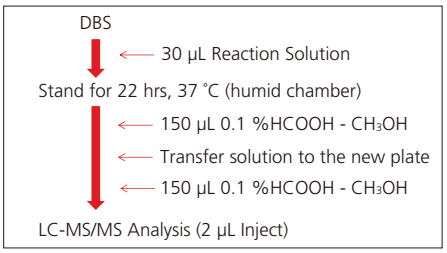

Fig. 1 Preparation Process Flowchart

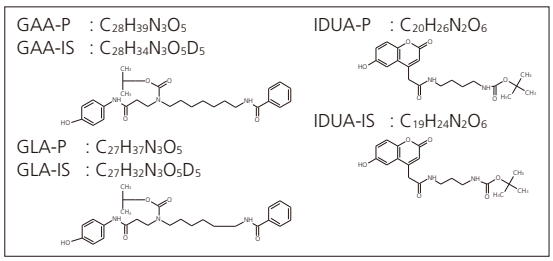

Fig. 2 Structures of Enzymatic Reaction Products and Internal Standards

Table 1 MRM Transitions

Compounds	Polarity	Precursor ion *m/z*	Product ion *m/z*
IDUA-P	+	391.2	291.2
IDUA-IS	+	377.3	277.2
GAA-P	+	498.4	398.3
GAA-IS	+	503.4	403.3
GLA-P	+	484.3	384.3
GLA-IS	+	489.3	389.3

•Note: P: Product, IS: Internal Standard

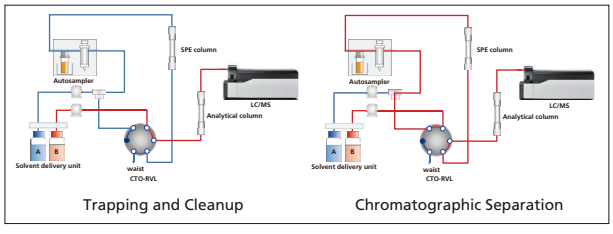

Fig. 3 MRM Chromatograms of Each Target Compound

Table 2 Analytical Conditions

<LC>

Analytical Column	: Shim-pack XR-ODS (75 mm L. × 2.0 mm I.D., 2.2 µm)
Trapping Column	: POROS® R1 (30 mm L. × 2.1 mm I.D., 20 µm)
Solvent A	: 0.05 % HCOOH-5 mM $HCOONH_4$-H_2O
Solvent B	: 0.1 % HCOOH-CH_3OH
Ratio	: 50 %B
Flowrate	: 0.6 mL/min
Oven Temperature	: 30 ˚C
Injection Volume	: 2 µL
Analysis Time	: 5.5 min

<MS>

Instrument	: LCMS-8050
Ionization Mode	: ESI (+)
Interface Temperature	: 100 ˚C
DL Temperature	: 100 ˚C
Heat Block Temperature	: 100 ˚C
Nebulizing Gas Flow	: 3 L/min.
Heating gas Flow	: 5 L/min.
Drying Gas Flow	: 15 L/min.

Measurement Results

The enzymatic activities of IDUA, GLA, and GAA were measured. Examples of enzymatic activity measurement results are shown in Table 3 and MRM chromatograms of each target compound in Fig. 4. Filter paper without any blood was used as blanks. Sample A is a sample that contains enzymes with each activity, Sample B is missing IDUA, Sample C is missing GLA, and Sample D is missing GAA. Whereas peaks were clearly detected for enzyme decomposition products in Sample A, there was a decrease in target peaks in Samples B to D.

Table 3 Example of Enzymatic Activity Measurement Results

	IDUA (μmol/h/L)	GLA (μmol/h/L)	GAA (μmol/h/L)
Blank	0.3	0.1	0.1
Control	15.2	10.1	6.0
Sample A	13.2	6.6	7.3
Sample B	1.8	6.9	5.8
Sample C	17.6	0.5	9.8
Sample D	8.4	2.0	0.8

Activity (μmol/h/L) = [(P/IS) × [IS] × 30/3.4] / 22
· (P/IS): Area ratio between product (P) and internal standard (IS)
· [IS]: Concentration (μmol/L) of internal standard (IS)
· 30: Volume of solution added (μL)
· 3.4: Volume of blood in DBS (μL)
· 22: Reaction time (hr)

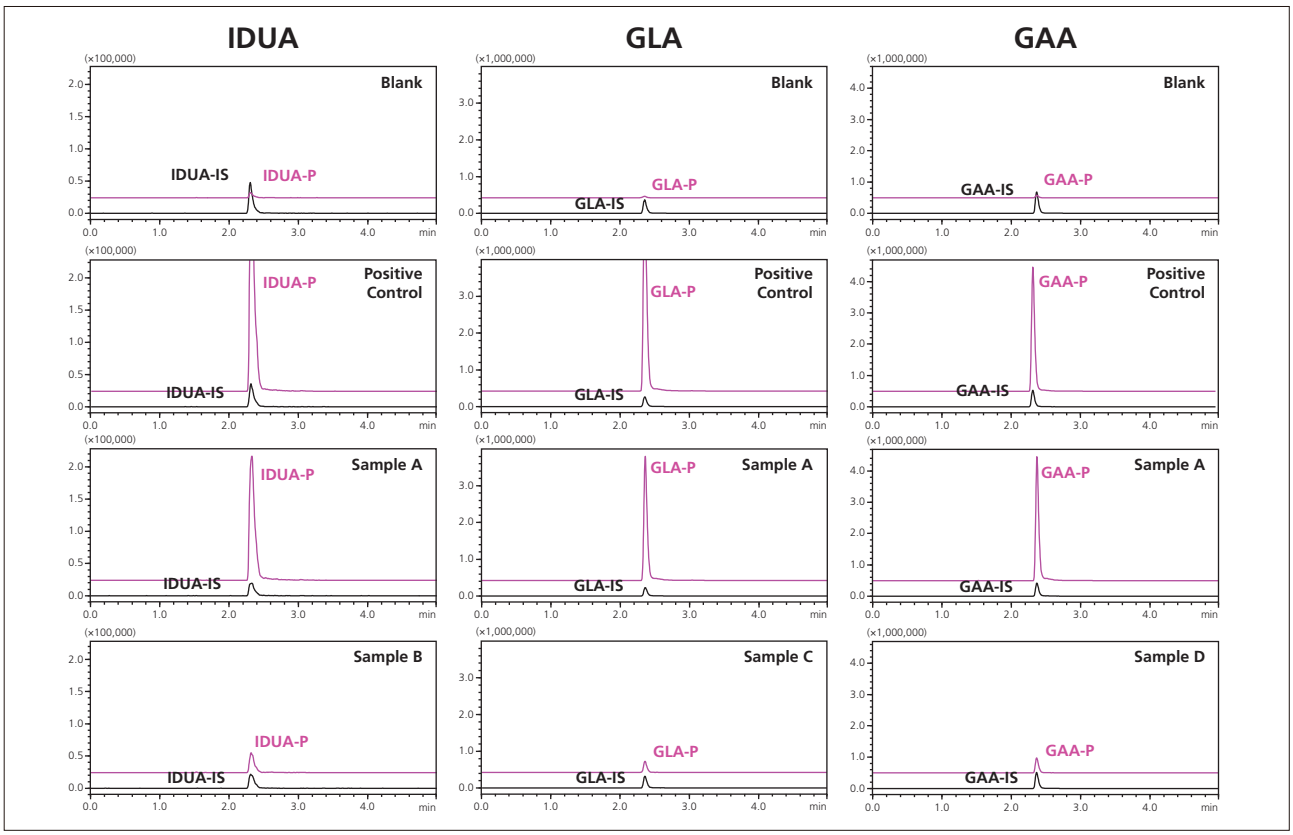

Fig. 4 MRM Chromatograms of Each Target Compound
Blank: Sample filter paper containing no blood; Sample A: Sample containing enzymes with all activities; Sample B: Sample missing IDUA; Sample C: Sample missing GLA; and Sample D: Sample missing GAA

[References]
la Marca G *et al., Anal. Chem.* 81 (2009) 6113-6121
Ombrone D. *et al., Eur. J. Mass Spectrom. 19,* 497-503 (2013)

[Acknowledgments]
This Application News bulletin was prepared based on guidance and samples provided by Dr. G. la Marca (Meyer Children's Hospital, Mass Spectrometry, Clinical Chemistry and Pharmacology Laboratory, Florence, Italy). We are sincerely grateful for his help.

Notes: The equipment mentioned in this article has not been approved/certified as medical equipment under Japan's Pharmaceutical and Medical Device Act.
The analytical methods described in this article cannot be used for diagnostic purposes.

First Edition: Dec. 2016

SHIMADZU
Shimadzu Corporation
www.shimadzu.com/an/