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Short Communication

Enzyme activities of α-glucosidase in Japanese neonates with pseudodeficiency alleles

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A B S T R A C T

Lysosomal storage disorders (LSDs) are caused by defective enzyme activities in lysosomes, characterized by the accumulation of sphingolipids, glycolipids, oligosaccharides, mucopolysaccharides, the oxidation products of cholesterol, and other biological substances. A growing number of clinical studies have suggested the enhanced efficacy of existing therapies, including enzyme replacement therapy, which is effective when it is initiated during the presymptomatic period. Thus, the identification of disease-affected individuals by newborn screening has been considered an effective platform. Previous studies have suggested that the discrimination of infantile-onset Pompe disease (IOPD) requires multi-step examination of GAA enzyme activity using the fluorometric technique. In sharp contrast, the MS/MS-based technique can identify the population of IOPD and the pseudodeficiency alleles of the GAA enzyme [Liao HC et al. Clin Chem (2017) in press; doi: http://dx.doi.org/10.1373/clinchem.2016.269027]. To determine whether MS/MS-based assay can identify these two populations in Japanese neonates, we first performed a validation study of this assay using flow-injection analysis (FIA)-MS/MS and liquid chromatography (LC)-MS/MS followed by examination of GAA enzyme activity in our population. By minimizing the effect of substrate-derived in-source decomposition products, the activities of 6 LSD enzymes were quantified in FIA-MS/MS and LC-MS/MS. The mean value of GAA activity with IOPD, pseudodeficiency alleles, and healthy controls by FIA-MS/MS were 1.0 ± 0.3 μmol/h/L (max, 1.3; min, 0.7; median, 1.2; n = 3), 2.7 ± 0.7 μmol/h/L (max, 4.5; min, 1.5; median, 2.5; n = 19), and 12.9 ± 5.4 μmol/h/L (max, 29.6; min, 2.5; median, 11.0; n = 83), respectively. These results suggest that the population of GAA with pseudodeficiency alleles has approximately 20% of GAA enzyme activity compared to controls, providing the preliminary evidence to estimate the cut-off values in the Japanese population using this technique.

1. Introduction

Lysosomal storage disorders (LSDs) are caused by defective enzyme activities in lysosomes, characterized by the accumulation of oligosaccharides, glycolipids, mucopolysaccharides, sphingolipids, the oxidation products of cholesterol, and other biological substances [1–2]. Although the prevalence of these diseases is rare, many revolutionary therapies have been developed. Due to its high effectiveness, enzyme replacement therapy is one of the most well-appreciated treatments for these disorders. Accumulating evidence has suggested that the treatment for LSDs has, in general, maximal benefit to the disease-affected individuals. To achieve this benefit, newborn screening for LSD has been considered a promising platform [3].

A tandem mass spectrometry (MS/MS)-based enzyme assay for LSDs was first reported in 2004 ([4], reviewed in [5]). Since then, several newborn screening programs have been performed based on this assay [6–8]. The advantage of this method is strongly associated with the inclusion of individual, internal standards for multiple enzymes in each assay reaction, which enhances the assay's accuracy dramatically. Furthermore, this MS/MS-based assay usually gives lower background compared to fluorometric assay because the accumulating enzyme reaction product of each reaction can be selectively quantified using the mixed reaction monitoring mode. These two advantages provide MS/MS-based assay technique with a wider range of enzyme activity quantification. The analytical range is a measure defined as the ratio of enzyme activity of healthy controls to that of blank [9]. This analytical range is closely associated with the lowest limit of enzyme activity quantification [8–10]. The analytical ranges for the MS/MS-based method are normally 3- to 10-fold higher than those of fluorometric assay, suggesting that the disease-affected population can be directly identified by enzyme activity using a DBS, rather than a leukocyte concentrate. Based on the abovementioned advantage, the number of diseases by which enzyme activity can be quantified using MS/MS-based methodology is now expanding [9].

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In Pompe disease, at least two types of phenotype have been established [11]. One is an infantile-onset Pompe disease (IOPD), which limits the life expectancy of the affected individual by two years when the appropriate treatment has not been provided. The major manifestations include cardiomegaly, hypotonia, muscular weakness, and hepatomegaly. The other phenotype is late-onset Pompe disease (LOPD), which is usually recognized in adults because of the elevated enzyme activity of creatinine kinase [12,13]. For newborn screening, the population of IOPD must be identified from that of healthy subjects. It is known that there is a minor, but distinct sub-population with low enzyme activity associated with the pseudodeficiency alleles of the c.1726G > A (p.G576S) mutation of α-glucosidase (GAA) [14,15]. This mutation has been found in the populations of Asian countries such as Japan and Taiwan. Overall, the mutation occurs in ~3% of the total Asian population [14,16,17]. Importantly, a recent study clearly demonstrated that a population with IOPD/LOPD has approximately 1% or even less enzyme activity when the MS/MS-based method was employed, whereas the sub-population of the GAA pseudodeficiency alleles has nearly 5–10% enzyme activity [18]. In this study, we first validated this assay using flow-injection analysis (FIA)-MS/MS and liquid chromatography (LC)-MS/MS, respectively. Then, we further demonstrated the levels of GAA enzyme activity in populations with IOPD and the GAA pseudodeficiency alleles using this technique.

2. Experimental procedure

2.1. Reagents

The substrates and internal standards for α-glucosidase (GAA), α-galactosidase A (GLA), α-L-iduronidase (IDUA), glucocerebrosidase (ABG), acid sphingomyelinase (ASM), and galactosylceramidase (GALC) were purchased from PerkinElmer (Waltham, MA). Acetonitrile and methanol were purchased from Fischer Scientific (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). Ammonium acetate and ethyl acetate were purchased from Wako Pure Chemicals (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 (Tokyo, Japan).

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

The DBSs for QC were kindly provided by Dr. Jonathan Rehnberg at Diagnostics Division, PerkinElmer (Turku, Finland) and Dr. Anna Potier at Diagnostics Division, PerkinElmer (Waltham, MA).

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

The preparation of enzyme reaction used to determine 6 LSD enzyme activities has been previously reported [19]. In brief, the enzymes were extracted from the DBSs (3 mm in diameter) using an automated puncher (model 1296–071 DELFIA® Dried blood Spot Punch, PerkinElmer) and reacted with substrates 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 and reconstituted with the mobile phase (150 μL, H_2O/CH_3CN/formic acid = 20/80/0.002).

2.5. Analytical procedure

Flow-injection analysis (FIA)-MS/MS: The sample solution was delivered into a LCMS8030plus MS/MS spectrometer (Shimadzu, Kyoto, Japan) using an HPLC system Nexera (Shimadzu) at a flow rate of 0.1 mL/min. Typically, an aliquot (1–5 μL) was injected onto FIA-MS/MS. MS/MS conditions were optimized, as described in Supplementary Procedure.

Liquid chromatography (LC)-MS/MS: The levels of enzyme reaction products were analyzed using the same LCMS8030plus mass spectrometer and Nexera HPLC system. We used a MonoTower C18 (3 × 50 mm) or an InertSustainSwift C18 (2.1 × 30 mm, 3 μm) analytical column purchased from GL Sciences (Tokyo, Japan). Details of instrumental parameters are available in Supplementary Tables 1–5.

2.6. Determination of enzyme activities in DBSs

The activity of each enzyme was determined by examining the accumulation of the reaction product using the corresponding internal standard in μmol/h/L of blood, where each 3-mm DBS punch contained 3.1 μL of blood. Both the positive mode of electrospray ionization and multiple-reaction monitoring mode were used to quantify the enzyme reaction products.

2.7. Identification of GAA c.1726G > A alleles

GAA enzyme activity was screened using 4MU as a substrate in the presence of acarbose [15]. Then, the specimen with low enzyme activity was further examined through the PCR-based technique using the sequences 5′-AGG GAG GGC ACC TTG GAG CCT G-3′ and 5′-GGG AGG CGA TGG CTT CGG TCA AG-3′ as the forward and reverse primers, respectively [15]. All individuals with pseudodeficiency alleles of GAA in this study had homozygous c.1726G > A alleles.

3. Results

To validate GAA assay using FIA-MS/MS- and LC-MS/MS-based protocols, we first examined the CV values of GAA activity for intraday

Table 1
Intraday and interday assay precision for multiple analyses of control samples.

Method	Mode of elution	Solvent	Intraday CV (%)							Interday CV (%)						
			GAA	GLA	IDUA	ABG	ASM	GALC	*n*	GAA	GLA	IDUA	ABG	ASM	GALC	*n*
FIA-MS/MS	Isocratic	CH_3CN	9.1	24.7	21.6	1.5	2.5	3.0	5	14.4	14.2	19.0	14.5	19.9	18.0	4
FIA-MS/MS	Isocratic	CH_3OH	4.5	9.0	15.2	2.9	5.6	2.2	5	8.8	12.9	24.6	12.6	18.9	12.4	4
LC-MS/MS	Isocratic	CH_3CN	3.8	3.4	6.1	2.4	0.8	2.4	5	11.9	16.6	13.7	24.0	27.8	16.5	4
LC-MS/MS	Gradient	CH_3OH	8.4	2.8	7.6	7.8	7.3	5.6	5	13.9	19.1	19.8	10.6	6.0	12.3	4

CH_3CN, acetonitrile; CH_3OH, methanol; CV, coefficient of variation.