Simplified Newborn Screening Protocol for Lysosomal Storage Disorders

Thomas F. Metz, Thomas P. Mechtler, Joseph J. Orsini, Monica Martin, Bori Shushan, Joseph L. Herman, Rene Ratschmann, Chike B. Item, Berthold Streubel, Kurt R. Herkner, and David C. Kasper

BACKGROUND: Interest in lysosomal storage disorders, a collection of more than 40 inherited metabolic disorders, has increased because of new therapy options such as enzyme replacement, stem cell transplantation, and substrate reduction therapy. We developed a high-throughput protocol that simplifies analytical challenges such as complex sample preparation and potential interference from excess residual substrate associated with previously reported assays.

METHODS: After overnight incubation (16–20 h) of dried blood spots with a cassette of substrates and deuterated internal standards, we used a TLX-2 system to quantify 6 lysosomal enzyme activities for Fabry, Gaucher, Niemann-Pick A/B, Pompe, Krabbe, and mucopolysaccharidosis I disease. This multiplexed, multidimensional ultra-HPLC–tandem mass spectrometry assay included Cyclone P Turbo Flow and Hypersil Gold C8 columns. The method did not require offline sample preparation such as liquid–liquid and solid-phase extraction, or hazardous reagents such as ethyl acetate.

RESULTS: Obviating the offline sample preparation steps led to substantial savings in analytical time (approximately 70%) and reagent costs (approximately 50%). In a pilot study, lysosomal enzyme activities of 8586 newborns were measured, including 51 positive controls, and the results demonstrated 100% diagnostic sensitivity and high specificity. The results for Krabbe disease were validated with parallel measurements by the New York State Screening Laboratory.

CONCLUSIONS: Turboflow online sample cleanup and the use of an additional analytical column enabled the implementation of lysosomal storage disorder testing in a nationwide screening program while keeping the total analysis time to <2 min per sample.

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In recent years various approaches for high-throughput tandem mass spectrometry (MS/MS) screening of lysosomal storage disorders (LSDs) have been developed. The first assays were developed for the screening of Pompe, Gaucher, Niemann-Pick A/B, Fabry, and Krabbe disease in 2004 (1). The protocols have continuously evolved, and have been refined and optimized for high-throughput analysis (2, 3). The screening panel for LSDs was recently expanded and currently includes MS/MS assays for mucopolysaccharidosis (MPS) I, II, IVA, and VI (4–7).

However, newborn screening for LSDs is still a technological challenge. One of the major technical challenges in implementing MS/MS-based multiplex enzyme assays by using the currently available substrates is the complexity of sample preparation. Specimens collected from newborns are incubated with enzyme-specific buffers containing substrates for each lysosomal enzyme, followed by MS/MS detection of all respective enzymatic products and internal standards. The technology for screening several enzyme activities at once from more or less 1 single blood punch is complicated, time-consuming, and laborious, and includes the handling of hazardous reagents such as ethyl acetate (1, 3).

Currently, routine newborn screening for LSDs has been introduced for Pompe disease in Taiwan (8)
and by the state of New York for Krabbe disease (9). Other US states such as Washington (10) and countries such as Austria started the first pilot studies for multiplex MS/MS screening, in which a variety of LSDs are screened within a single assay (11–13). For future implementation of high-throughput LSD assays in routine clinical diagnostics, sample handling and MS analysis must be simplified; specifically, sample pretreatment, separation, and finally detection must become more integrated (14). The implementation of online multidimensional chromatography combining sample preparation with analysis in 1 protocol facilitates ease-of-use sample introduction and increases speed of analysis (15).

Turbulent flow chromatography (TurboFlow) involves the use of large-diameter particles (approximately 30 μm) with a high surface area packed into narrow-bore columns (0.5–1 mm i.d.) and available in a variety of stationary phases. Relatively crude samples such as dilute serum, cerebrospinal fluid, plasma, urine, blood-spot extracts, and whole blood can be injected directly into these columns under high mobile-phase linear velocities, inducing turbulent-flow conditions. TurboFlow simultaneously eliminates matrix interferences from salts and proteins, the most common suppressors of MS/MS performance, and eliminates the need to perform liquid–liquid extraction (LLE) and solid-phase extraction (SPE) cleanup steps of previous methods (3, 16). The TurboFlow cleanup is accomplished online in a matter of seconds and the analytes of interest, after cleanup of potential matrix interferences, are subsequently transferred to an analytical column for ultra-HPLC (UHPLC) separation before MS/MS analysis (17).

We recently introduced a method for screening for LSDs that uses TurboFlow chromatography technology (18). In the current study, we modified and optimized the system for high-throughput screening of 6 different LSDs in a single assay. To demonstrate the technical feasibility and robustness of this novel method, we applied it within a comprehensive pilot screening program that runs up to several thousand samples in a routine newborn screening laboratory, including samples from affected patients. Moreover, the protocol was expanded for the screening of MPS I, and parallel measurements were performed in collaboration with the newborn screening (NBS) laboratory in New York state, New York State Department of Health, Albany, NY, for evaluation of its use with their Krabbe assay (9).

**Material and Methods**

**MATERIALS**

Deionized water (18 MΩ) produced by a Millipore Milli-Q Reference A+ System. Methanol (#106035) and isopropanol (#109634) were purchased from Merck Chemicals. Acetone (#650501), formic acid (#94318), and trifluoroacetic acid (#T62200) were purchased from Sigma Aldrich. Acetonitrile (#A/0627/17), Cyclone P 0.5 × 50–mm Turbo Flow HTLC columns, and Hypersil Gold C8 2.1 × 50–mm, 1.9-μm particle size columns were purchased from Thermo Fisher Scientific. Microplates 96/U (#0030 601.203), microplates 96/F (#0030 601.106) and deep well plates (#0030 505.301) were purchased from Eppendorf.

After we received informed consent from the parents, we collected dried blood spots (DBS) consecutively from 8586 newborns during the national routine Austrian NBS program. In Austria, NBS is centralized and conducted by one single laboratory located at the Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, for the entire population (approximately 78 000 births/year). In addition, NBS samples and 51 samples from patients with known LSDs were analyzed anonymously for 6 different lysosomal enzyme activities beyond the current screening panel for endocrine and metabolic disorders, including cystic fibrosis (19). The study was approved by the local ethics committee (EK 478/2009) and conducted in accordance with the institutional guidelines. The expanded screening for LSDs included the analysis of acid β-glucocerebrosidase (ABG), α-galactosidase (GLA), α-glucosidase (GAA), acid sphingomyelinase (ASM), galactocerebrosidase (GALC), and iduronidase (IDUA) enzyme activities in the anonymized DBS samples.

**SAMPLE PREPARATION**

Sample preparation was performed according to a 2-day working protocol. On day 1, the protocol for the incubation of DBS with enzyme reagent cocktails was adapted from Zhang et al. (3) for ABG, ASM, GAA, and GLA and from Blanchard et al. (20) for IDUA. The GALC protocol was modified from Orsini and his co-workers (9). All protocols for day 1 and 2 are described in detail in the Supplementary Materials 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue9. In brief, on day 1 aliquots of a single blood spot were incubated with enzyme-specific buffers containing substrates and internal standard for each lysosomal enzyme. On day 2, after an overnight incubation of 20–22 h, the enzymatic reactions for ABG, ASM, GAA, GLA, and IDUA were quenched with 100 μL stop solution (80 mL acetonitrile/19.8 mL water/0.2 mL formic acid). After an overnight incubation of 19.5–21.5 h (30-min shorter than the previous incubation), the GALC enzyme reaction was quenched with 100 μL stop solution, sealed with silicone covers, and incubated again (30 min, 37 °C, 750 rpm) to maximize the product outcome. Finally, all 6 assays were transferred...
into 1 single deep well plate, sealed with aluminum foil, centrifuged for 15 min at 3320 g, and measured with the TLX2 system by using online sample cleanup. Table 1 provides a short overview of the Zhang et al. protocol with the use of LLE and SPE compared with the new simplified protocol.

**Ultra-HPLC**

Transcend UHPLC systems (part of the TLX-2) with Allegro quaternary pumps were used (ThermoFisher Scientific). The mobile phases were A: 0.1 mL formic acid/0.01 mL trifluoroacetic acid/99.89 mL water; B: 0.1 mL formic acid/0.01 mL trifluoroacetic acid/99.89 mL acetonitrile; C: 45 mL isopropanol/45 mL acetonitrile/10 mL acetone; D: 80 mL acetonitrile/19.8 mL water/0.2 mL formic acid. Mobile phase C was used as a wash solvent and mobile phase D was used to prevent air bubbles in the pump system. The injection volume was 10 μL. The Transcend system employs 2 HPLCs and two 6-port valves per channel, which were configured in the standard focus mode (17). The online sample cleanup and separation performed by use of the

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**Table 1. Stepwise comparison of a protocol that includes liquid–liquid and solid-phase extraction and the new Turboflow chromatography online cleanup protocol (~400 newborns per day).**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>5-Plex protocol*</th>
<th>6-Plex protocol (TLX2 system)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gaucher, Niemann-Pick A/B, Pompe, Fabry, and Krabbe disease</td>
<td>Gaucher, Niemann-Pick A/B, Pompe, Fabry, Krabbe, and MPS I disease</td>
</tr>
<tr>
<td><strong>Punching of DBS in duplicates (2 × 4 MTPs)</strong></td>
<td>Punching of DBS in duplicates (2 × 4 MTPs)</td>
<td>Punching of DBS in duplicates (2 × 4 MTPs)</td>
</tr>
<tr>
<td><strong>Reelute with 70 μL extraction buffer (first spot)</strong></td>
<td>Reelute with 70 μL extraction buffer (first spot)</td>
<td>Reelute with 70 μL extraction buffer (first spot)</td>
</tr>
<tr>
<td><strong>Incubate (1 h, 37 °C, 750 rpm)</strong></td>
<td>Incubate (1 h, 37 °C, 750 rpm)</td>
<td>Incubate (1 h, 37 °C, 750 rpm)</td>
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<tr>
<td><strong>Incubation of 10-μL DBS extraction aliquots from first spot with 15 μL reaction cocktail, separately for ABG, ASM, GAA, and GLA</strong></td>
<td>Incubation of 10-μL DBS extraction aliquots from first spot with 15 μL reaction cocktail, separately for ABG, ASM, GAA, and GLA</td>
<td>Incubation of 10-μL DBS extraction aliquots from first spot with 15 μL reaction cocktail, separately for ABG, ASM, GAA, and GLA</td>
</tr>
<tr>
<td><strong>Centrifuge all plates (1 min, 1000g)</strong></td>
<td>Centrifuge all plates (1 min, 1000g)</td>
<td>Centrifuge all plates (1 min, 1000g)</td>
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<tr>
<td><strong>Incubation of the second spot with 30 μL GALC reaction cocktail</strong></td>
<td>Incubation of the second spot with 30 μL GALC reaction cocktail</td>
<td>Incubation of the second spot with 30 μL GALC reaction cocktail</td>
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<tr>
<td><strong>Centrifuge (1 min, 1000g)</strong></td>
<td>Centrifuge (1 min, 1000g)</td>
<td>Centrifuge (1 min, 1000g)</td>
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<tr>
<td><strong>Incubate (20–24 h, 37 °C, 250 rpm)</strong></td>
<td>Incubate (20–24 h, 37 °C, 250 rpm)</td>
<td>Incubate (20–24 h, 37 °C, 250 rpm)</td>
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<tr>
<td><strong>Time: 2.7 h</strong></td>
<td>Time: 2.7 h</td>
<td>Time: 2.7 h</td>
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<tr>
<td><strong>Day 2</strong></td>
<td><strong>Transfer all corresponding specimens into 1 deep well MTP</strong></td>
<td><strong>Transfer all corresponding specimens into 1 deep well MTP</strong></td>
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<tr>
<td></td>
<td><strong>+100 μL 1:1 methanol/ethyl acetate</strong></td>
<td><strong>+80 mL acetonitrile/19.8 mL water/0.2 mL FA to all GALC plates</strong></td>
</tr>
<tr>
<td></td>
<td><strong>+400 μL ethyl acetate</strong></td>
<td><strong>+80 μL acetonitrile/water + 0.2% FA to all other plates</strong></td>
</tr>
<tr>
<td></td>
<td><strong>+400 μL HPLC Water</strong></td>
<td><strong>Transfer all corresponding specimens into 1 deep well MTP</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Shake (5 min, ~500 rpm)</strong></td>
<td><strong>Shake GALC plate (10 min, 750 rpm)</strong></td>
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<tr>
<td></td>
<td><strong>Centrifuge (~3000g, 10 min)</strong></td>
<td><strong>Centrifuge (~3000g, 15 min)</strong></td>
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<tr>
<td></td>
<td><strong>LLE; transfer into deep well MTP</strong></td>
<td><strong>LLE; transfer into deep well MTP</strong></td>
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<tr>
<td></td>
<td><strong>N2 dry (10–15 min)</strong></td>
<td><strong>N2 dry (10–15 min)</strong></td>
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<tr>
<td></td>
<td><strong>Reelute with 100 μL methanol/ethyl acetate</strong></td>
<td><strong>Reelute with 100 μL methanol/ethyl acetate</strong></td>
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<td></td>
<td><strong>SPE with silica gel</strong></td>
<td><strong>SPE with silica gel</strong></td>
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<td></td>
<td><strong>N2 dry (20–25 min)</strong></td>
<td><strong>N2 dry (20–25 min)</strong></td>
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<tr>
<td></td>
<td><strong>Reelute with 50:50 acetonitrile/water</strong></td>
<td><strong>Reelute with 50:50 acetonitrile/water</strong></td>
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<td><strong>MS analysis</strong></td>
<td><strong>MS analysis</strong></td>
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<td></td>
<td><strong>Repeat this procedure for all 4 plates</strong></td>
<td><strong>Repeat this procedure for all 4 plates</strong></td>
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<tr>
<td></td>
<td><strong>Total time: 4–5 h</strong></td>
<td><strong>Total time: 50 min</strong></td>
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</tbody>
</table>

*a Adapted from Zhang et al. (2).

*b MTP, 96-well microtiter plate; FA, formic acid; N2, nitrogen.*
TLX-Turboflow System was recently described by Kasper et al. (18). The cleanup and chromatographic sequence are described in detail in the online Supplementary Materials 1.

MULTIPLEXED SAMPLE INTRODUCTION

The total time for the multidimensional TurboFlow UHPLC experiment was 4 min. The analytes of interest emerged from the analytical column at around 2.15 min in a window that was 1.30-min wide. MS/MS data acquisition started 2.15 min after injection and continued for 1.3 min until all analyte signals were recorded (see Fig. 1). In the current experimental setup, a dual-channel TLX-2 system was used (2 TurboFlow and 2 analytical columns). We used staggered injections, so that while one sample was being cleaned up on the Turboflow column another was being chromatographed into the MS/MS analyzer. Hence, the effective analysis time for each sample was <2 min instead of the 4 min required if samples were run serially.

MASS SPECTROMETRY

MS was performed on a Thermo Scientific TSQ Quantum Ultra with an HESI-2 heated electrospray probe. We monitored single-reaction–monitoring transitions for the 6 products and their respective internal standards. A chromatogram that includes the retention and peaks for substrates, products, and internal standards for all 6 lysosomal enzymes is shown in Fig. 1. The MS settings for each compound of interest are provided in Table 2. In the final step, the amount of product was calculated from the ion abundance ratio of the product to the internal standard for a sample multiplied by the amount of added internal standard, time of incubation, and blood spot volume, according to the method reported by Li et al. (16). Blank values (obtained with extracts of 3.2-mm blood-free filter disks) were subtracted to give the final values of lysosomal enzyme activity.

Results

DEVELOPMENT OF A HIGH-THROUGHPUT HEXAPLEX SCREENING ASSAY FOR LSDs

The multiplexed, multidimensional UHPLC MS/MS screening method was optimized (Table 2) without the need for time-consuming offline sample preparation such as LLE and SPE (see online Supplementary Material 1). The assays for Fabry, Gaucher, Niemann-Pick A/B, Pompe, Krabbe, and MPS I disease were less laborious, which led to time savings of 4–5 h per 400 samples on day 2 (Table 1). In a first pilot study, all 6 lysosomal enzyme activities for 8586 newborns and 51 patients with known LSDs were analyzed (Table 3). The cutoff values were chosen as $\leq 3.2 \mu$mol·L$^{-1}$·h$^{-1}$ for Gaucher, $\leq 0.9 \mu$mol·L$^{-1}$·h$^{-1}$ for Niemann-Pick A/B, $\leq 3.3 \mu$mol·L$^{-1}$·h$^{-1}$ for Pompe, $\leq 2.9 \mu$mol·L$^{-1}$·h$^{-1}$ for Fabry, and $\leq 0.7 \mu$mol·L$^{-1}$·h$^{-1}$ for MPS I disease, respectively, according to the 0.1 percentile of the normal population. The cutoff for Krabbe disease was below 20% of the daily mean activity according to Orsini et al. (9). We did not observe any statistical difference in lysosomal enzyme activities between single and multiplex analysis with the use of the combined online sample cleanup and the separation of substrates and products with an analytical column (see online Supplementary Fig. 1).

Fig. 1. Chromatogram including the retention time and peaks for substrates, products, and internal standards for all 6 lysosomal enzymes.
DETECTION OF AFFECTED PATIENTS WITH LSDs

For method evaluation, we analyzed a total of 51 patients with known LSDs (12 patients with Pompe, 13 with Gaucher, 22 with Fabry, 1 with Niemann-Pick A/B, 1 with Krabbe, and 2 with MPS I disease). All disease control patients had diminished enzyme activities that showed little or no overlap with enzyme activity values obtained from the normal reference population (Table 3; also see online Supplemental Table 3).

METHOD COMPARISON FOR THE KRABBE DISEASE ASSAY

The GALC enzyme activities for 176 newborns were measured in parallel by the New York screening laboratory to evaluate the Turboflow assay (Table 4). The coefficient of determination ($R^2$) between both methods was 0.79. The mean activity measured with the Turboflow assay was lower, but SDs and CVs were similar (Table 4). Both assays clearly differentiated the affected and nonaffected newborns.

Discussion

LSDs result in the accumulation of macromolecular substrates that would normally be degraded by lysosomal enzymes. The combined incidence has been estimated at 1 per 7700 live births for whites (21, 22). The relatively high combined incidence, availability of effective therapies for some of these diseases, and dire
Substantial medical challenges still remain for the development of successful therapies for many of the other LSDs, as well as challenges regarding the availability of those therapies (23). The screening for several LSDs simultaneously in a single sample can be time-consuming, expensive, and work-intensive without the use of highly automated online systems. Current limitations include the requirement to perform LLE and SPE cleanup, and the use of hazardous reagents such as ethyl acetate (1–3).

The introduction of online sample cleanup that uses multidimensional chromatography essentially eliminates the use of such organic compounds from the screening of LSDs by MS/MS (Table 1), and reduces the need for large amounts of consumables (see online Supplemental Material 2).

Table 3. Enzymatic activities (μmol·L⁻¹·h⁻¹) in affected patients and healthy controls determined by the Turboflow chromatography method.

<table>
<thead>
<tr>
<th></th>
<th>ABG</th>
<th>ASM</th>
<th>GAA</th>
<th>GLA</th>
<th>GALC</th>
<th>IDUA</th>
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<tbody>
<tr>
<td><strong>Mean</strong></td>
<td></td>
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<tr>
<td>Newborns (n=8586)</td>
<td>16.8</td>
<td>8.9</td>
<td>22.5</td>
<td>10</td>
<td>1.27</td>
<td>7.9</td>
</tr>
<tr>
<td>Affected patients (n=13)</td>
<td>0.8</td>
<td>0.4</td>
<td>0.5</td>
<td>1.0</td>
<td>0.08</td>
<td>0.3</td>
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<tr>
<td><strong>% Maximum activity of affected patients/mean of newborns activity</strong></td>
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<tr>
<td>—</td>
<td>13.1</td>
<td>4.5</td>
<td>6.2</td>
<td>22.0</td>
<td>6.31</td>
<td>5.1</td>
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<tr>
<td><strong>Percentile 0.1%</strong></td>
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<tr>
<td>—</td>
<td>3.2</td>
<td>0.2</td>
<td>3.3</td>
<td>2.9</td>
<td>0.04</td>
<td>0.7</td>
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<tr>
<td><strong>Percentile 1.0%</strong></td>
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<td>—</td>
<td>4.6</td>
<td>2.5</td>
<td>6.2</td>
<td>3.0</td>
<td>0.14</td>
<td>1.5</td>
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<tr>
<td><strong>Percentile 25%</strong></td>
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<tr>
<td>—</td>
<td>11.7</td>
<td>5.8</td>
<td>15.3</td>
<td>5.5</td>
<td>0.43</td>
<td>5.2</td>
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<tr>
<td><strong>Median</strong></td>
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<tr>
<td>—</td>
<td>15.6</td>
<td>8.0</td>
<td>20.9</td>
<td>8.0</td>
<td>0.78</td>
<td>7.4</td>
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<tr>
<td><strong>Percentile 75%</strong></td>
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<tr>
<td>—</td>
<td>20.5</td>
<td>11.0</td>
<td>27.7</td>
<td>11.9</td>
<td>1.53</td>
<td>10.0</td>
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<tr>
<td><strong>Percentile 99%</strong></td>
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<td>—</td>
<td>41.8</td>
<td>23.8</td>
<td>55.7</td>
<td>38.8</td>
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<td>20.4</td>
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<td><strong>Percentile 99.9%</strong></td>
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<tr>
<td>—</td>
<td>76.5</td>
<td>43.2</td>
<td>75.9</td>
<td>73.0</td>
<td>16.35</td>
<td>28.7</td>
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<tr>
<td><strong>Minimum</strong></td>
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<tr>
<td>—</td>
<td>2.6</td>
<td>0.3</td>
<td>1.3</td>
<td>1.5</td>
<td>0.02</td>
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<td><strong>Maximum</strong></td>
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<td>—</td>
<td>92.9</td>
<td>62.6</td>
<td>86.8</td>
<td>91.0</td>
<td>16.91</td>
<td>44.1</td>
</tr>
</tbody>
</table>

To illustrate this point, we integrated the use of published sample preparation approaches (18) into a method that we described in a report by la Marca et al. (24), in which they suggested that online multidimensional HPLC sample preparation eliminated the need for laborious offline cleanup steps with the use of a homebuilt multidimensional IC method in which a POROS cleanup column (Applied Biosystems) and an analytical C18 column were used. A similar protocol that included the use of a Turboflow cleanup column was recently reported by our group (18); this protocol was expanded to a hexaplex assay for the screening of MPS I in the present work. A serious problem with some published LSD assays is the potential for interference of the enzyme product signal from excess substrate due to insource fragmentation (14, 15). In these previous studies, interference was minimized by either detuning the ion source, i.e., purposing making the instrument less sensitive, or adding off-line cleanup steps such as SPE. We found that the use of an online analytical column in conjunction with the Turboflow cleanup column, and the higher resolving powers of UHPLC, completely eliminated such interference while keeping the total analysis time to 2 min per sample and facilitated the expansion of the screening panel. This expansion has practical value because new substrates for the screening for MPS II, IVA, and VI are on the horizon (4–7). To estimate this point, we integrated the screening of MPS II, IV, and VI into the Turboflow cleanup column and evaluated the resulting enzymatic activities (16.8 μmol·L⁻¹·h⁻¹) in a panel of newborns and affected patients. Although our GALC activities were lower than those measured by the New York laboratory, we could clearly differentiate these two groups.

Table 3. Enzymatic activities (μmol·L⁻¹·h⁻¹) in affected patients and healthy controls determined by the Turboflow chromatography method.

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<td>15.3</td>
<td>5.5</td>
<td>0.43</td>
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<td>15.6</td>
<td>8.0</td>
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<td>—</td>
<td>20.5</td>
<td>11.0</td>
<td>27.7</td>
<td>11.9</td>
<td>1.53</td>
<td>10.0</td>
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<tr>
<td>—</td>
<td>76.5</td>
<td>43.2</td>
<td>75.9</td>
<td>73.0</td>
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<td>28.7</td>
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<tr>
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<td>92.9</td>
<td>62.6</td>
<td>86.8</td>
<td>91.0</td>
<td>16.91</td>
<td>44.1</td>
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To illustrate this point, we integrated the screening of GALC activity with our published pentaplex assay (18) and evaluated the resulting GALC activities in collaboration with the New York screening laboratory by perform[ing parallel measurements. Although our GALC enzyme activities were lower than those measured by the New York laboratory, we could clearly differentiate these two groups.
the affected Krabbe patient and healthy newborns [patient’s GALC activity was below the cutoff for retesting of <12% according to Orsini et al. (9)]. The lower GALC activity might be due to slightly different sample workup. These preliminary results demonstrated that both assays are comparable, but further studies with more positive controls are required.

We minimized a potential source of ion suppression and residual substrate interference due to insulin fragment by using the analytical column to separate the substrate from the product. Other sources of ion suppression such as salt, proteins, and phospholipids were eliminated by using Turboflow chromatography (18).

The purchase of a commercial 2-channel online sample cleanup system (TLX-2) is approximately €150 000, and screening for LSDs requires one additional mass spectrometer for the high-throughput analysis of approximately 100 000 samples per year. A detailed overview of cost calculations for both LLE and SPE screening and a Turboflow assay is provided in the online Supplementary Materials 2. The latter protocol has lower run-time costs (<€0.80) and fewer personnel requirements, and does not require the purchase of liquid-handling stations for automated processing of SPE.

One limitation of the current protocol is the separate incubations on day 1, which require several additional microtiter plates and sample handling. However, Gelb et al. recently demonstrated that these preanalytical steps can be simplified by combining incubation buffers for Pompe, Fabry, and MPS I diseases; hence additional time and expenses for consumables can be saved (2).

With a biochemical assay it is not possible to discriminate between pseudodeficiency genes that result in low enzyme activities and genes that lead to a severe phenotype. Prospective screening for LSDs has to be accompanied by genetic confirmatory testing [e.g., Krabbe screening in New York state (9)]. Results of this and other studies have demonstrated that enzymatic assays that use MS are robust and accurate for the detection of known symptomatic LSD patients (2, 11, 12). The rarity of some of these diseases will, however, necessitate more extensive population-based studies to accurately evaluate the true frequency of both false-negative and false-positive results that occur with the use of this biochemical screening approach.

Quality of screening is an important issue, and hence certified substrates and QCs must be produced under good manufacturing practices with regulatory approvals. Quality assurance programs, e.g., the Newborn Screening Quality Assurance Program at the CDC (Atlanta, GA) provides QC standards that are essential to maintain a high quality of screening (25). Worldwide collaborative projects such as the Region 4 Stork website (http://www.region4genetics.org) are necessary to ensure safety and consistency of screening between states and even within countries (26).

The availability of suitable treatments for some of these disorders has resulted in increased efforts to develop new, reliable, and robust methods that can be used to perform high-throughput population screening through established NBS programs worldwide. New protocols and technologies are now available that permit higher efficiency (2, 27, 28). Nonetheless, the current experience of nationwide screening for LSDs is limited. Several small pilot projects have been started, but whole-population LSD NBS has been implemented on a routine basis in only 2 jurisdictions. Screening for Pompe disease was introduced in Taiwan because of the high population prevalence of this disease (8), and screening for Krabbe disease was started in 2006 in New York state (9) because hematopoietic stem cell transplantation during the newborn stage demonstrated improved outcomes (30).

Table 4. Parallel measurement results for Krabbe disease in 176 newborns by 2 screening laboratories.

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>1 Newborns</th>
<th>2 Newborns</th>
<th>1 Affected patients</th>
<th>2 Affected patients</th>
</tr>
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<tbody>
<tr>
<td>Mean ratios of product/internal standard</td>
<td>0.74</td>
<td>0.22</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean activity, μmol·L⁻¹·h⁻¹</td>
<td>2.66</td>
<td>0.77</td>
<td>0.23</td>
<td>0.08</td>
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<tr>
<td>SD activity, μmol·L⁻¹·h⁻¹</td>
<td>0.05</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>21.74</td>
<td>12.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum activity, μmol·L⁻¹·h⁻¹</td>
<td>0.69</td>
<td>0.17</td>
<td>0.19</td>
<td>0.07</td>
</tr>
<tr>
<td>Maximum activity, μmol·L⁻¹·h⁻¹</td>
<td>5.02</td>
<td>2.29</td>
<td>0.27</td>
<td>0.09</td>
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<tr>
<td>% Daily mean activity according to Orsini et al. (9)</td>
<td>5.50</td>
<td>6.31</td>
<td></td>
<td></td>
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</tbody>
</table>

a Laboratory 1: Albany, New York; laboratory 2: Austrian Newborn Screening Laboratory, Vienna, Austria.

b 4 Replicates.
We are faced with rapidly advancing technical possibilities and industry-driven development of enzyme replacement therapies that are still limited to just a small number of LSDs, a situation that is raising controversial discussions worldwide. These disorders were low-priority targets in the American College of Medical Genetics expert panel, in part because detection was not considered feasible, cost-effective, and simple. This viewpoint has changed owing to recent developments regarding high-throughput screening technologies that allow simplified, less laborious, and more cost-effective screening of several LSDs from a single blood spot.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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References


