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Short Communication

Levels of enzyme activities in six lysosomal storage diseases in Japanese neonates determined by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Lysosomal storage disorders (LSDs) are caused by defective enzyme activities in lysosomes, characterized by the accumulation of glycolipids, oligosaccharides, mucopolysaccharides, sphingolipids, and other biological substances. Accumulating evidence has suggested that early detection of individuals with LSDs, followed by the immediate initiation of appropriate therapy during the presymptomatic period, usually results in better therapeutic outcomes. The activities of individual enzymes are measured using fluorescent substrates. However, the simultaneous determination of multiple enzyme activities has been awaited in neonatal screening of LSDs because the prevalence of individual LSDs is rare. In this study, the activities of six enzymes associated with LSDs were examined with 6-plex enzyme assay using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The accumulation of enzyme products was almost linear for 0–20 h at 37 °C. Dried blood spots (DBSs) provided by the Centers for Disease Control and Prevention (CDC) were used for quality control (QC). The intraday and interday coefficient of variance values were <25%. The enzyme activities of healthy individuals were higher than those of LSD-confirmed individuals. These results suggest that the levels of enzyme activities of six LSDs in a Japanese population were comparable to those of a recent report [Elliott et al. Mol Genet Metab 118 (2016) 304–309], providing additional evidence that the 6-plex LSD enzyme assay is a reproducible analytical procedure for neonatal screening.

1. Introduction

Lysosomal storage disorders (LSDs) are a group of congenital metabolic disorders caused by the accumulation of glycolipids, oligosaccharides, mucopolysaccharides, sphingolipids, and other biological substances induced by the defective activity of lysosomal enzymes [1–2]. Lysosomal enzymes are found in nearly all mammalian cells. Thus, lysosomal enzyme deficiency can lead to systemic manifestations of LSD symptoms. As regards therapy, a prior study demonstrated the efficacy of hematopoietic stem cell transplantation [3]. In this therapy, the wild-type enzyme in the transplanted hematopoietic cells is delivered to affected cells via a mechanism called cross-correction [4]. Enzyme replacement therapy plays an important role in the treatment of several LSDs, such as Pompe, Fabry, and Gaucher disease and mucopolysaccharidosis (MPS) I, II, and VI [5]. Substrate reduction therapy is also used to treat Gaucher disease [6].

Neonatal screening of six LSDs involving Pompe, Fabry, MPS I, Gaucher, Krabbe, and Niemann–Pick disease type A/B has attracted much attention [7]. Pompe disease is associated with defective α-glucosidase (GAA) activity, leading to muscle weakness [8]. Fabry disease is an X-linked disorder caused by a deficiency of α-galactosidase A (GLA), resulting in the accumulation of glycosphingolipids in biological fluids and tissues [9]. MPS I is linked to a deficiency of α-L-iduronidase (IDUA) [9]. There are three disease subtypes based on the phenotype: Hurler (severe), Hurler–Scheie (intermediate), and Scheie (mild) [10]. Gaucher disease is characterized by an accumulation of galactosylceramide due to a glucocerebrosidase (ABG) defect [11]. Krabbe disease leads to severe neurological manifestations and is linked to a galactosylceramidase (GALC) deficiency [11]. Currently, hematopoietic cell transplant therapy is used to treat Krabbe disease [3]. Niemann–Pick disease type A/B is caused by a deficiency of acid sphingomyelinase (ASM) [12]. Enzyme replacement therapy for the disease is currently under development (ClinicalTrials.gov: NCT01722526) [12].

To identify individuals with LSDs during the presymptomatic period, neonatal screening for LSDs has been performed [13–15]. In addition to neonatal screening, increasing evidence indicates that liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based technology may be superior to previously performed assays for LSDs (Reviewed in [7]). Furthermore, accumulating evidence

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suggests that the MS/MS-based method seems to be feasible for neonatal screening [16–20]. In this study, we examined the ability of LC-MS/MS to detect 6-plex LSD enzyme activity and applied the methodology to random Japanese neonates.

2. Experimental procedure

2.1. Reagents

The substrates and internal standards for GAA, GLA, IDUA, ABG, ASM, and GALC were purchased from PerkinElmer (Waltham, MA). Acetonitrile and methanol were purchased from Fischer Scientific (Tokyo, Japan). Isopropanol was purchased from Wako Pure Chemicals (Tokyo, Japan). Deionized water was obtained through a Milli-Q water system from Millipore (Milford, MA). Formic acid was purchased from Kanto Chemical (Tokyo, Japan). The other reagents used in this study were of the highest grade commercially available.

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. Dried blood spot (DBS) specimens for quality control (QC)

The DBSs for QC were kindly provided by Dr. Hui Zhou at the Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention (CDC) (Atlanta, GA) [21].

2.4. Determination of enzyme activities of six LSDs by LC-MS/MS

The preparation and analysis of the enzyme activities of the six LSDs by LC-MS/MS has been previously reported [22]. In brief, the enzymes were extracted from the DBSs (3 mm in diameter) using a punch and reacted with substrate in a buffer (30 μL) for 20 h at 37 °C in a 96-well plate. The concentrations of the substrates and internal standards were as follows: GAA, 0.35 mM, 24 μM; GLA, 1.2 mM, 24 μM; IDUA, 0.25 mM, 15 μM; ABG, 0.5 mM, 20 μM; ASM, 0.75 mM, 15 μM; and GALC, 0.85 mM, 10 μM. To terminate the reaction, a mixture of ethyl acetate/methanol (50/50, 100 μL) was added. This reaction mixture was then transferred to a 96-well deep plate, and ethyl acetate (400 μL) and water (200 μL) were added. After mixing and centrifugation, the supernatant (75 μL) was transferred to a 96-well shallow plate. This organic solution was then dried under an N_2 stream, reconstituted with the mobile phase (150 μL, H_2O/CH_3CN/formic acid in a 20/80/0.002 ratio), and analyzed using an LC-MS/MS equipped with a Quattro Premier mass spectrometer and an ACQUITY ultra-high performance liquid chromatograph (Waters, Milford, MA) system. The activity of each enzyme was determined by the accumulation of the reaction product using the corresponding internal standard in μmol/h/L of blood, where each 3-mm DBS punch contained 3 μL of blood. The following analytical columns were tested: an ACQUITY BEH C18 (1.7 μm, 100 × 2.1 mm) from Waters, a Chromolith RP-2 (3 μm, 100 × 2.1 mm) from Merck-Millipore (Tokyo, Japan), and a MonoTower C18 (3 μm, 100 × 2.1 mm) from GL Sciences (Tokyo, Japan). Multiple-reaction monitoring (MRM) was used for the quantitation of enzyme reaction products. Details of the methods are available in Supplementary Tables 1–4.

2.5. Determination of GAA enzyme activity using a fluorometric substrate

The measurement of GAA enzyme activity was performed using 4-methylumbelliferone-labeled substrate, as reported previously [15]. In brief, the GAA enzyme was extracted overnight from the 3-mm DBS punch, and the aliquot was reacted with the enzyme substrate in the presence of acarbose at 37 °C for 20 h. The reaction was then terminated by the addition of 150 mM EDTA solution (pH 11.3–12.0). Finally, the accumulation of reaction products was determined using an ARVO fluorometer (PerkinElmer) (λex = 355 nm, λem = 460 nm).

3. Results

The LSD assay was validated in terms of (1) chromatographic separation of the enzyme reaction products using several commercially available reversed-phase columns, (2) QC validation using CDC-provided QC DBSs, (3) analysis of the enzyme activity in random neonates and LSD-confirmed individuals in a Japanese population, and (4) correlation of the activity of the GAA enzyme using LC-MS/MS and fluorometric methods.

3.1. Chromatographic separation

First, the chromatographic separation of the reaction products of the 6-plex LSD assay system was examined. Three commercially available analytical columns were tested: a silica-based conventional BEH C18 column (Waters) that is widely available worldwide and two silica-based monolith columns compatible with higher flow rates (a Chromolith RP-2 column [Merck-Millipore] and a MonoTower C18 column [GL Sciences]). Overall, the chromatographic properties of the three columns were almost the same. A BEH C18 column was used in a subsequent study of this assay due to its commercial availability, as the enzyme reaction products of GAA, GLA, IDUA, ABG, ASM, and GALC were readily detected under the analytical conditions tested (Fig. 1A). The peaks of the enzyme reaction products, indicated by arrows in Fig. 1A, were baseline separated from the corresponding in-source degraded compounds of the substrates of GAA, GLA, ABG, ASM, and GALC, leading to minimal inaccuracy in the measurement of the enzyme reaction products. The other two columns showed similar chromatographic behavior. The only difference in separation among the three columns was that the peak of substrate and internal standard for GALC migrated before those for ABG in the MonoTower C18 column (Supplementary Table 4). The accumulation of the enzyme reaction products of the six enzymes was almost linear over 20 h at 37 °C (Fig. 1B).

3.2. Validation of the assay using CDC-provided QC DBSs

Second, the 6-plex LSD assay was validated using QC DBSs provided by the CDC [21]. For this purpose, the enzyme activities of the six LSDs were determined in high/middle/low/baseline QC DBSs containing 100, 50, 5, and 0% control enzyme activity, respectively. As shown in Fig. 2A, the linear correlation between the measured enzyme activity and nominal enzyme content in the QC DBSs was acceptable. Notably, we regularly obtained a good linear correlation (i.e. $R^2 > 0.95$), which is within the acceptable level in clinical laboratory medicine. The intra-day and interday coefficient of variation values for the high and middle CDC-provided QC DBSs were within 25% when assessed using the BEH C18 column (Table 1). On average, the activity of the enzymes in the CDC-provided QC DBSs by our measurement was 15.3 μmol/h/L for GAA (92% of the reported enzyme activity in CDC analytical information), 9.9 μmol/h/L for GLA (105%), 5.8 μmol/h/L for IDUA (43%), 8.4 μmol/h/L for ABG (74%), 1.7 μmol/h/L for ASM (56%), and 4.6 μmol/h/L for GALC (86%), respectively ($n = 6$). To ensure that the each measured enzyme activity derived from a single adult donor does not depend on the position in a 96-well plate, the enzyme activities in 24 different wells in the plate were examined. Overall, the difference in enzyme activities of the six LSDs from mean value was within 20% (Supplementary Fig. 1). The linearity of enzyme activity in the QC DBSs and intraday and interday coefficient variation values were also measured using the Chromolith column and found these were similar (data not shown; see Supplementary Table 5).