Simultaneous Testing for 6 Lysosomal Storage Disorders and X-Adrenoleukodystrophy in Dried Blood Spots by Tandem Mass Spectrometry

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BACKGROUND: Newborn screening for lysosomal storage disorders (LSD) has revealed that late-onset variants of these conditions are unexpectedly frequent and therefore may evade diagnosis. We developed an efficient and cost-effective multiplex assay to diagnose six LSDs and several peroxisomal disorders in patients presenting with diverse phenotypes at any age.

METHODS: Three 3-mm dried blood spot (DBS) punches were placed into individual microtiter plates. One disc was treated with a cocktail containing acid sphingomyelinase-specific substrate and internal standard (IS). To the second DBS we added a cocktail containing substrate and IS for β-glucosidase, acid α-glucosidase, α-galactosidase A, galactocerebrosidase, and α-L-iduronidase. The third DBS was extracted with methanol containing d4-C26 lyso-phosphatidylcholine as IS and stored until the enzyme plates were combined and purified by liquid–liquid and solid-phase extraction. The extracts were evaporated, reconstituted with the extract from the lysosphatidylcholine plate, and analyzed by flow injection tandem mass spectrometry.

RESULTS: Reference intervals were determined by analysis of 550 samples from healthy controls. DBS from confirmed patients with 1 of the 6 LSDs (n = 33), X-adrenoleukodystrophy (n = 9), or a peroxisomal biogenesis disorder (n = 5), as well as carriers for Fabry disease (n = 17) and X-adrenoleukodystrophy (n = 5), were analyzed for assay validation. Prospective clinical testing of 578 samples revealed 25 patients affected with 1 of the detectable conditions.

CONCLUSIONS: Our flow injection tandem mass spectrometry approach is amenable to high-throughput population screening for Hurler disease, Gaucher disease, Niemann-Pick A/B disease, Pompe disease, Krabbe disease, Fabry disease, X-adrenoleukodystrophy, and peroxisomal biogenesis disorder in DBS.

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diseases, screening may include Fabry disease (α-galactosidase A deficiency; OMIM 301500), Niemann–Pick type A and B disease (NPA/B) (acid sphingomyelinase deficiency; OMIM 257200 and 607616), mucopolysaccharidosis type II (iduronate-2-sulfatase deficiency; OMIM 309900), and Gaucher disease (β-glucosidase deficiency; OMIM 230800). Outside the US, screening programs in Taiwan have implemented newborn screening for Pompe and Fabry diseases (2). A major finding from past and ongoing newborn screening projects is the much higher than expected prevalence of infants with low lysosomal enzyme deficiencies and genotypes suggestive of later onset disease variants (2). Notably, these LSDs are likely underdiagnosed in adult patients because they present with symptoms different from the classic, earlier onset phenotypes.

Given this context, our aim was to develop an assay that would allow for the simultaneous high-throughput testing of dried blood spots (DBS) for 6 of the above-mentioned LSDs, ALD, and peroxisomal biogenesis disorders (PBD) by combining previously described methods for the measurement of lysosomal enzyme activities and lysophosphatidylcholines (7–12). Following validation of this multiplex assay, we have applied it for screening of at-risk patients of any age.

Materials and Methods

Materials

All lysophosphatidylcholine (LPC) species were purchased from Avanti Polar Lipids. Chloroform (ACS reagent grade) and methanol (≥99.9% purity) were purchased from EMD chemicals. Ammonium formate, silica, sodium phosphate monobasic, sodium citrate trihydrate, sodium acetate, taurocholic acid sodium salt hydrate, N-acetylgalactosamine and D-saccharic acid 1,4 lactone monohydrate were purchased from Sigma-Aldrich. Glacial acetic acid and zinc chloride were purchased from Mallinckrodt. Acarbose was purchased from Toronto Research Chemicals. Substrates (S) and internal standards (IS) for the 6 lysosomal enzymes were manufactured by Genzyme Pharmaceutical and provided by the Newborn Screening Branch of the CDC (8, 13).

Preparation of calibrators and controls. LSD controls at 4 levels of enzyme activity were prepared and supplied by the CDC (10, 13). These controls include a base pool control (CDC BP) deficient in enzyme activities, a low control (CDC low) with reduced enzyme activities, a medium control (CDC med) with moderate enzyme activities and a high control (CDC high) with normal enzyme activities. QC materials with LPCs at 2 levels were prepared in-house. For level 1 (ALD QC 1), EDTA blood was spotted on Whatman 903 filter paper and allowed to dry for at least 3 h at ambient conditions. For level 2 (ALD QC 2), EDTA blood was enriched with C25-, C22-, C24-, and C26-LPC, spotted on Whatman 903 filter paper, and allowed to dry for at least 3 h at ambient conditions. The spotted cards were then transferred to a zip-lock bag with desiccator and stored at −20 °C. Liquid calibrators with product (P) to IS ratios (P/IS) of 0.00, 0.05, 0.5, 1.0, 2.0 and 5.0 for each enzyme supplied by the CDC, as well as liquid calibrators corresponding to 0.00, 0.58, 0.97, 1.16, 1.74 and 2.32 mg/L of C25-, C22-, C24-, and C26-LPC in a 3-mm DBS were used for linearity studies.

Samples. Reference intervals were established from DBS of 550 controls. In addition, DBS samples from confirmed cases of MPS I (n = 5), Gaucher disease (n = 5), NPA/B disease (n = 2), Pompe disease (n = 5), Krabbe disease (n = 5), Fabry disease (n = 11), Fabry carriers (n = 17), ALD (n = 9), ALD carriers (n = 5), and PBD (n = 5) were analyzed. These studies were approved by the Mayo Clinic Institutional Review Board (10-000292 and 10-006866).

Methods

A 5-plex cocktail to assay β-glucosidase (ABG), acid α-glucosidase (GAA), galactocerebrosidase (GALC), α-galactosidase A (GLA), and α-1-iduronidase (IDUA) activity was based on the method described by Orsini et al. (9). The vials containing S and IS for ABG and GAA were reconstituted with methanol and transferred to the GALC S+IS vial followed by evaporation. The GLA S+IS vial was reconstituted with 1.8 mL of 96 g/L sodium taurocholate in water and then transferred to the dry GALC S+IS vial, which was then heated to 60 °C to dissolve all solids. To the GALC S+IS vial the following reagents were added: 0.3 mL of 0.8 mmol/L acarbose in water (GAA inhibitor) and 2.88 mL of 1 mol/L N-acetylgalactosamine (GLA inhibitor) in buffer (0.2 mol/L sodium phosphate + 0.1 mol/L sodium citrate, pH = 4.4). The IDUA S+IS vial was then reconstituted with 12.52 mL of buffer (0.2 mol/L sodium phosphate + 0.1 mol/L sodium citrate, pH = 4.4) and 0.5 mL of 3 mmol/L D-saccharic acid, 1,4-lactone in water. The reconstituted IDUA S+IS vial was transferred to the GALC S+IS vial. An acid sphingomyelinase (ASM) cocktail was prepared separately by reconstituting the ASM S+IS vial with 0.15 mL of 96 g/L sodium taurocholate in water and then adding 17.85 mL of buffer (0.85 mol/L sodium acetate + 0.604 mmol/L zinc chloride, pH = 5.7). After mixing, 1-mL aliquots of these reagents were stored at −20 °C for up to 2 months.

Three 3-mm discs were excised from a DBS and placed into individual microtiter plates. The first disc was treated with 30 μL of ASM cocktail, the second one with 30 μL 5-plex cocktail containing S and IS for ABG, GAA, GLA, GALC, and IDUA. The 2 enzyme plates
were sealed, centrifuged for 2 min at 493g, and incubated for 19 h at 37 °C. The third disc was extracted with 300 μL of methanol containing 12 ng/mL d₄-C₂₆ LPC as previously described (11). The extract was then evaporated under heated nitrogen, reconstituted in 130 μL of mobile phase (800 mL methanol/200 mL water with 5 mmol/L ammonium formate) and stored refrigerated. Following the 19-h incubation, the 5-plex and ASM reactions were stopped by adding 200 μL of 1:1 ethyl acetate:methanol to each plate. The 5-plex and ASM plates were combined into a single deep-well plate and liquid–liquid extraction was performed by adding 400 μL of ethyl acetate and then 400 μL of water to each well. The plate was sealed and centrifuged at 493g for 2 min, and 150 μL of the organic layer was transferred to a new plate, evaporated under nitrogen, and reconstituted in 150 μL of 19:1 ethyl acetate:methanol. The reconstituted extract was added to a silica-containing filter plate that had been washed previously with 200 μL of 19:1 ethyl acetate. The sample moved through the filter plate via positive pressure and the plate was eluted with an additional 200 μL of 19:1 ethyl acetate. The 350 μL of eluent collected was evaporated under a stream of nitrogen and then reconstituted with the stored ALD plate. The samples were subjected to flow injection tandem mass spectrometry (FIA-MS/MS) analysis. The total time to process a plate of 96 samples was 60 and 70 min, respectively, before and after the 19 h incubation.

**MS/MS procedure.** A triple-quadrupole MS/MS system (Applied Biosystems/MDS Sciex API3200) operated in positive ion mode (source voltage, 5500 V) was used. Mass calibration and resolution of both resolving quadrupoles were optimized with a poly(propylene)glycol solution introduced by an infusion pump. Method optimization was performed by infusing a solution containing the measured enzyme products, LPC species and IS at 0.6 mL/h. The instrument was optimized to monitor the transitions (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue9). The single reaction monitoring experiments (100 ms dwell, each experiment) were added to the MS/MS method. Sample introduction into the Turbo V™ atmospheric pressure ionization source was achieved by a CTC Leap autosampler and Shimadzu HPLC system. Autosampler injections of 20 μL per sample were made into the liquid chromatography (LC) mobile phase (800 mL methanol/200 mL water with 5 mmol/L ammonium formate) flow of 0.250 mL/min without the use of a chromatographic column. Between injections, analysis time was 1 min per sample. Because an unidentified substance that was inconsistently present in DBS interfered with accurate quantification of C₂₆-LPC by the flow injection tandem mass spectrometry (FIA-MS/MS) method. Each prepared sample with an apparently increased concentration of C₂₆-LPC was verified by a reflex analysis by use of LC-MS/MS as described previously (11, 12) to minimize false-positive results.

**Results**

**LINEARITY AND IMPRECISION**

Liquid calibrators for ABG, ASM, GAA, GALC, GLA, and IDUA at 7 different P/IS ratios (0, 0.05, 0.10, 0.50, 1.0, 2.0, and 5.0 P/IS) showed reproducible signals with a linear response (mean n = 3 each analyte; ABG, m = 1.021, b = 0.047, R² = 0.999; ASM, m = 1.338, b = 0.028, R² = 0.999; GAA, m = 0.965, b = 0.036, R² = 0.999; GALC, m = 0.788, b = 0.033, R² = 0.998; GLA, m = 0.900, b = 0.045, R² = 0.999; IDUA, m = 0.990, b = 0.039, R² = 0.999). Liquid calibrators for C₂₀⁻, C₂₂⁻, C₂₄⁻, and C₂₆-LPC at 6 different concentrations (0.000, 0.581, 0.968, 1.161, 1.742, and 2.322 μg/mL) also showed reproducible signals with a linear response (mean n = 3 each analyte, C₂₀-LPC, m = 0.707, b = 0.029, R² = 0.994; C₂₂-LPC, m = 0.740, b = 0.018, R² = 0.995; C₂₄-LPC, m = 0.700, b = 0.022, R² = 0.994; C₂₆-LPC, m = 0.785, b = 0.020, R² = 0.993). Intra- and interassay imprecision data for each enzyme activity and LPC concentration are provided in online Supplemental Tables 2 and 3.

**SAMPLE STABILITY**

The stability of prepared samples was assessed by analysis of 6 QCs and 10 randomly selected DBS samples, 1, 2 and 3 days after preparation. Prepared samples returned the same clinical result after 2 days of storage at 10 °C or after 3 days of storage at −20 °C.

**METHOD COMPARISON**

A method comparison was performed by use of DBS from previously diagnosed individuals with MPS I (n = 5), Gaucher disease (n = 5), NPA/B disease (n = 2), Pompe disease (n = 5), Krabbe disease (n = 5), Fabry disease (n = 11), ALD (n = 9), PBD (n = 5), and female carriers of GLA (n = 17) and ABCD1 (n = 5) mutations, as well as controls (n = 20). Alternative assays replicated in our laboratory were another MS/MS multiplex method for ABG, ASM, GAA, GALC, and GLA analysis (10), a fluorometric method for IDUA analysis (14), and an LC-MS/MS method for C₂₀⁻, C₂₂⁻, C₂₄⁻, and C₂₆-LPC analysis (11, 12). Analytical agreement between methods varied by analyte (see online Supplemental Table 4), however, all methods were clinically concordant with the exception of Fabry carriers, for which the selected cutoff value resulted in lower clinical sensitivity (35% vs 82%) when compared to the reference method. False-negative results for Fabry carriers were not surpris-
ing because Fabry carriers may have considerable residual activity that overlaps with unaffected controls (15).

INTERFERENCE
Potential interference due to the presence of anticoagu-
lants (EDTA, acid citrate dextrose and sodium heparin) was evaluated. Blood was collected from 2 healthy donors via a finger stick and from vein into tubes containing acid citrate dextrose, EDTA and sodium heparin. The blood was spotted on filter paper, allowed to dry and then analyzed. Results were less than ±20% different between these collection methods.

FILTER PAPER STUDY
Whatman 903, Alhstrom 226, and Munktell filter papers were compared by spotting blood collected from 10 healthy donors on each type of paper. The mean difference from the mean value in each donor was measured 0.8%, 5.0%, −6.5% (donor mean range = 2.8–7.3 nmol·mL⁻¹·h⁻¹) for ABG; 2.2%, 8.2%, −10.4% (4.9–15.2; range and unit as shown above) for ASM; 6.8) for IDUA; 3.0%, 12.1%, 0.5%, 1.6%, 0.08) for C22-LPC; 1.0%, 10.6%, −11.5% (0.05–0.09) for C24-LPC; and 5.3%, 6.1%, −11.4% (0.09–0.22 mg/L) for C26-LPC in Whatman 903, Alhstrom 226 and Munktell filter papers, respectively. These findings were consistent with other enzyme assays measured in DBS on filter paper from different manufacturers (16).

REFERENCE RANGES
Reference intervals were determined according to Morkrid et al. (17) following the analysis of 550 DBS samples collected from healthy adult donors (n = 280 male; n = 270 female; age range: 1 day to 86 years, median age 23 years). Cutoff values for ABG (<3.0 nmol·mL⁻¹·h⁻¹), ASM (<5.5 nmol·mL⁻¹·h⁻¹), GAA (2<4.0 nmol·mL⁻¹·h⁻¹), GLA (2<4.0 nmol·mL⁻¹·h⁻¹), GLC (2<4.0 nmol·mL⁻¹·h⁻¹), IDUA (2<2.0 nmol·mL⁻¹·h⁻¹), C20-LPC (2>0.26 mg/L), C22-LPC (2>0.11 mg/L), C24-LPC (2>0.20 mg/L) and C26-LPC (20.30 mg/L) were determined by comparing relevant markers measured in patient samples to those observed in controls (Fig. 1).

PROSPECTIVE CLINICAL TESTING
Following the analytical and clinical validation of the assay using samples from known patients, we began testing clinical samples prospectively with the goal of providing an efficient and cost-effective diagnostic approach to patients who present with signs and/or symptoms that could be caused by deficiencies of one of the LSD enzymes analyzed or by a peroxisomal disorder. Table 1 summarizes the assay’s performance after testing 578 samples between September 2011 and December 2015.

Discussion
To facilitate the rapid diagnosis of patients of all ages with treatable LSDs and ALD, we developed and implemented a multiplex MS/MS assay to screen for 6 LSDs, ALD, and PBD in DBS. Late-onset variants of these conditions often exhibit a nonspecific phenotype, making it particularly difficult to consider or justify a request for several single enzyme activity tests or single gene analyses. Compared to single-enzyme assays or molecular genetic analysis of relevant genes or gene panels, the DBS-based, multiplex-test approach is less expensive and allows for fast turnaround time and laboratory efficiency (approximately 3 h of technologist time for up to 96 samples) because patients with various differential diagnoses can be tested simultaneously and a sufficient number of samples can be batched more quickly for analysis. Furthermore, the ability to compare enzyme and LPC results to each other has advantages because disease and control profiles can be determined and compared, which has been shown to markedly improve clinical specificity in newborn screening (18, 19). The finding of multiple low enzyme activities in the same specimen can also help avoid false-positive results because such findings suggest an artifact likely caused by improper handling or shipment of the specimen (i.e., exposure of the sample to heat during transit to the laboratory).

Between September 2011 and December 2015 we analyzed a total of 578 samples from patients of all ages (median 2.8 years; range birth to 86 years; female: n = 256; male: n = 322). A majority of samples were submitted because these patients displayed symptoms suggestive of one of the lysosomal or peroxisomal conditions detected by this assay. Abnormal results were reported for all disorders tested, and the number of male and female patients was similar for the autosomal recessive conditions. Except for 18 cases that were lost to follow up, the tentative diagnoses were confirmed or excluded by additional biochemical and/or molecular genetic testing. Excluding the 18 cases lost to follow up, clinical sensitivity, clinical specificity, and the positive predictive value were very high for a screening test, but the false-positive rate was high at 4% (Table 1). The latter is not entirely surprising because unaffected carriers and patients deemed pseudo-deficient are considered false positive. The identification of carriers and pseudo-deficient patients is expected because it reflects both the known limitation of enzyme assays and the experience from new-
Fig. 1. Reference ranges (n = 550) for 6 lysosomal enzyme activities and relevant lysophosphatidylcholines (C\textsubscript{24}, C\textsubscript{26}) compared to results measured for samples from patients with relevant disorders. The whisker ends represent the 1st and 99th percentiles, the lower end of the boxes represent the 10th percentile, the upper end of the boxes represent the 90th percentile, and the horizontal line in the boxes is the 50th percentile. Het, heterozygous.
born screening for these conditions (2). Abnormal results must be followed up with additional confirmatory testing, such as molecular genetic analysis of relevant genes or testing of other disease-specific biomarkers. The same DBS specimen can be used for such analyses. Except for Pompe disease, biomarkers for the other LSDs included in this assay can be measured in DBS, specifically glycosaminoglycans for MPS I, psychosine for Krabbe disease, glucosylsphingosine for Gaucher disease, globotriaosylsphingosine for Fabry disease, and sphingomyelin for NPA/B disease (20–23). Because C26-LPC can be falsely increased in DBS due to an unknown compound in some specimens, we apply reflex testing of samples with increased C26-LPC by LC-MS/MS as previously reported (11), which explains the absence of false-positive results for ALD and other peroxisomal conditions (Table 1).

In conclusion, the assay described here allows for a more efficient and cost-effective evaluation of patients of all ages with symptoms suggestive of the tested conditions.

### Table 1  Assay performance based on prospective clinical testing of 578 patients.a

<table>
<thead>
<tr>
<th>Condition</th>
<th>Count</th>
<th>LTFUb (age range, years)</th>
<th>FP</th>
<th>FN</th>
<th>TN</th>
<th>TOT – LTFU</th>
<th>PPV, %</th>
<th>NPV, %</th>
<th>FPR, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>578</td>
<td>24 (0.02–62.1)</td>
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<td>560</td>
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<td>100</td>
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<td>63</td>
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<td>POX</td>
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<td>3 (0.6–15.7)</td>
<td>0</td>
<td>0</td>
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<td>3</td>
<td>100</td>
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<tr>
<td>Fabry (m)</td>
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<td>1 (17.8)</td>
<td>2</td>
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<td>7</td>
<td>9</td>
<td>66.7</td>
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<td>1 (30.6)</td>
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<td>7</td>
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<td>Pompe</td>
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<td>4 (0.4–62.1)</td>
<td>2</td>
<td>0</td>
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<td>7</td>
<td>66.7</td>
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<td>22.2</td>
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<td>7</td>
<td>14</td>
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<td>7</td>
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<td>18</td>
<td>31</td>
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<td>1</td>
<td>1</td>
<td>100</td>
<td></td>
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</table>

a Excluding the 18 cases lost to follow-up overall clinical sensitivity was 100% and clinical specificity 95.7%.
b LTFU, lost to follow up; TP, true positive; FP, false positive; FN, false negative; TN, true negative; TOT, total cases; PPV, positive predictive value; NPV, negative predictive value; FPR, false-positive rate [FP/(TOT – LTFU)]; POX, X-linked ALD or peroxisomal biogenesis disorder, m, male.
c Artifacts were those samples that yielded multiple low enzyme activities most likely due to heat exposure of the samples during transport to the laboratory.

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