High-Throughput Assay of 9 Lysosomal Enzymes for Newborn Screening

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BACKGROUND: There is interest in newborn screening of lysosomal storage diseases (LSDs) because of the availability of treatments. Pilot studies have used tandem mass spectrometry with flow injection of samples to achieve multiplex detection of enzyme products. We report a multiplexing method of 9 enzymatic assays that uses HPLC-tandem mass spectrometry (MS/MS).

METHODS: The assay of 9 enzymes was carried out in 1 or 2 buffers with a cassette of substrates and internal standards and 1 or 2 punches of a dried blood spot (DBS) from a newborn screening card as the source of enzymes. The pre–HPLC-MS/MS sample preparation required only 4 liquid transfers before injection into a dual-column HPLC equipped with switching valves to direct the flow to separation and column equilibration. Product-specific and internal standard–specific ion fragmentations were used for MS/MS quantification in the selected reaction monitoring mode.

RESULTS: Analysis of blood spots from 58 random newborns and lysosomal storage disease–affected patients showed that the assay readily distinguished affected from nonaffected individuals. The time per 9-plex analysis (1.8 min) was sufficiently short to be compatible with the workflow of newborn screening laboratories.

CONCLUSIONS: HPLC-MS/MS provides a viable alternative to flow-injection MS/MS for the quantification of lysosomal enzyme activities. It is possible to assay 9 lysosomal enzymes using 1 or 2 reaction buffers, thus minimizing the number of separate incubations necessary.

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Treatments for a subset of lysosomal storage disorders (LSDs) have become available, and in many cases early initiation of therapy leads to clinical improvement. This has spawned widespread interest in newborn screening of a subset of LSDs. New York state now provides Krabbe disease screening (1), and recent legislation for LSD expanded newborn screening has passed in several states. Newborn screening for Pompe and Fabry diseases is carried out in Taiwan (2).

We have shown that tandem mass spectrometry (MS/MS) can be used together with designed enzyme substrates to quantify the rates of lysosomal enzymes in dried blood spots (DBSs) from newborn screening cards (3–8). Here we report a simplified assay of 9 lysosomal enzymes that makes use of 1 or 2 DBS punches in 1 or 2 assay cocktails that is appropriate for newborn screening. Assays are described for acid sphingomyelinase (ASM) (Niemann-Pick A/B disease), α-acid-glucosidase (GAA) (Pompe disease), α-galactosidase A (GLA) (Fabry disease), acid α-glucosidase (AGB) (Gaucher disease), galactocerebrosidase (GALC) (Krabbe disease), α-iduronidase (IDUA) (mucopolysaccharidosis I), iduronate-2-sulfatase (ID2S) (mucopolysaccharidosis II), N-acetyl-galactosamine-6-sulfatase (GAL6S) (mucopolysaccharidosis IVA), and N-acetyl-galactosamine-4-sulfatase (GAL4S) (mucopolysaccharidosis VI).

Methods

MATERIALS
The sources of materials and preparation of the 9-plex assay cocktail are listed in the Supplemental Material, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue3.
ASSAY INCUBATION AND WORKUP
DBSs were punched into a 96-well plate (0.5 mL, Axogen Scientific, VWR International). We used a standard pipette with 1, 6, 12, or 96 channels to pipette an aliquot (15 μL) of 9-plex assay cocktail into each well. The plate was sealed with film (AxySeal, VWR International) for overnight (16-h) incubation at 37 °C with orbital shaking (250 rpm). We added acetonitrile (90 μL) by multichannel manual pipette to precipitate proteins (addition of acetonitrile was sufficient to ensure mixing). The plate was covered with sealing film and centrifuged at 1600g for 5 min to pellet the precipitate. The sealing film was removed immediately, since prolonged exposure to acetonitrile softens the acrylic adhesive. The supernatant aliquots (80 μL) were removed from the plate, avoiding dislodgement of the pellet, and transferred into a new 96-well plate. We added deionized water at 120 μL per well. The plate was wrapped with aluminum foil and subjected to 9-plex MS/MS analysis.

ULTRA-HPLC SEPARATION METHOD
We used the ultra-HPLC (UHPLC) system (Acquity UPLC with 2D technology, Waters) equipped with an analytical column and a guard column (Acquity CSH C18; 2.1×50 mm, 1.7 μm; Acquity UPLC CSH C18 VanGuard precolumn; 2.1×5 mm, 1.7 μm; Waters).

As in a previously reported HPLC system (9), the UHPLC system was capable of parallel column regeneration, but the LC separation can be performed at ultrahigh pressures (up to 15 000 psi). Thus sub–2-micron particle sorbents could be used to increase separation efficiency and analytical throughput. The UHPLC column was maintained at 40 °C, and 10-μL aliquots were injected. We mixed the mobile phase from solvent A (water, 0.1% formic acid vol/vol) and solvent B (50% acetonitrile, 50% methanol, 0.1% formic acid vol/vol) at a flow rate of 0.8 mL/min according to a linear gradient elution program: initial 50% B; 0.69 min, 100% B; 1.49 min, 100% B; 1.50 min, 50% B; 2.50 min, 50% B. The UHPLC linear gradient included an analytical part (initial 1.5 min) accompanied by a column reequilibration step (1.5–2.5 min); thus 1.5 min/per sample was achieved with parallel column regeneration.

ELECTROSPRAY IONIZATION-MS/MS SELECTED REACTION MONITORING
We performed selected reaction monitoring (SRM)-based tandem mass spectrometry in the positive ion mode on a triple quadruple mass spectrometer (Xevo TQ MS, Waters) with Mass Lynx software version 4.1. Electrospray source parameters are given in online Supplemental Table 1, and compound specific SRM ion transitions are listed in online Supplemental Table 2. Multiple reaction monitoring (MRM) was performed with a 5-ms dwell time per SRM channel and an interchannel delay time of 5 ms, resulting in a duty cycle of 0.285 ms for the 9-plex assay (27 SRM channels monitored). We monitored SRM channels corresponding to substrates during assay development, but since they were not required to calculate enzyme activity, they could be omitted to decrease the duty cycle. However, it was important to establish that substrate and product peaks were resolved well by LC because insource fragmentation of some substrates, especially those for ID2S, GAL4S, and GAL6S, would give ions identical to those of products, thus increasing the background signal seen in blank samples (filter paper only without blood). Therefore, monitoring of substrate was required during the initial setup of the assay to ensure that substrate and product were well resolved by UHPLC. This presumably would not have to be routinely checked, since conigration of substrate and product (if the UHPLC column started to fail) would result in an abnormally high background rate (high product in the filter paper–only punch sample). In addition, when substrate and product are resolved by UHPLC, 2 product signals can be seen in the MRM channel.

ENZYME ACTIVITY CALCULATIONS
We calculated enzyme activity (Ae) in micromoles h⁻¹ L⁻¹ from the ratio of product to internal standard with the formula below, assuming that a 3-mm DBS punch contains 3.1 μL blood:  

\[ Ae = \frac{(P/IS) \times [IS] \times V}{(3.1 \times t_i)} \]

where P/IS is the product-to-internal-standard peak area ratio from the MS/MS data; [IS] is the concentration of internal standard in the assay in micromoles; V is the assay volume in microliters, and ti is the assay incubation time in hours.

Results
The common structural feature of the substrates was a group that was specifically recognized by the enzyme, a hydrophobic carbon chain as part of the enzyme-generated product (to allow interaction with the reversed-phase LC column to permit chromatographic separation), and a readily fragmentable functional group that directed ion collision–induced dissociation along a dominant fragmentation pathway in the mass spectrometer (improving assay sensitivity) (Fig. 1). t-Butyl–containing carbamates provided a readily fragmentable group (loss of CO₂ and isobutylene) in the case of GLA, GAA, IDUA, ID2S, GAL4S, and GAL6S products (10). For ABG, ASM, and GALC, the ceramide readily fragmented to give a common iminium ion containing the sphingosine moiety (3, 11). GAL6S substrate was unique in having a d9-t-butyl group, because the nondeuterated version was iso-
baric with IDUA substrate. An alternative approach of increasing the carbon chain of the linker to >5 carbons led to a drop in solubility in the assay mixture. The additional cost of the deuterated reagents does not add markedly to the overall cost of reagent synthesis. Synthetic procedures for all 27 reagents have been developed (see online Supplemental Material).

We hypothesized that the LC-MS/MS assay was sensitive enough that some of the enzymes could be assayed at a pH shifted from their pH optimum, thus minimizing the number of buffers needed. Detergent was required to solubilize ceramide-containing substrates, and detergent was well tolerated by the other lysosomal enzymes that acted on water-soluble substrates. We previously reported that acarbose inhibits maltase glucoamylase, an enzyme in blood that has α-glucosidase activity in the acid pH range and thus interferes with the analysis of GAA (3, 12). The sulfatases ID2S, GAL6S, and GAL4S are inhibited by the relatively high concentrations of free sulfate and phosphate in blood, and therefore the buffer con-

Fig. 1. Structures of the 9 substrates, products, and internal standards (ISs) used to assay 9 lysosomal enzymes. BOC, di-t-butyl dicarbonate.
tained metal cations that caused precipitation of these anions without reducing the activity of the nonsulfatases. In this way, we were to assay the 9 lysosomal enzymes in 2 buffers (3-plex + 6-plex; see online Supplemental Material) or in a single buffer (9-plex, Methods). The former requires 2 punches of a DBS, whereas the latter requires only a single punch.

Online Supplemental Fig. 1 shows the UHPLC-MS/MS system. UHPLC provided an automated and fast way to process the samples (13, 14), thus eliminating the need for liquid–liquid and solid-phase extraction steps used in our earlier assays. With 2 UHPLC columns and switching valves, 1 column was used to perform the analyte separation while the other column was being equilibrated with solvent for the next sample injection. This protocol doubled the throughput.

We explored a version of the multiplex assay in which the 3 sulfatases were incubated in a single buffer (3-plex) and the 6 other enzymes were assayed in a different buffer (6-plex), thus requiring 2 3-mm DBS punches. Fig. 2A and 2B shows the HPLC ion traces for the 3-plex and 6-plex, respectively. For the 3-plex, the products and internal standards eluted well before the corresponding substrates. We were interested in quantifying only the enzyme-generated products corresponding to the ion reactions occurring at the UHPLC retention times of the products. The retention time of the ID2S product and internal standard were identical, since the internal standard was the deuterated analog of the product. In the case of GAL4S and GAL6S, there was a slight retention time shift for product vs internal standard, since the number of methylenes in the linker arms differed by 1 (Fig. 1). Fig. 2B shows the ion traces for the 6-plex. A small amount of substrate-to-product conversion due to insource fragmentation was seen for ABG, GALC, GLA, and GAA. We also explored a multiplex assay in which product and internal standard from assay incubations for the 3-plex and 6-plex reactions were combined and submitted to a single HPLC-MS/MS analysis (Fig. 3). Again, chromatographic separation of substrate and product was achieved in all 9 cases. These HPLC runs required up to 4 min.
A substantial improvement of separation speed was achieved by UHPLC (Fig. 4). Insourcing fragmentation of substrate to product was seen in the case of ABG, GALC, GAL4S, and GAL6S, and to a lower extent in GLA and GAA. Analytes were well resolved in only 1.4 min, and the separation between substrate and product was superior to that obtained in HPLC for all analytes. This robust separation with UHPLC ensured that there was no contamination of the product peaks from insourcing fragmentation of the substrates. By use of dual columns with switching valves (online Supplemental Fig. 2), we achieved an inject-to-inject time of only 1.8 min.

Online Supplemental Table 2 lists the mass-to-charge ratios ($m/z$) of precursor and product ions corresponding to monoisotopic molecular mass of substrates, products, and internal standards plus a proton or sodium cation (ID2S-S and GAL6S-S only). The fact that both protonated and sodiated ions were seen for ID2S-S and GAL6S-S was of no consequence, since we quantified only the product and internal standard species to measure the activity of the lysosomal enzymes. Substrate monitoring in the mass spectrometer was done only during performance analysis of the chromatography. The substrate channels can be turned off during routine assays, where only the product and internal standard ion species are monitored.

High reproducibility of the UHPLC analytical system was documented by results from a system suitability test (online Supplemental Table 3), which demonstrated typical retention time and peak area CVs $<1\%$ and $<15\%$, respectively, for all products and internal standards.

We also measured the degree of MS/MS signal suppression by blood components. We added assay buffer containing a mixture of substrates, products, and internal standards at assay-relevant concentrations to wells containing a DBS punch or a filter paper–only punch. Mixtures were quenched without incubation and processed and analyzed by UHPLC-MS/MS. Online Supplemental Table 4 gives the ion peak areas for the blood-containing samples divided by that for the filter paper–only samples (a ratio $<1$ means blood components caused suppression). For the 9-plex analysis, the suppression ratios ranged from 0.79 to 1.11 for the 9 products, from 0.78 to 1.30 for the 9 internal standards, and from 0.89 to 1.0 for the 9 product/internal standard ratios. The internal standards helped correct for any suppression. The 6 + 3-plex showed higher levels of suppression than the 9-plex, reaching a ratio as low as 0.57 for the ABG product. Again, the internal standard helped correct for suppression, since the suppression ratio on the ABG product/internal standard ratio was 0.97 (see online Supplemental Table 4).

Fig. 3. HPLC-MS/MS chromatogram of combined 9-plex assay of a healthy individual. ▼, chromatographic peak of P; ▼, chromatographic peak of S; ▲, peak of IS; IS, internal standard; P, product; S, substrate.
The next step was to evaluate the 6 + 3-plex and 9-plex assays on the QC samples. The CDC distributes blood spots made from leukocyte-depleted blood (base pool) mixed with various amounts of unprocessed cord blood (pooled from many donors). As shown in online Supplemental Fig. 2, linear responses were obtained for all 9 enzymes (specific activity of each enzyme in micromoles product h\(^{-1}\) (L blood)\(^{-1}\) vs the fraction of whole blood in the CDC QC DBS), showing that the assay response was proportional to the amount of enzyme in the DBS.

Fig. 5 shows the distribution of specific activity for GLA, GAA, and IDUA enzymes when measured with the 9-plex assay and 80 DBSs from random newborns and 3 LSD-affected individuals (not newborns but older patients). Plots for the other 6 LSDs are provided in online Supplemental Fig. 3. For all 9 LSDs, the samples from the diagnosed patients showed enzyme activities below those from the random newborns.

Plots corresponding to Fig. 5 and online Supplemental Fig. 3, obtained for the 6 + 3-plex assay, are shown as online Supplemental Fig. 4. In all cases the affected individuals had specific activities well below those of the 58 random newborns.

Table 1 summarizes the enzyme specific activity data obtained for 9 enzymes with the 9-plex or 6 + 3-plex UHPLC-MS/MS assays. We analyzed 58 randomly selected newborn DBSs, DBSs from a few affected patients (covering all 9 lysosomal storage diseases), a few CDC QC samples, and blanks (filter paper only).

Because the CDC QC samples are made from pooled blood, we used multiple punches of these samples (n = 12) to examine the interassay imprecision (CV) for the 9 enzyme assays (online Supplemental Table 5). For the 9-plex, the CV ranged from 3% to 11% for the low, medium, and high samples. Similar results were obtained for the 6 + 3-plex.

**Discussion**

Table 1 reveals, in some cases, differences in absolute enzyme specific activity measured for the 9-plex assay compared to the 6-plex and 3-plex. This is because the assay buffers differ in pH, additives, and volume used.
for incubation. Hence the absolute enzyme activity of a healthy sample always must be compared to enzyme activity of affected individuals, which determines the analytical resolution of the enzyme assay. Our results show that sufficient analytical resolution is feasible for either the 9-plex or 6 + 3-plex enzyme assays. The 6 + 3-plex assay provides somewhat higher resolution for ID2S compared to 9-plex, whereas the mean (SD) reference DBS enzyme specific activity is 0.98 (0.298) and 0.23 (0.069) mmol h⁻¹(L blood)⁻¹, respectively.

The LC-MS/MS platform is transferrable across commercially available instrumentation. This transfer-
<table>
<thead>
<tr>
<th>Enzyme Specific Activity [μmol h⁻¹ (L blood)⁻¹]</th>
<th>IDUA</th>
<th>GLA</th>
<th>GAA</th>
<th>ASM</th>
<th>GALC</th>
<th>ABG</th>
<th>GAL4S</th>
<th>GAL6S</th>
<th>ID2S</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>9-plex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>6</td>
<td>0.09 (0.025)</td>
<td>0.01 (0.001)</td>
<td>0.02 (0.011)</td>
<td>0.01 (0.005)</td>
<td>0.00 (0.002)</td>
<td>0.04 (0.023)</td>
<td>0.04 (0.025)</td>
<td>0.01 (0.004)</td>
</tr>
<tr>
<td>CDC QC Base</td>
<td>2</td>
<td>1.08 (0.117)</td>
<td>0.02 (0.002)</td>
<td>0.05 (0.000)</td>
<td>0.03 (0.004)</td>
<td>0.01 (0.003)</td>
<td>0.22 (0.046)</td>
<td>0.06 (0.020)</td>
<td>0.04 (0.011)</td>
</tr>
<tr>
<td>CDC QC Low</td>
<td>2</td>
<td>1.84 (0.305)</td>
<td>0.08 (0.001)</td>
<td>0.19 (0.002)</td>
<td>0.06 (0.002)</td>
<td>0.03 (0.001)</td>
<td>0.81 (0.020)</td>
<td>0.09 (0.006)</td>
<td>0.06 (0.008)</td>
</tr>
<tr>
<td>CDC QC Medium</td>
<td>2</td>
<td>7.42 (1.727)</td>
<td>0.55 (0.008)</td>
<td>0.61 (0.015)</td>
<td>0.30 (0.017)</td>
<td>0.32 (0.023)</td>
<td>2.19 (0.033)</td>
<td>0.48 (0.058)</td>
<td>0.39 (0.073)</td>
</tr>
<tr>
<td>CDC QC High</td>
<td>2</td>
<td>13.98 (0.564)</td>
<td>0.91 (0.075)</td>
<td>1.06 (0.074)</td>
<td>0.81 (0.005)</td>
<td>0.63 (0.031)</td>
<td>3.40 (0.096)</td>
<td>0.76 (0.043)</td>
<td>0.62 (0.013)</td>
</tr>
<tr>
<td>Single DBS⁶</td>
<td>6</td>
<td>12.37 (0.859)</td>
<td>1.17 (0.033)</td>
<td>1.54 (0.066)</td>
<td>0.84 (0.096)</td>
<td>4.30 (0.237)</td>
<td>6.12 (0.300)</td>
<td>0.87 (0.072)</td>
<td>0.71 (0.056)</td>
</tr>
<tr>
<td>Normal DBS³</td>
<td>58</td>
<td>14.05 (3.621)</td>
<td>0.82 (0.378)</td>
<td>3.04 (1.157)</td>
<td>1.62 (0.579)</td>
<td>0.48 (0.368)</td>
<td>9.89 (3.925)</td>
<td>1.16 (0.424)</td>
<td>1.54 (0.915)</td>
</tr>
<tr>
<td>Affected DBS</td>
<td>3</td>
<td>1.43 (0.267)</td>
<td>0.03 (0.004)</td>
<td>0.15 (0.019)</td>
<td>ND³</td>
<td>0.01 (0.003)</td>
<td>1.41 (1.128)</td>
<td>0.06³</td>
<td>0.01 (0.003)</td>
</tr>
<tr>
<td><strong>6 + 3-plex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>6</td>
<td>0.23 (0.300)</td>
<td>0.02 (0.032)</td>
<td>0.05 (0.087)</td>
<td>0.02 (0.03)</td>
<td>0.01 (0.005)</td>
<td>0.02 (0.044)</td>
<td>0.17 (0.084)</td>
<td>0.01 (0.021)</td>
</tr>
<tr>
<td>CDC QC Base</td>
<td>2</td>
<td>1.36 (0.56)</td>
<td>0.10 (0.036)</td>
<td>0.11 (0.067)</td>
<td>0.03 (0.016)</td>
<td>0.02 (0.006)</td>
<td>0.06 (0.018)</td>
<td>0.24 (0.015)</td>
<td>0.05 (0.015)</td>
</tr>
<tr>
<td>CDC QC Low</td>
<td>2</td>
<td>1.92 (0.305)</td>
<td>0.36 (0.199)</td>
<td>0.43 (0.344)</td>
<td>0.06 (0.008)</td>
<td>0.11 (0.036)</td>
<td>0.19 (0.072)</td>
<td>0.36 (0.090)</td>
<td>0.10 (0.000)</td>
</tr>
<tr>
<td>CDC QC Medium</td>
<td>2</td>
<td>5.77 (0.166)</td>
<td>1.61 (0.301)</td>
<td>1.10 (0.074)</td>
<td>0.25 (0.004)</td>
<td>0.71 (0.200)</td>
<td>0.99 (0.013)</td>
<td>0.46 (0.173)</td>
<td>0.27 (0.081)</td>
</tr>
<tr>
<td>CDC QC High</td>
<td>2</td>
<td>10.65 (0.651)</td>
<td>2.93 (0.169)</td>
<td>1.63 (0.041)</td>
<td>0.61 (0.137)</td>
<td>1.45 (0.249)</td>
<td>2.30 (0.064)</td>
<td>0.84 (0.195)</td>
<td>0.47 (0.111)</td>
</tr>
<tr>
<td>Single DBS⁶</td>
<td>6</td>
<td>16.72 (0.613)</td>
<td>5.00 (0.519)</td>
<td>3.78 (0.318)</td>
<td>0.81 (0.098)</td>
<td>3.94 (0.650)</td>
<td>4.88 (0.619)</td>
<td>0.89 (0.122)</td>
<td>0.52 (0.133)</td>
</tr>
<tr>
<td>Normal DBS³</td>
<td>58</td>
<td>5.77 (1.771)</td>
<td>1.68 (0.535)</td>
<td>2.59 (0.662)</td>
<td>1.39 (0.526)</td>
<td>0.74 (0.382)</td>
<td>4.95 (1.935)</td>
<td>2.03 (0.705)</td>
<td>0.83 (0.330)</td>
</tr>
<tr>
<td>Affected DBS</td>
<td>3</td>
<td>1.95 (0.663)</td>
<td>0.33 (0.115)</td>
<td>0.31 (0.197)</td>
<td>ND³</td>
<td>0.09 (0.090)</td>
<td>0.58 (0.409)</td>
<td>0.28³</td>
<td>0.08 (0.029)</td>
</tr>
</tbody>
</table>

*Data are mean (SD). The CDC QC samples were run 2 times, and the blank (filter paper only) was run 6 times.

⁶ Single healthy newborn, DBS punched 6 times.

³ Fifty-eight randomly selected newborns, each DBS punched once.

³ ND, no data.

³ n = 1.
ability is demonstrated by similar optimized parameters, e.g., collision energy, for various triple quadrupole mass analyzers in online Supplemental Tables 1 and 2.

Most newborn screening laboratories use flow-injection MS/MS rather than LC-MS/MS. We believe it is possible to implement LC-MS/MS in newborn screening laboratories. First, la Marca et al. (13) and Metz et al. (14) have already shown that LC-MS/MS works well in newborn screening laboratories. Second, UHPLC-MS/MS is used on thousands of samples daily in the pharmaceutical industry, mostly for pharmacokinetic and QC analyses. Third, our experimental results show that LC resolution and column backpressure are stable after at least 3000 runs of the assay. Thus, UHPLC column cost will not substantially add to the cost of newborn screening, and columns can be changed in <10 min. Fourth, the void volume from the LC column, where most of the ionic and polar blood-derived components are present, is diverted to waste rather than the mass spectrometer, thus minimizing contamination of the electrospray source. We believe that the addition of LC to the MS/MS platform will ramp up in newborn screening laboratories over the next few years. It provides much more information than fluorometric assays.

The issue of enzyme stability in DBS remains to be carefully evaluated. It is possible that poorly controlled environmental factors may reduce enzyme specific activities. One nice feature of our multiplex assay is that a drop in activity of several enzymes would indicate a poorly handled DBS. A drop in activity of 3 sulfatases measured in the multiplex assay would suggest the possibility of multiple sulfatase deficiency (16), but we have not tested this with DBSs from appropriate patients.

Fluorometric methods are also being developed for newborn screening of LSDs (17, 18). It is possible that both MS/MS and fluorometric methods will be implemented in newborn screening laboratories for LSDs. One study claimed that the fluorometric method was more rapid than the MS/MS method for assaying ID2S, since the fluorometric method used a 1-h incubation and the MS/MS method used an overnight incubation (18). However, there is no basis for the statement at this time that the fluorometric assay is faster. After a 16-h incubation, we achieved an activity for the ID2S enzyme that was approximately 20-fold above the signal measured in the no-blood control, whereas the increase was approximately 10-fold above the signal measured in the no-blood control. Thus, it appears that the 2 assays have comparable sensitivities and that a few hours’ incubation time is also achievable with the current LC-MS/MS assay.

The major advantage of the LC platform over flow injection is that the pre-MS steps for the former are reduced to a minimum, whereas flow injection requires either liquid–liquid extraction of assay mixture with organic solvent or additional solid-phase extraction by the use of silica gel or ion-exchange resin to remove substrates and buffer salts (3, 6, 7, 19). Relatively large amounts of substrate compared to product and internal standard are injected into the LC column, and substantial ionization suppression of products and internal standards by substrates will occur if adequate LC resolution is not obtained. On the other hand, most of the substrates are removed by the pre-MS sample processing in our previous methods involving flow injection. In flow injection, a single solution of uniform composition is infused into the mass spectrometer, and products and internal standards are quantified over a relatively long period of time (typically tens of seconds). In LC-MS/MS, products and internal standards elute over a relatively short time period of about a few seconds, and the composition of the eluent entering the mass spectrometer source changes over time. It is difficult to say at this time whether the current LC-MS/MS assay is more robust than assays that use flow injection into the mass spectrometer. The previous comparative study of a triplex assay performed with HPLC-MS/MS and flow injection-MS/MS gave encouraging results (14). It is likely that both methods will become available in newborn screening laboratories, allowing a degree of flexibility that fits with the different preferences among the large number of screening laboratories worldwide.
References