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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

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ABSTRACT

Background: We sought to modify a previously published tandem mass spectrometry method of screening for 5 lysosomal storage disorders (LSDs) in order to make it better suited for high-throughput newborn screening.
Methods: Two 3-mm dried blood spot (DBS) punches were incubated, each with a different assay solution. The quadruplex solution was used for screening for Gaucher, Pompe, Krabbe and Fabry diseases, while a separate solution was used for Niemann–Pick A/B disease.
Results: The mean activities of acid-β-glucocerebrosidase (ABG), acid sphingomyelinase (ASM), acid glucosidase (GAA), galactocerebroside-β-galactosidase (GALC) and acid-galactosidase A (GLA) were measured on 5055 unidentified newborns. The mean activities (compared with their disease controls) were, 15.1 (0.35), 22.2 (1.34), 16.8 (0.51), 3.61 (0.23), and 20.7 (1.43) (μmol/L/h), respectively. The number of specimens that fell below our retest level cutoff of <20% daily mean activity (DMA) for each analyte is: ABG (6), ASM (0), GAA (5), GALC (17), and GLA (2).
Conclusions: This method provides a simplified and reliable assay for screening for five LSDs with clear distinction between activities from normal and disease samples. Advantages of this new method include significant decreases in processing time and the number of required assay solutions and overall decreased complexity.

1. Introduction

Therapies for some lysosomal storage diseases (LSDs), including Pompe, Fabry and Gaucher diseases are available. Early presymptomatic detection and initiation of therapy reduce disease-related morbidity and mortality [1–3]. Infants with infantile Krabbe disease may benefit from a cord blood transplant, but only if transplanted within the first or second month of life [4]. Previously, Li et al. developed the use of electrospray ionization tandem mass spectrometry (ESI-MS/MS) with dried blood spots (DBS) for the multiplex analysis of a panel of lysosomal enzymes (acid-β-glucocerebrosidase, ABG; acid-galactosidase A, GLA; acid α-glucosidase, GAA; acid sphingomyelinase, ASM; galactocerebroside-β-galactosidase, GALC) that when deficient cause Gaucher, Fabry, Pompe, Niemann–Pick A/B and Krabbe diseases, respectively [5]. This method was refined by Zhang et al. to make it more suitable for newborn screening laboratories [6]. Variations of Zhang's method have been used to screen for each of these LSDs, except for Niemann–Pick A/B [7–10]. Scott et al. simplified the ESI-MS/MS method to yield a triplex assay of Pompe, Fabry and mucopolysaccharidosis-I (MPS 1) [11]. Scott's approach was to combine all substrate internal standard pairs into a single buffer to perform the enzyme functional test. This simplified Zhang's method, which required a single buffer for each enzyme reaction. More recently, Metz et al. simplified Zhang's assay by eliminating the liquid/liquid and solid phase extraction steps by utilizing online column clean-up capabilities of HPLC–MS/MS instrumentation [12]. Shortly thereafter they reported their results of neonatal screening for five lysosomal storage disorders [13]. In the US, Illinois, Missouri, New Jersey and New Mexico all have mandates to screen for Gaucher, Fabry, Pompe, Niemann–Pick A/B and Krabbe diseases. New York currently screens for Krabbe disease and we sought to develop a

Abbreviations: LSD, lysosomal storage disorder; DBS, dried blood spot; DMA, daily mean activity; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; ABG, acid β-glucocerebrosidase; ASM, acid sphingomyelinase; GAA, acid α-glucosidase; GALC, galactocerebrosidase; GLA, α-galactosidase; IS, internal standard; S, substrate; P, product.

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similar assay to Scott and coworkers ESI-MS/MS multiplexed assay. Our approach was to use our current GALC assay conditions as a foundation for the multiplex assay and to add sequentially as many of the other enzymes as possible while maintaining good separation between normal and diseased control specimens. Additionally, we wanted to minimize the number of buffers and DBS required for this assay. We then performed a small population study to determine enzyme activities for the associated enzymes.

2. Materials and methods

2.1. Samples and substrate

All experiments were conducted in compliance with Institutional Review Board guidelines. Vials of pre-mixed substrates (S) and internal standards (IS) for each enzyme and quality control DBS samples (low, medium and high) were provided by Dr. Hui Zhou (Centers for Disease Control and Prevention, Atlanta, Georgia). DBSs received for routine newborn screening purposes were used for the population study. Disease-positive DBSs were received from outside sources [Dr. X. Kate Zhang (Genzyme Corp.), Dr. C. Ron Scott (Univ. of Washington), Dr. David Kasper (Austria) and Dr. Joel Charrow (IL)], and consist of both newborn and adult samples from clinically diagnosed individuals. Due to limited availability of these positive controls, (only 1 punch provided) ASM controls were not tested for ABG, GAA, GALC or GLA, and one GLA control was not tested for ASM, as this would have required a second punch. All newborn DBS were shipped at room temperature and stored at 4 °C in plastic bags with desiccant until tested. Quality control DBS (LSD low, med and high) were provided by Dr. Hui Zhou (Centers for Disease Control and Prevention) and stored at −20 °C until tested [14]. Blank specimen cards were punched and assayed on each plate to perform blank subtraction. Two punches of each quality control DBS and 3 punches from blank specimen cards were included in each assay plate.

2.2. Preparation of assay cocktail solutions

We used two 3 mm DBS punches, one for the quadruplex assay (GALC, ABG, GAA and GLA) and one for the ASM assay. To prepare the quadruplex assay solution the contents of an ABG-S/IS, and a GAA-S/IS vial were dissolved in methanol (3 mL each) (JT Baker, NJ) and transferred to a GALC-S/IS vial, and the methanol was evaporated using in-house compressed air. The contents of a GLA-S/IS vial were dissolved in 1.8 mL of 96 g/L sodium taurocholate solution (Sigma cat. no. T4009, USA) and transferred to the ABG/GAA/GALC combination vial.

The vial was vortexed and heated in a hot tap water (~50 °C) bath until the contents completely dissolved. Acarbose (0.3 mL, 0.8 mmol/L, Toronto Research Chemicals, Ontario, Canada) and 0.2 mol/L sodium phosphate/0.10 mol/L citrate buffer (15.9 mL, pH 4.4, Sigma-Aldrich, St. Louis, MO), were added to the vial and the resulting solution was vortexed.

GLA inhibitor (1 M N-acetyl-D-galactosamine) was not included in the multiple analyte solution used in this study due to recent data showing it was unnecessary [11]. The ASM solution was prepared in the same manner as described previously using sodium acetate (Sigma-Aldrich), zinc chloride (Sigma), glacial acetic acid (Fluka, Seelze, Germany) and sodium hydroxide (Krackeler Scientific #11-3722-04, NY, USA) [6]. Final assay cocktail compositions are in Table 1. For complete list of reagents used, solution recipes and storage conditions see Supplementary data.

Table 1
Final assay solution component concentrations using 4-plex + 1 method.

Quadruplex solution	ASM
6.67 μM GALC-IS, GAA-IS and GLA-IS, 13.33 μM ABG-IS	6.67 μM ASM-IS
1.0 mM GALC-S, 0.667 mM GAA-S, 3.33 mM GLA-S and 0.33 mM ABG-S	0.33 mM ASM-S
0.013 mM acarbose	0.8 g/L sodium taurocholate
9.6 g/L sodium taurocholate	0.83 M sodium acetate
0.18 M sodium phosphate and 0.09 M citrate	0.60 mM zinc chloride

2.3. Sample processing

DBSs were punched into duplicate 96-well v-bottom microtiter plates (Costar V-Bottom #3363, Corning NY) using a Wallac DBS Puncher (Perkin Elmer, Waltham, MA). One plate received 30 μL of quadruplex assay cocktail using a 12-channel pipette (Biohit Proline 25–250 μL), while the other plate received 30 μL of ASM cocktail. Plates were sealed with a polypropylene plate sealer (Costar #3080, NY) and centrifuged at 2000 RPM (Eppendorf, Centrifuge 5810) for 2 min prior to being incubated at 37 °C for 19 h with shaking at ~160 RPM (Barnstead Titer Plate Shaker #4625, Dubuque, IA).

All post-incubation processing, including all pipetting steps and the solid phase extraction (SPE), was performed using specially programmed Biomek® NX Laboratory Workstations (Beckman Coulter, USA) and Cytomat hotels (Kendro, Langenselbold, Germany), which allowed for maximum processing efficiency.

Reactions were quenched with 100 μL of 1:1 methanol/ethyl acetate per well (Axygen, 250 μL pre-sterilized tips, LRS, Union City, CA); and 100 μL aliquots from duplicate wells on each plate were combined into a single well of a deep-well plate. Ethyl acetate (400 μL per well, JT Baker, Phillipsburg, NJ) was added followed by 400 μL of water (Barnstead Filtration System, Nanopure Diamond Filter), and the plates were centrifuged (2 min, 2000 RPM). The upper layer (150 μL) was transferred to a new 96-well plate, and solvent was evaporated at 35 °C using a TurboVap 96 (Caliper Life Science, Hopkington, MA). Samples were reconstituted in 150 μL of 19:1 ethyl acetate:methanol and transferred for solid phase extraction (SPE) to a 96-well filter plate (E & K Scientific Santa Clara, CA) prepacked with 90–100 mg of silica (Sigma-Aldrich #227196, 230–400 mesh, 60A, Merck, Grade 9385) per well. A vacuum manifold designed for use with the liquid handling equipment was used to perform the SPE; the eluant was collected in a 96-well deep-well plate (Costar). Solvent was evaporated as above, and residues were reconstituted into 130 μL of 5 mmol/L ammonium formate in 80:20 methanol:water (Sigma-Aldrich) and transferred to a 96-well plate (Costar). The plate was wrapped with aluminum foil and placed into the auto-sampler for ESI-MS/MS analysis. For a complete list of the materials used, see Supplementary data.

2.4. LC–MS/MS enzyme analysis

The ESI-MS/MS was performed on a Quattro *micro*™ triple-quad mass spectrometer (Waters, Milford, MA) used in positive ion mode (see Supplementary data for specific instrument settings). Injections of 20 μL were done with a variable flow-rate using 80:20 methanol/water on a Waters 1525 μ binary HPLC coupled with a Waters 2777C sample manager. The following variable flow-rate was used: 0.1 mL/min for 0.05 min after injection, decreasing to 0.04 mL/min over 0.15 min, increasing to 0.08 mL/min over 0.55 min and then ramping up to 0.6 mL/min in 0.15 min for 0.1 min to completely flush the sample from the system before returning to 0.1 mL/min prior to the next sample injection. Data was collected during 1 min of infusion and signal returned to the background between injections. Data was acquired and analyzed by MassLynx™ 4.0 software.

2.5. Population study using Zhang method

Prior to creation of the 4 + 1 multiplex assay over 6700 anonymized specimens were tested (in 2008) using the previously