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Brief Communication

Improved sensitivity of an acid sphingomyelinase activity assay using a C6:0 sphingomyelin substrate

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ABSTRACT

Short-chain C6-sphingomyelin is an artificial substrate that was used in an acid sphingomyelinase activity assay for a pilot screening study of patients with Niemann–Pick disease types A and B. Using previously published multiplex and single assay conditions, normal acid sphingomyelinase activity levels (i.e. false negative results) were observed in two sisters with Niemann–Pick B who were compound heterozygotes for two missense mutations, p.C92W and p.P184L, in the *SMPD1* gene. Increasing the sodium taurocholate detergent concentration in the assay buffer lowered the activity levels of these two patients into the range observed with other patients with clear separation from normal controls.

1. Introduction

Acid sphingomyelinase (ASM) deficiency is a lysosomal storage disorder that historically is known as types A and B Niemann–Pick disease (NPD). ASM deficiency can result in a broad clinical spectrum, ranging from an early infantile-onset disease with hepatosplenomegaly, failure to thrive, and neurodegeneration that leads to death usually by age 3 (type A) to a childhood or adult-onset disease with a heterogeneous phenotype that includes hepatosplenomegaly, infiltrative lung disease, and liver disease with survival generally into adulthood (type B) [1]. Individuals with this disorder inherit two mutations in acid lysosomal sphingomyelin phosphodiesterase 1(*SMPD1*), which is located within a cluster of imprinted genes on chromosome 11p15.5 [2].

Over the years, a significant amount of effort has been dedicated to developing an ASM activity assay for the screening and diagnosis of NPD A and B. Early approaches employed artificial chromogenic or fluorogenic substrates, such as 2-hexadecanoylamino-4-nitrophenylphosphorylcholine (HDA-PC) and 6-hexadecanoylamino-4-methylumbelliferylphosphorylcholine (HMU-PC), to measure ASM activity [3]. However, a potential pitfall of such assays is a false negative result. For example, Harzer et al. found that NPD B patients with the p.Q292K mutation showed normal ASM activity using HDA-PC or HMU-PC as the substrate [4,5]. Currently, a mass spectrometry-based multiplex assay for measuring lysosomal enzyme activities has been used in multiple pilot population screenings for NPD A/B, Gaucher, Pompe, Fabry, and Krabbe and mucopolysaccharidosis type I diseases [6,7]. Short-chain sphingomyelin, C6:0 sphingomyelin (C6:0-SPM), is the artificial substrate used in the assay to measure ASM activity [8,9]. Recently we have experienced additional missense mutations in ASM gene giving rise to falsely normal ASM activity levels. Multiple detergents and their concentrations have been optimized to reduce the false negative rate, thereby increasing the sensitivity of the ASM activity assay.

2. Material and methods

A mixture of lyophilized C6:0-SPM substrate (ASM-S) and C4:0 ceramide internal standard (ASM-IS) was obtained from the Center for Disease Control and Prevention (CDC, Atlanta, Georgia). Sodium acetate, sodium taurocholate and zinc chloride were obtained from Sigma (St. Louis, MO). HPLC grade water, methanol, acetonitrile, and ethyl acetate were all procured from Honeywell Burdick and Jackson (Muskegon, MI). Whole blood from 34 NPD patients (2 type A, 31 type B, 1 type A or B) with a previously confirmed clinical diagnosis was obtained following informed consent. Whole blood from normal donors ($n = 136$) was purchased from ProMedDx, LLC (Norton, MA). Venous blood was drawn into Vacutainer® tubes containing EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ), shipped on cold packs overnight, and held at 4 °C. Within 48 h of collection, dried blood spot (DBS) samples were prepared by inverting the blood tubes several times, spotting 75 μL of blood onto Whatman 903® specimen collection paper, and drying at room temperature for at least 4 h. The DBS cards were stored in sealed plastic bags at −20 °C or below with desiccant and a humidity indicator card until analysis.

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The vial containing the ASM-S and -IS mixture was reconstituted in 18 mL of methanol, aliquoted, and dried under nitrogen gas. Enzyme reaction buffer (0.930 M sodium acetate and 0.604 mM zinc chloride, adjusted with acetic acid to pH 5.7) containing various concentrations of detergent (1.0, 5.0, 10.0, 15.0, 20.0 mg/mL sodium taurocholate, 0.3, 0.6, 0.9, 1.2, 3.0 mg/mL triton X-100, or 5.0, 10.0, 15.0, 20.0, 50.0 mg/mL CHAPS) was used to reconstitute the dried S and IS aliquots.

For the single enzyme assay, each 3.2 mm DBS punch was mixed with 50 μL of prepared assay cocktail and incubated for 22 h at 37 °C on an orbital shaker at 225 rpm. The reactions were quenched with 100 μL of 1:1 ethyl acetate:methanol (*v*/*v*), and 120 μL of each sample was transferred to a 2 mL centrifuge tube containing 400 μL ethyl acetate and 400 μL HPLC-grade water to perform liquid–liquid extraction. From the upper organic layer of each sample, 200 μL was transferred to a new vial and dried under nitrogen gas. Dried extracts were reconstituted in 200 μL of 80:15:5 methanol:acetonitrile:water (*v*/*v*/*v*) containing 0.5% trifluoroacetic acid for liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis.

Analyses were performed on an API 4000 tandem mass spectrometer (Applied Biosystems, Foster City, CA) interfaced with an Agilent 1200 Series binary pump (Santa Clara, CA). A normal-phase silica column was run in isocratic mode using 80:15:5 methanol:acetonitrile:water (*v*/*v*/*v*) as the mobile phase. Mass spectrometry was performed in the multiple reaction monitoring mode with the following transitions: m/z 398.4 > 264.3 for C6:0 ceramide product (ASM-P) and m/z 370.3 > 264.3 for C4:0 ceramide ASM-IS. Data were collected and analyzed using Analyst 1.5 software. The amount of product formed during the reactions was quantified by calculating the ratio of ion abundance of the product formed (P) versus the internal standard (P/IS) from background-subtracted data.

For the multiplex assay, the enzyme reaction method and LC/MS/MS analysis were performed as described in Zhang et al. [8]. Briefly, substrate and internal standard cocktails for each lysosomal enzyme were combined with DBS extract and incubated overnight. Following incubation, the reaction mixtures from each lysosomal enzyme assay were quenched, combined, and subjected to liquid–liquid and solid phase extraction steps before LC/MS/MS analysis. The only deviation was the new formulation of ASM cocktail that contained a higher concentration of sodium taurocholate as described above in the single enzyme assay.

3. Results and discussion

Two adult sisters (NP1 and NP2) were confirmed clinically, enzymatically, and genetically to have NPD B, but surprisingly were found to have normal ASM activity in a pilot screening study that used the multiplex assay format. The mean ASM activity in DBS from 22 other NPD patients was 0.4 ± 0.3 μmol/L/h versus 4.5 ± 2.0 μmol/L/h from 20 normal controls ($P < 0.0001$) (Fig. 1A). Genotyping showed that the two sisters were compound heterozygotes for the same two *SMPD1* missense mutations, p.C92W and p.P184L [2]. The levels of a newly reported biomarker for NPD A and B, lyso-sphingomyelin [10,11], were also highly elevated in both sisters compared to normal controls (data not shown). When C12:0-SPM and C16:0-SPM (Note: natural SPM has fatty acyl chain lengths from C16 to C24) were used as substrates in the ASM assay, the activity level from NP2 (NP1 not tested) could be successfully differentiated from a subset of normal controls (Supplemental Fig. 1). These data demonstrated that this mutant form of ASM could efficiently generate ceramide from the hydrolysis of the unnatural short-chain C6:0-SPM, but not from sphingomyelin with a fatty acyl chain length greater than C12:0.

Using NPD patient samples with the highest residual ASM activity levels, normal control samples with the lowest ASM activity levels, and a sample from NP2, we tested the effects of three individual detergents (CHAPS, sodium taurocholate and triton X-100) and their concentrations on ASM activity with C6:0-SPM as the substrate (Supplemental Fig. 2). At increasing concentration of CHAPS, the ASM activity in both the normal control and patient samples was rapidly reduced. At CHAPS concentrations ranging from 5 to 20 mg/mL, the ASM activity level in NP2 fell into the range observed for other NPD samples and could now be distinguished from normal controls. No ASM activity was observed at CHAPS concentrations above 50 mg/mL. For sodium taurocholate, increasing the concentration from the original 1 mg/mL to 5 mg/mL showed the best separation in ASM activity between normal control and patient samples, including NP2. Similarly, increasing the

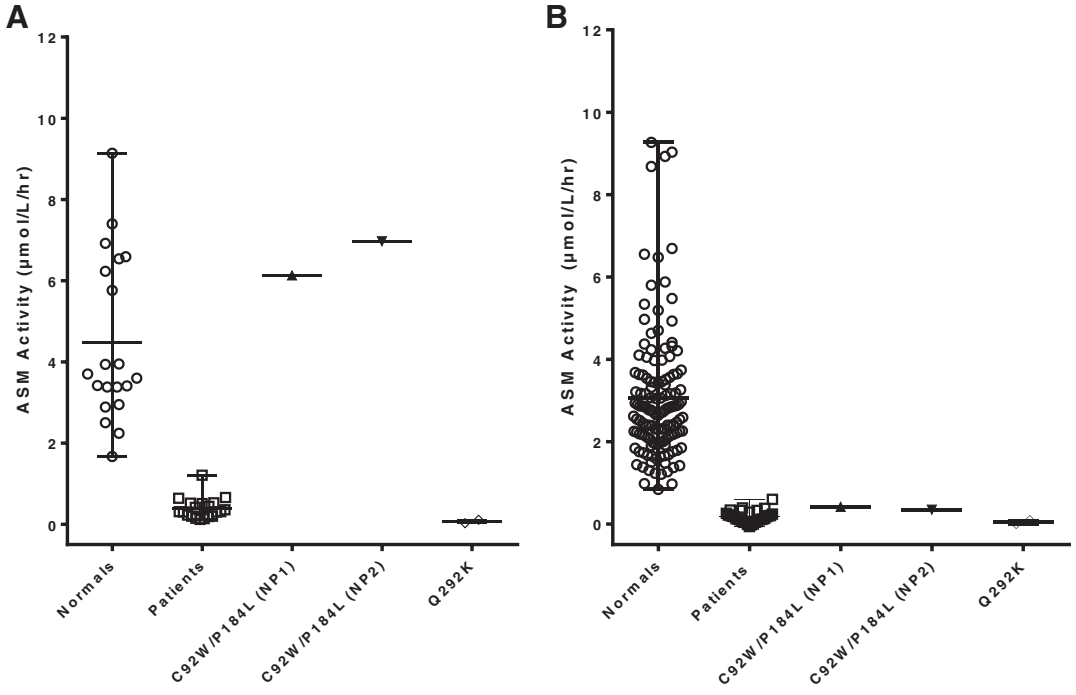

Fig. 1. Screening of acid sphingomyelinase activity in DBS using a multiplexed assay containing sodium taurocholate at concentrations of (A) 0.6 mg/mL and (B) 4.5 mg/mL in the final enzymatic reaction mixtures.

concentration of triton X-100 from 0.3 to 3 mg/mL resulted in a gradual decrease of ASM activity in both groups, but with slowly improving differentiation of activity levels between patients and normal controls.

Although Triton X-100 and sodium taurocholate showed similar separation, sodium taurocholate was chosen for further testing since it is currently used in the NBS ASM assay. A larger confirmatory study using a multiplexed enzyme activity assay was performed with a sodium taurocholate concentration of 4.5 mg/mL in the final enzymatic reaction mixture. DBS from 136 normal controls and 34 NPD patients (including NP1 and NP2) were tested. The results showed a clear separation in ASM activity between normal controls and NPD samples, which included not only NP1 and NP2, but also two other patient samples harboring the p.Q292K mutation (Fig. 1B). The mean activity in DBS for the NPD samples was 0.2 μmol/L/h (range 0 to 0.6 μmol/L/h) versus 3.0 μmol/L/h (range 0.8 to 9.3 μmol/L/h) for normal controls ($P < 0.0001$) (Fig. 1B). The higher sodium taurocholate concentration did not affect the results of the other lysosomal enzymes measured in the multiplex assay (data not shown).

ASM activity is known to be affected by the concentration of detergent in the assay reaction mixture [12]. Our results indicate that the SPM fatty acyl chain length, as well as the type of detergent and its concentration, are critical determinants of the sensitivity of the ASM activity screening assay. Similar testing should be performed on other new substrate, detergent, and buffer materials. We propose that ASM catalysis requires the formation of a critical enzyme–substrate complex in which the substrate is accessed from its detergent-bound state. At low detergent concentrations, we hypothesize that sphingomyelin containing longer (≥C12:0-SPM) but not shorter fatty acyl chains can form characteristically stable substrate/detergent complexes or so-called ultrastructures. The relative stability of these ultrastructures allows native ASM to catalyze substrate turnover, but prevents hydrolysis when the conformation of ASM is altered due to a mutation. In the case of the artificial substrates, HMU-PC and HDA-PC or C6:0-SPM, the tightly organized ultrastructure of the substrate/detergent complex does not exist at low detergent concentrations. This results in less discriminative hydrolysis of substrate by mutant forms of ASM, such as p.Q292K [4,5] or in our case, p.C92W and/or p.P195L, and generates a false normal ASM activity level in the assay. At higher detergent concentrations above the critical micelle concentration (approximately 5 mg/mL for taurocholate), the rigidity of the ultrastructure is restored and the substrate selectivity of ASM is re-established. The occurrence of false negative results with at least two different *SMPD1* missense mutations demonstrates the need for careful assay methodology development to prevent misdiagnosis, especially with respect to the substrate and detergent formulation used in in vitro assays. The results from this study are based on a small sample size, and it is possible that other mutations not yet tested could behave in a similar manner. Therefore, a large scale study is needed to fully test the robustness of the proposed assay conditions.

In conclusion, we optimized the detergent concentration of the assay reaction mixture for measuring ASM activity in DBS using C6:0-SPM as the substrate. This formulation will be beneficial for screening and diagnostic assays for NPD A and B for clinical laboratory and NBS center, as it allows for the differentiation of normal controls from NPD A and B patients with a reduced false negative rate.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2015.04.001.

References

[1] M.M. McGovern, A. Aron, S.E. Brodie, R.J. Desnick, M.P. Wasserstein, Natural history of type a Niemann–Pick disease — possible endpoints for therapeutic trials, Neurology 66 (2006) 228–232.
[2] C.M. Simonaro, J.H. Park, E. Eliyahu, N. Shtraizent, M.M. McGovern, E.H. Schuchman, Imprinting at the SMPD1 locus: implications for acid sphingomyelinase-deficient Niemann–Pick disease, Am. J. Hum. Genet. 78 (2006) 865–870.
[3] A.E. Gal, F.J. Fash, Synthesis of 2-N-(hexadecanoyl)-amino-4-nitrophenyl phosphorylcholine-hydroxide, a chromogenic substrate for assaying sphingomyelinase activity, Chem. Phys. Lipids 16 (1976) 71–79.
[4] K. Harzer, A. Rolfs, P. Bauer, M. Zschiesche, E. Mengel, J. Backes, B. Kustermann-Kuhn, G. Bruchelt, O.P. van Diggelen, H. Mayrhofer, I. Krageloh-Mann, Niemann–Pick disease type A and B are clinically but also enzymatically heterogeneous: pitfall in the laboratory diagnosis of sphingomyelinase deficiency associated with the mutation Q292K, Neuropediatrics 34 (2003) 301–306.
[5] O.P. van Diggelen, Y.V. Voznyi, J.L. Keulemans, K. Schoonderwoerd, J. Ledvinova, E. Mengel, M. Zschiesche, R. Santer, K. Harzer, A new fluorimetric enzyme assay for the diagnosis of Niemann–Pick A/B, with specificity of natural sphingomyelinase substrate, J. Inherit. Metab. Dis. 28 (2005) 733–741.
[6] J.J. Orsini, M.M. Martin, A.L. Showers, O.A. Bodamer, X.K. Zhang, M.H. Gelb, M. Caggana, Lysosomal storage disorder 4 + 1 multiplex assay for newborn screening using tandem mass spectrometry: application to a small-scale population study for five lysosomal storage disorders, Clin. Chim. Acta 413 (2012) 1270–1273.
[7] T.F. Metz, T.P. Mechtler, J.J. Orsini, M. Martin, B. Shushan, J.L. Herman, R. Ratschmann, C.B. Item, B. Streubel, K.R. Herkner, D.C. Kasper, Simplified newborn screening protocol for lysosomal storage disorders, Clin. Chem. 57 (2011) 1286–1294.
[8] X.K. Zhang, C.S. Elbin, W.L. Chuang, S.K. Cooper, C.A. Marashio, C. Beauregard, J.M. Keutzer, Multiplex enzyme assay screening of dried blood spots for lysosomal storage disorders by using tandem mass spectrometry, Clin. Chem. 54 (2008) 1725–1728.
[9] M.H. Gelb, F. Turecek, C.R. Scott, N.A. Chamoles, Direct multiplex assay of enzymes in dried blood spots by tandem mass spectrometry for the newborn screening of lysosomal storage disorders, J. Inherit. Metab. Dis. 29 (2006) 397–404.
[10] W.L. Chuang, J. Pacheco, S. Cooper, M.M. McGovern, G.F. Cox, J. Keutzer, X.K. Zhang, Lyso-sphingomyelin is elevated in dried blood spots of Niemann–Pick B patients, Mol. Genet. Metab. 111 (2014) 209–211.
[11] A. Rolfs, H. Mascher, Method For the Diagnosis of Niemann–Pick Disease. International Patent Application No. PCT/EP2014/001306, 2014.
[12] M. Tayama, S. Soeda, Y. Kishimoto, B.M. Martin, J.W. Callahan, M. Hiraiwa, J.S. O'Brien, Effect of saposins on acid sphingomyelinase, Biochem. J. 290 (Pt 2) (1993) 401–404.