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Short Communication

Quantification of 11 enzyme activities of lysosomal storage disorders using liquid chromatography-tandem mass spectrometry

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A S T R A C T

Lysosomal storage disorders (LSDs) are characterized by the accumulation of lipids, glycolipids, oligosaccharides, mucopolysaccharides, and other biological substances because of the pathogenic deficiency of lysosomal enzymes. Such diseases are rare; thus, a multiplex assay for these disorders is effective for the identification of affected individuals during the presymptomatic period. Previous studies have demonstrated that such assays can be performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) detection. An assay procedure to quantify the activity of 11 enzymes associated with LSDs was provided. First, a validation study was performed using dried blood spot (DBS) samples with 100% and 5% enzyme activity for quality control (QC). Under the assay condition, the analytical range, defined as the ratio of the peak area of the enzyme reaction products from the DBS for QC with 100% enzyme activity to that from the filter paper blank sample, was between 14 for GALN and 4561 for GLA. Based on these results, the distribution of the enzyme activity for the 11 LSD enzymes was further examined. Consistent with the previous data, the enzyme activity exhibited a bell-shaped distribution with a single peak. The averaged enzyme activity for the healthy neonates was as follows: GLA, 3.80 ± 1.6; GAA, 10.6 ± 4.8; IDUA, 6.4 ± 2.3; ABG, 8.6 ± 3.1; ASM, 3.3 ± 1.1; GALC, 2.8 ± 1.3; ID2S, 16.7 ± 6.1; GALN, 1.2 ± 0.5; ARSB, 17.0 ± 8.7; NAGLU, 4.6 ± 1.5; and GUSB, 46.6 ± 19.0 μmol/h/L (mean ± SD, n = 200). In contrast, the enzyme activity in disease-affected individuals was lower than the minimum enzyme activity in healthy neonates. The results demonstrate that the population of disease-affected individuals was distinguished from that of healthy individuals by the use of LC-MS/MS.

1. Introduction

Lysosomal storage disorders (LSDs) are characterized by the accumulation of biological substances, such as glycolipids, lipids, oligosaccharides, and mucopolysaccharides, in the lysosomes as a result of the pathogenic defects of the lysosomal enzymes [1,2]. The accumulating evidence suggests that the efficacy of existing therapies for the treatment of LSDs is related to newborn screening [3,4]. The best known example includes a study of Pompe disease in Taiwan, which showed that the survival rate was significantly improved without ventilator use [5]. In this case, the treatment was initiated during the presymptomatic period immediately after newborn screening. Among the currently available therapies, enzyme replacement therapy (ERT) has been widely accepted as an effective treatment option. It is known that several LSDs, including Pompe disease; Fabry disease; mucopolysaccharidosis (MPS) I, II, IVA, and VI; and Gaucher disease can be treated with ERT [2].

Mass spectrometry (MS)-based LSD assays were first reported by Dr. M.H. Gelb at the University of Washington [6,7]. The principle of this method is the inclusion of an internal standard (IS) for each assay to quantify the accumulation of enzyme reaction products using tandem mass spectrometry (MS/MS) with multiple reaction monitoring (MRM). The IS for each enzyme reaction was synthesized using deuterium-labeled compounds, leading to the higher accuracy of the quantitative results [8]. Based on this, this technique has been significantly improved over the more than one decade that it has been studied. First, the substrates for enzyme assay are synthetic thus non-endogenously existing, leading to the accurate quantification of elevating enzyme reaction products with minimal background. Secondly, this MRM-based assay can perform several assay in a single buffer while each of these assay needs to be performed individually using fluorescence-active substrate [9]. Thirdly, to increase the number of enzymes, an initial study established an assay for 5-plex LSD enzymes, including α-glucosidase (GAA) for Pompe disease, α-galactosidase A (GLA) for Fabry disease, acid β-glucosidase (ABG) for Gaucher disease, acid sphingomyelinase (ASM) for Niemann-Pick disease type A/B, and galactosylcerebrosidase (GALC) for Krabbe disease [7], followed by the inclusion of α-L-iduronidase (IDUA) for mucopolysaccharidosis (MPS) I in a subsequent study [10]. Finally, to accommodate the large number of DBS samples under current NBS platform, the assay was performed using flow-injection analysis under high-throughput assay conditions (8). Based on these innovations, these reagents were used for several pilot studies reported in Austria [11], Taiwan [12], and the United States (U.S.) [10]. Based on these studies, NBS for LSD has been implemented in Taiwan [12] and some state in the U.S. [13].

Because the number of new therapies has been emerging based on

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new technologies, the number of assay reagents for LSD enzyme activity has also been growing. Assay reagents for iduronate-2-sulfatase (ID2S) for MPS II, *N*-acetylgalactosamine-6-sulfatase (ARSB) for MPS IVA, *N*-acetylgalactosamine-4-sulfatase (GALN) for MPS VI, *N*-acetyl-α-D-glucosaminidase (NAGLU) for MPS IIIB, and β-glucuronidase (GUSB) for MPS VII [14], as well as lysosomal acid lipase and biotinidase for the associated deficiencies [15,16] were developed in previous studies. These enzyme activities can be quantified with a combination of enzymes. For example, a previous study reported that the enzyme activities for the above-mentioned 6 LSDs were quantified with ID2S, ARSB, and GALN using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [17]. Subsequently, the same group developed another 6-plex assay for ID2S, ARSB, GALN, NAGLU, GUSB, and tripeptidyl peptidase 1 [14]. Previous studies have reported that enzyme assays for ID2S, ARSB, GALN, NAGLU, GUSB were performed using LC-MS/MS [14,17]. One reason for this choice is the accumulation of artifactually produced heat-labile compounds from the enzyme substrate during MS detection [14,17]. For the accurate quantification of the enzyme activity, these peaks should be minimized either by chromatographic separation or optimizing MS detection. Although this assay lasts 2 min, this method can be used for NBS. The assay time in this study was close to that in the recently published assay procedure that targets NBS [14,16,17]. A chromatographic condition that enables the quantification of enzyme activity for ID2S, ARSB, and GALN aiming for high throughput quantification was reported in a previous study [18]. The current study has extended that previous study on the multiplex quantification of LSD enzymes involving GAA, GLA, IDUA, ABG, ASM, GALC, NAGLU, and GUSB by using LC-MS/MS.

2. Experimental procedures

2.1. Materials

The reagents required for the 6-plex LSD enzyme assay for the GAA, GLA, IDUA, ABG, ASM, and GALC were purchased from PerkinElmer (Waltham, MA). The reagents for the ID2S, GALN, and ARSB were purchased from PerkinElmer under a custom manufacturing agreement. The reagents for the NAGLU and GUSB were provided by Prof. Michael H. Gelb (University of Washington, WA). The *N*-Acetylglucosamine thiazoline (NAG-thiazoline) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). The acetonitrile was purchased from Thermo Fischer Scientific (Tokyo, Japan). The deionized water was obtained from a Milli-Q water system (Millipore, Milford, MA). The formic acid was purchased from Kanto Chemical (Tokyo, Japan). A set of DBS samples with high (100%) and low (5%) activity was provided by PerkinElmer for QC. The other reagents used in this study were of the highest commercially available grade.

2.2. Approval by institutional research ethics board

This study was approved by the Research Ethics Board of the National Center for Child Health and Development.

2.3. DBS

The DBS samples were stored at −20 °C according to previous procedures [19]. One untreated MPS I-affected individual, four MPS II-affected individuals with ERT, one untreated MPS IIIB-affected individual, two untreated MPS IVA-affected individuals, and two MPS VI-affected individuals with ERT were examined. We used newborn specimens for all controls.

2.4. Reaction-for GAA, GLA, IDUA, ABG, ASM, and GALC (Plate A)

The assay cocktail contained the known concentrations of substrate for the GAA for Pompe disease (0.35 mM), the GLA for Fabry disease (1.2 mM), the IDUA for MPS I (0.25 mM), the ABG for Gaucher disease (0.5 mM), the ASM for Niemann-Pick disease type A/B (0.75 mM), and the GALC for Krabbe disease (0.85 mM) and of IS for the GAA (24 μM), GLA (24 μM), IDUA (15 μM), ABG (20 μM), ASM (15 μM), and GALC (10 μM) [20,21]. All of the assays were carried out with a 3-mm punch in 30 μL of assay cocktail in a polypropylene 96-well plate (#260252, Thermo Fisher Scientific, Tokyo) and incubated at 37 °C for 20 h. To terminate this enzyme reaction, a mixture of methanol/ethyl acetate (50/50, 100 μL) was added. Next, to extract the enzyme reaction products, ethyl acetate (400 μL) and 0.5 M sodium chloride (200 μL) were added and mixed vigorously using a pipette. After the centrifugation of these plates at 700 ×*g* for 5 min at room temperature using a plate centrifuge (model PlateSpinII, Kubota, Tokyo, Japan), an aliquot of the organic layer (200 μL) was transferred to a fresh 96-well plate.

2.5. Reaction for ID2S, ARSB, GALN (Plate B)

The assay cocktail contained the known concentrations of substrate for the ID2S for MPS II (0.5 mM), the GALN for MPS IVA (1 mM), and the ARSB for MPS VI (1 mM) and of IS for the ID2S (5 μM), GALN (5 μM), and ARSB (5 μM) [18]. The enzyme reactions were performed in 50 mM ammonium acetate (pH 5.0) containing 7.5 mM barium acetate, 5 mM cerium acetate, and 2 mM (*Z*)-Pugnac. All of the assays were carried out with a 3-mm punch in 30 μL of the assay cocktail in a polypropylene 96-well plate and incubated at 37 °C for 20 h. To terminate this enzyme reaction, a mixture of methanol/ ethyl acetate (50/50, 100 μL) was added. Next, to extract the enzyme reaction products, ethyl acetate (400 μL) and 0.5 M sodium chloride (200 μL) were added and mixed vigorously using a pipette. After the centrifugation of these plates at 700 ×*g* for 5 min at room temperature using a plate centrifuge, an aliquot of the organic layer (200 μL) was transferred to a fresh 96-well plate.

2.6. Reaction for NAGLU and GUSB (Plate C)

The assay cocktail contained the known concentrations of substrate for the NAGLU for MPS IIIB (0.5 mM) and the GUSB for MPS VII (0.5 mM) and of IS for the NAGLU (10 μM) and GUSB (10 μM) [14]. The enzyme reactions were performed in 50 mM ammonium acetate (pH 5.0) containing 7.5 mM barium acetate, 5 mM cerium acetate, and 0.1 mM NAG-thiazoline (Toronto Research Chemicals, Ontario, Canada) [14]. All of the assays were carried out with a 3-mm punch in 30 μL of assay cocktail in a polypropylene 96-well plate (#260252, Thermo Fisher Scientific, Tokyo) and incubated at 37 °C for 20 h. To terminate this reaction mixture, a mixture of methanol/ethyl acetate (50/50, 100 μL) was added. Next, to extract the enzyme reaction products, ethyl acetate (400 μL) and 0.5 M sodium chloride (200 μL) were added and mixed vigorously using a pipette. After the centrifugation of these plates at 700 ×*g* for 5 min at room temperature using a plate centrifuge, an aliquot of the organic layer (200 μL) was transferred to a fresh 96-well plate.

2.7. Reconstitution of enzyme reaction products for 11-plex complete assay and 6/3/2-plex separate assay

For the 11-plex assay, an aliquot of the ethyl acetate layer from Plates A (200 μL), B (200 μL), and C (200 μL) were combined in a deep-well plate. Next, the solvent of the extracts was removed under a nitrogen stream. Finally, the enzyme reaction products were reconstituted with a reconstitution solvent (acetonitrile/water/formic acid = 80/20/0.1, 300 μL). In this study, it was estimated that a 3-mm DBS punch contained 3.1 μL of whole blood. Enzyme activity was calculated in μmol/h/L blood as was previously reported [22].