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Comparative Triplex Tandem Mass Spectrometry Assays of Lysosomal Enzyme Activities in Dried Blood Spots Using Fast Liquid Chromatography: Application to Newborn Screening of Pompe, Fabry, and Hurler Diseases

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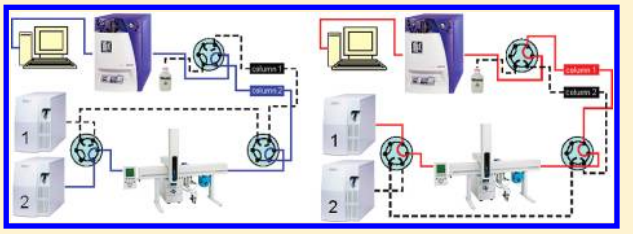
Supporting Information

ABSTRACT: We report a comparative study of triplex tandem mass spectrometry (MS/MS) based assays of lysosomal enzymes in dried blood spots for the early detection of Pompe, Fabry, and Hurler diseases in newborns. Four methods have been evaluated that differed in sample handling and the equipment used. A newly developed method uses assay quenching with acetonitrile to precipitate blood proteins followed by analysis on an LC−electrospray/MS/MS system capable of multiple consecutive sample injections on two parallel chromatographic columns. This method requires 1.5 min per a triplex analysis of enzyme products and internal standards, which matches the throughput of the previously reported flow injection method. LC separation reduces matrix effects and allows for more facile sample workup. The new LC-based method showed figures of merit that were superior to those of the currently used method based on liquid−liquid extraction into ethyl acetate and flow injection into the mass spectrometer. The other methods we investigated for comprehensive comparison involved liquid−liquid extraction into ethyl acetate followed by LC−ESI-MS/MS and acetonitrile quenching followed by direct flow injection. Both methods using acetonitrile quenching were found to be robust and provide good quality data while requiring fewer liquid transfer steps and less disposable material and labor than did the extraction methods. The individual merits of the new methods are discussed to present an evaluated alternative approach to high-throughput analysis in newborn screening laboratories.



Tandem mass spectrometry (MS/MS) has become an established tool for the detection of rare congenital metabolic disorders by newborn screening laboratories.[1] In addition to the direct detection of metabolites as disease markers,[2] new methods have been developed that quantitate lysosomal enzyme activity in dried blood spots (DBS), as reviewed in refs 3 and 4. Enzyme activity determination as a method for newborn screening is based on the observation by Chamoles and co-workers[5−7] that lysosomal enzymes retain latent activity in DBS and can be assayed after rehydration in a suitable buffer. Both direct measurements of metabolite levels and enzyme assays have to meet strict requirements of quality and high throughput.[8,9] Enzyme assays have been shown to be highly specific[10] and have very low rates of false positives, as confirmed by pilot programs of newborn screening for deficiencies of the lysosomal enzymes galactocerebroside β-galactosidase (Krabbe disease)[8] and acid α-glucosidase (Pompe disease).[9] An advantage of MS/MS-based enzyme assays is the capacity for multiplexing the analytical process by simultaneous introduction of products and internal standards into the mass spectrometer.[10] To achieve this, synthetic enzyme substrates, products, and internal standards are designed so as to have mutually exclusive molecular masses to allow for measurement in separate channels by selected reaction monitoring (SRM). This has been demonstrated by a pilot study of simultaneous (triplex) screening for deficiencies of acid α-glucosidase (GAA, the cause of Pompe disease), α-galactosidase (GLA, the cause of Fabry disease), and α-L-iduronidase (IdA, the cause of mucopolysaccharidosis I) that has been carried out on 20 000 DBS from random newborns in the Washington State Newborn Screening Laboratory[11] and by another study of screening for Fabry disease in Austria.[12]

The results of previous studies have identified sample handling as the major bottleneck in the screening procedure.[8,11] Our previous research explored both an automated serial sample workup using a lab-on-valve apparatus[13] and a parallel workup using microtiter multiwell plates.[3,4] The latter approach involves sample purification by solid-phase or liquid−liquid extraction to remove the substrate, buffer salts, and other assay additives that

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Scheme 1. Working Scheme Representing the Function of the LC System with Two Parallel Flow Channels for Multiple Consecutive Sample Injections[a]

	Position 1	Position 2	Position 1	Position 2	Position 1	Position 2
Column 1						
	Separation	Equlibration	Separation	Equlibration	Separation	Equlibration
	90 sec	90 sec	90 sec	90 sec	90 sec	90 sec
Column 2						
	Equlibration	Separation	Equlibration	Separation	Equlibration	Separation
	90 sec	90 sec	90 sec	90 sec	90 sec	90 sec
Inj. #	1.	2.	3.	4.	5.	6.
Time		1.5 min	3 min	4.5 min	6 min	7.5 min 9 min

[a] For the first injection, the switching valves are in position 1 and LC separation take place on column 1 connected to the flow channel leading to the mass analyzer. Simultaneously, column 2 is being equilibrated with the initial mobile phase. After 90 seconds, the valves are switched to position 2, and the second injection is performed on column 2, now eluting to the mass analyzer.

could impede electrospray ionization or cause interferences. The multiwell plate approach has been adopted, with modifications,[8,9] by the newborn screening programs. Recently, three research groups reported developments of liquid chromatography-based methods to introduce samples from lysosomal enzyme assays into the tandem mass spectrometer.[14−16] Some of those methods relied on an LC column switching to speed up the separation of substrates, products, and internal standards in the previously developed quintuplex assay.[10] The use of a fast LC to introduce samples is of interest because it minimizes the major bottleneck of the assays, which is liquid handling and transfers, to an injection onto the LC column. However, because LC separation is an innately serial operation, the time per injection becomes a major issue for the LC−MS/MS approach to be competitive with the multiwell plate based one.

In this study, we compare a newly developed LC−ESI-MS/MS method for the quantitation of GAA, GLA, and IdA activities in a single incubation and injection into the mass spectrometer. The results from the LC−ESI-MS/MS measurements are compared to data obtained by the recently reported liquid−liquid extraction-based ESI-MS/MS[11] and also to its simplified modification for the same sets of DBS from random anonymous newborns. The main goal of this comparative study was to evaluate the time per analysis and result quality to provide data that could assist newborn screening laboratories in deciding which analytical method would be more appropriate for their operations.

EXPERIMENTAL SECTION

Materials, Assay Incubation, and Sample Work Up. These followed previously reported procedures.[10,11,17,18] The materials used (Scheme S1 in the Supporting Information) and all experimental details are described in the Supporting Information. In addition to the standard workup protocol involving liquid−liquid extraction into ethyl acetate,[11] we used a direct quench with acetonitrile, as described in the Supporting Information.

Continuous-Flow Methods. Samples that were processed by the assay protocols were further analyzed by continuous-flow methods coupled to MS/MS for enzyme activity quantitation. The first method used standard flow injection. The newly developed method relied on separation by liquid chromatography (LC) in a dual-column setup shown in Figure 1. The mobile phase flow was maintained by a pair of 1525 Micro Binary HPLC Pumps operated simultaneously in order to create parallel flow channels for a pair of identical analytical columns (Hypersil GOLD C_{18}; 50 mm × 2.1 mm, 3 μm). Each analytical column was equipped with a Uniguard precolumn filled by a Hypersil GOLD C_{18} cartridge (10 mm × 2.1 mm, 3 μm; Thermo Scientific, San Jose, CA) and a precolumn microfilter containing a 0.5 μm pore size stainless steel frit (Idex Health & Science, Oak Harbor, WA). Sample aliquots of 10 μL were injected using the 2777C Sample Manager. The LC system consists of two parallel columns (1 and 2, Figure 1) and three switching valves. The timing of the separation and equilibration steps is shown in Scheme 1. The described approach assumes isochronal duration of the LC separation and column re-equilibration, which is feasible in rapid LC separations.[19] The mobile phase was mixed from solvent A (95% water, 5% acetonitrile, 0.1% formic acid v/v/v) and solvent B (100% acetonitrile, 0.1% formic acid v/v) and eluted at a flow rate of 0.6 mL/min according to the following linear gradient program: initial 30% B; 0.99 min 70% B; 1.00 min 30% B; 3.00 min 30% B. MXP 7900 2-position, 6-port valves (Western Analytical Products, Wildomar, CA) were used to direct the mobile phase flow through the injection port and to divert the inlet to the mass analyzer. The LC system dwell volumes were minimized by using narrow-bore PEEK tubing (0.005 in. × 1/16 in. mm; Idex Health & Science, Oak Harbor, WA).

ESI-MS/MS Selected Reaction Monitoring. Mass spectrometry analyses were performed in positive ion mode on Waters Quattro Micro and Acquity TQD tandem quadrupole mass spectrometers (Waters, Milford, MA). Instrument settings are given as Supporting Information. Enzyme activities were calculated as described in the Supporting Information.[4]

LC Method Performance. The system reproducibility for enzyme assays was evaluated by CV of retention times and peak areas by injecting a mixture of standards (6 replicates) after every 100 injections of acetonitrile quenched enzyme assays (600 total). Furthermore, 80 individual acetonitrile quenched enzyme assays were prepared and measured on two consecutive days (160 assays total) to assess intra- and interday variations of IS and enzyme product retention times and IS peak areas. Effects on the LC separation of the strong solvent were investigated by diluting the acetonitrile-quenched assay samples into 70:30 water/acetonitrile (v/v), which corresponds to the initial mobile phase composition.

Method Comparison. A sample set of DBS from 31 healthy individuals, 4 Pompe patients, 3 Fabry patients, 4 MPS-I patients, and a blank (filter paper), were assayed using the acetonitrile mediated protein precipitation (APP) and liquid−liquid extraction into ethyl acetate (LLE) sample workups in combination with liquid chromatography (LC) and flow injection analysis (FIA). Thus, the above sample set was analyzed by all four methods labeled as LC-APP, FIA-APP, LC-LLE, and FIA-LLE, and the data were compared. The last method, which includes liquid−liquid extraction with ethyl acetate followed by flow injection (FIA-LLE) into ESI-MS/MS, is currently used in the pilot study carried out at the Department of Health (DOH) for the newborn screening program in Washington state.[11] The mean and median activities of GLA, GAA, and IdA were calculated[4] for the 31 healthy specimens and compared to the pilot study data. Peak areas and retention times were compared for all four methods. The differences in enzyme activities between affected patients and the mean of normal patients were evaluated for all 4 methods. The robustness of FIA-APP for use in a newborn screening laboratory was evaluated by consecutively

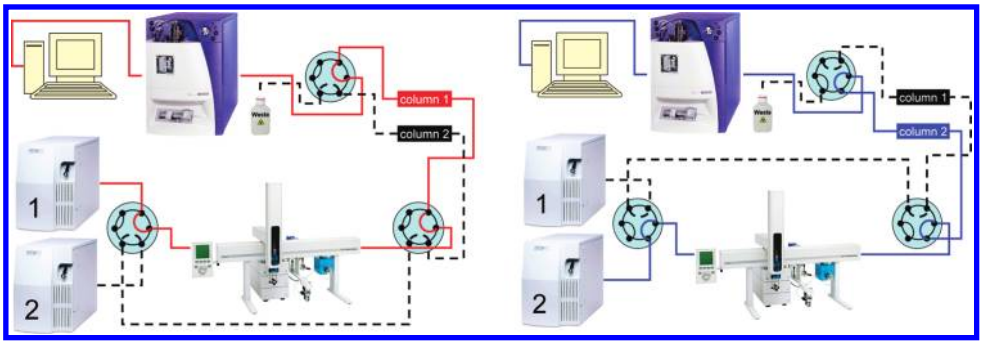

Figure 1. LC system with two parallel flow channels used for multiple consecutive sample injections. The left panel represents the state where 6-port 2-position valves are switched in position 1. Sample injection and LC separation are performed on column 1; the analytical channel is marked in red. The right panel represents the state where the valves are in position 2, with sample injection and separation being performed on column 2; the analytical channel is marked in blue.

running ten 96-well plates (800 DBS, 100 QC samples, and 60 blanks).

RESULTS AND DISCUSSION

LC Separation versus Flow Injection. The new LC-APP method was evaluated by system performance tests, protein assay, sample matrix elution profiles, and reproducibility of the entire assay protocol. Since the LC column-based method involving multiple consecutive sample injections has not been used in large-scale newborn screening, potential issues with LC system reproducibility (retention and response), column stability, and matrix effects have to be carefully addressed.[20,21] The current method in use in a pilot study in the Washington State Department of Health Newborn Screening Laboratory uses liquid−liquid extraction with ethyl acetate to remove most of the polar matrix components, including assay buffer and IdA-S as well as other polar components from the DBS. Extraction requires several manual sample handling operations by a technician and is avoided when LC separation is performed because polar components elute shortly after the void volume of the reverse-phase LC column. To simplify the manual sample handling, we quenched the assays with acetonitrile and centrifuged the blood protein precipitate. This simple procedure removed >99% of protein as summarized in Table S1 (Supporting Information). The intra- and intercolumn coefficients of variation (CV) for retention times were typically ≤1%. The peak areas showed intra- and intercolumn CV values at 8−12% but mostly stayed below 10% (Table S2, Supporting Information). The system carry-over in consecutive injections was determined by repeated injections of standard mixtures at concentrations corresponding to the assay cocktail followed by injection of a mobile phase blank (n = 6). The observed carry-over values for products and internal standards in blank injections, respectively, were very low, e.g., 0.10% and 0.12% for GAA, 0.12% and 0.11% for GLA, and 0.07% and 0.18% for IdA. These are comparable to the carry-over specifications for the injection autosampler (0.05%).

Matrix Effect Considerations. One main issue regarding LC−MS/MS is matrix effects caused by the assay and DBS components that may alter the ionization efficiency in the electrospray interface and affect the response factors of coeluting analytes.[22] The factors contributing to the matrix effects can be identified as blood cell constituents (proteins, membrane phospholipids, etc.), polar extracellular proteins (e.g., serum albumin), glucose, and various electrolytes. Note that the concentrations of residual enzyme substrates present in the assay exceed those of the products/IS by about 2 orders of magnitude. Such abundant components will presumably affect ion formation by electrospray if they were coeluted with products and IS. Therefore, the linear gradient elution program was designed to separate enzyme products and their IS from the residual substrates and sample matrix components on a time scale comparable to that for flow injection analysis.

Matrix effects were studied using the method of postcolumn infusion,[23] where a mixture of GAA-P/IS, GLA-P/IS, and IdA-P/IS was steadily infused into the mobile phase eluting from the LC column, and the SRM signals were monitored during the on column injection of a triplex assay sample (Figure 2). Ion suppression due to overlapping components is manifested as a dip in the particular SRM trace. Figure 2a shows the separation after a direct injection of an acetonitrile quenched sample, while Figure 2c shows the separation of the same sample that was diluted with water to 70:30 water/CH_3CN before injection. The overall matrix effects taking place during the LC analysis are demonstrated by Figure 2b,d, which show LC−SRM chromatograms acquired in the presence of postcolumn infusion. There are noticeable signal drops in each SRM ion trace at times when substrates are eluted from the column. The other signal loss occurs right after the elution of the void volume and is presumably due to ion suppression by polar components such as assay buffers and sample matrix that are not retained on the reversed-phase column. However, all of the major signal losses occur at elution times which are distinct from the analyte elution window. The results indicate that the separation of products and IS from substrates and matrix components is essential to preclude ion suppression of the analytes and that separation is achieved during the short LC run time. This represents a major advantage of the LC-based method when compared to simple flow injection of the mixture. The consecutive ionization of compounds as they emerge from the LC column also eliminates the issue of nonenzymatic product formation by substrate in-source fragmentation. The internal standards (IS) for GAA and GLA are deuterated analogues of the respective products (P) and therefore the IS and P coelute. In contrast, IdA-IS is a homologue of IdA-P and the CH_2 group difference results in partial separation by LC (Figure 2a,c). Given this, the possibility of different matrix effects and their impact on the method accuracy[24] was investigated, as discussed below.

Ion suppression due to the presence of a DBS matrix was observed for GAA-P and IS (12.1 and 8.2%, respectively), which correlated with lipid (lysoPC, C18:0) coelution (Figure S1, Supporting Information). Less ion suppression was observed

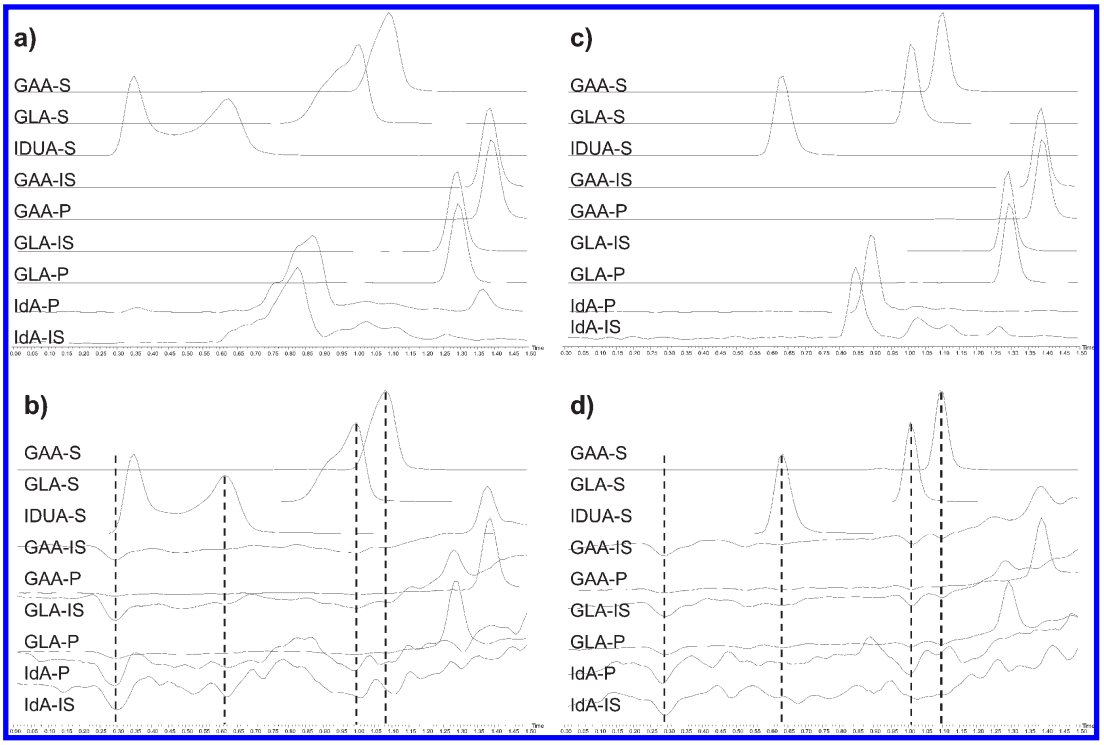

Figure 2. LC chromatograms with nine channels of SRM ion traces (each SRM represents one substrate, one product, or one internal standard) (a) Healthy individual specimen after acetonitrile mediated protein precipitation and (b) the same chromatogram combined with the steady infusion of GAA/GLA/IdA products and internal standards. The chromatogram in panel c shows the same specimen after acetonitrile protein precipitation. The sample volume was diluted to the initial mobile phase solvent composition. Panel d shows an identical LC run which was combined with steady infusion of GAA/GLA/IdA products and internal standards. The dashed lines indicate the main ion suppression regions.

for GLA-P and IS (5.5 and 6.9%, respectively) when compared to the procedural blank (no DBS matrix present). Interestingly, IdA-P and IS followed an opposite trend in that the ion counts were 19.9 and 19.0% higher in the presence of the sample matrix when compared to the procedural blank. However, the values of signal suppression/enhancement (Table S3, Supporting Information) do not indicate severe matrix effects, and both the products and IS are affected to the same extent within experimental error.

Optimization of LC Separation. The solvent effects observed after direct injection of acetonitrile quenched sample (Figure 2a) can be eliminated by adjusting the sample solvent (water/acetonitrile 15:85 v/v) to match the mobile phase composition at injection (water/acetonitrile 70:30 v/v). LC of samples treated this way resulted in baseline LC separation of all substrates, products, and internal standards (Figure 2c). The LC separation performed under nonideal conditions exhibited substantial broadening of the early eluting peaks, as shown in Figure 2a. In particular, the peak of IdA-S was affected, but this was inconsequential because this substrate peak area is not used for the enzymatic activity calculation. LC separation can be improved by optimizing the injection solvent (Figure 2c); however, this would involve two additional liquid transfer steps that would diminish the advantages of the LC−MS/MS approach. The quality of the LC separation (Figure 2a,c) has to be balanced against the number of steps in the assay sample manipulation that affects the sample throughput. Figure S2 in the Supporting Information illustrates that sufficient separation of substrates from the products and internal standards is achieved even under nonideal conditions. Hence, the quenched samples were directly injected from a shallow 96-well plate into the LC−MS/MS system so that four liquid transfers and a drying step used in the pilot study assay[11] were eliminated. The time per an LC−MS/MS analysis using parallel columns was ~90 s, which was equal to the time for a single infusion in the pilot study assay.[11] An added benefit of the LC−MS/MS method is the reduced cost of labor, disposables, and solvents. The disadvantage is the increased cost of equipment and column replacement costs. However, this cost increase is minimal compared to the overall cost of the autosampler and mass spectrometer.

Enzyme Activity Measurements. We further evaluated the LC−MS/MS method reproducibility and column lifetime by running several hundred DBS assays. Figure S3 in the Supporting Information shows the combined chromatograms of mixtures containing equimolar amounts of substrates, products, and internal standards injected in the course of 600 triplex enzyme assays (1 injection of standard per every 100 assays). These conditions were chosen to simulate the daily work load of a newborn screening laboratory and they document the excellent reproducibility of retention times (CV 0.38−0.85%) and peak areas (CV 7.49−12.89%). The intra- and interday reproducibility of retention times and areas were calculated using 80 assays analyzed on two consecutive days (corresponding to intra- and interday precision). The results (Table S4 and Figure S4a,b in the Supporting Information) indicate that the intra- and interday CVs for the retention times of the GAA and GLA product and IS were below 1%, which complies with the most rigorous quality criteria. The IdA-P and IdA-IS retention times CV (2−3%) were somewhat higher but satisfactory. The IS peak areas of GAA and GLA were reproducible with CVs (typically 13−15%), which were only slightly higher than the measured SST values (8−12%).

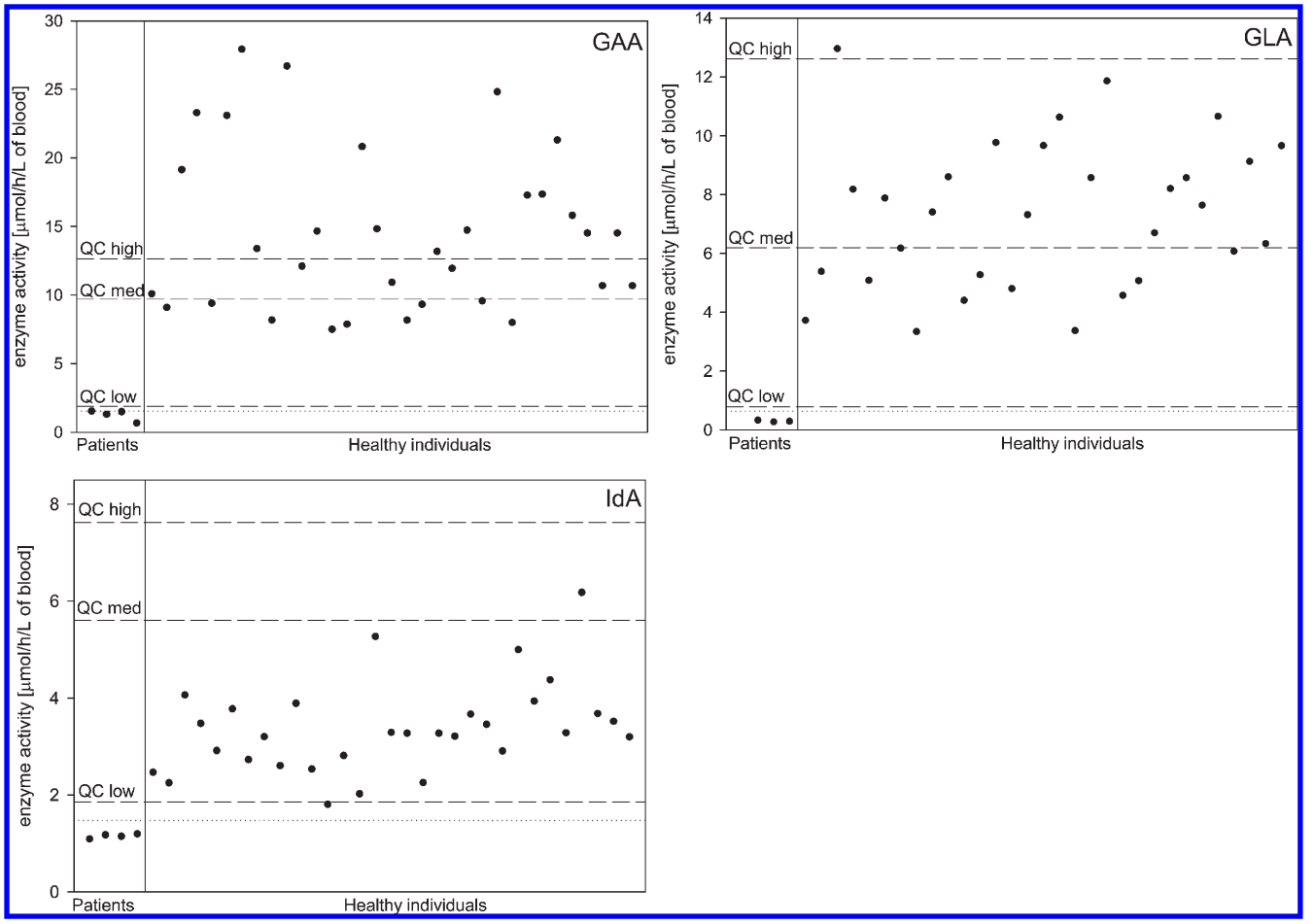

Figure 3. Enzyme activity distribution determined by LC−ESI-MS/MS after acetonitrile mediated protein precipitation for individual sample measurements (the individual values are given in Table S8 in the Supporting Information). The dotted lines correspond to QC base activity, and the dashed lines indicate QC low, QC medium, and QC high levels, respectively.

Again, somewhat higher CVs were obtained for IdA-IS (16.7; 22.7% intraday and 27.5% interday), which was due to the relatively low ion counts (Table S3 in the Supporting Information) and the above-mentioned strong solvent effects. Validation studies were performed concerning linearity of response, accuracy, precision, selectivity, and sensitivity, as summarized in Tables S5−S7 of the Supporting Information.

A sample set of DBS from 31 healthy individuals, 4 Pompe patients, 3 Fabry patients, and 4 MPS-I patients (Table S8 in the Supporting Information) was used for comparison of the continuous flow methods (LC and FIA) and sample workups using the acetonitrile mediated protein precipitation and liquid−liquid extraction with ethyl acetate. The sample set was analyzed by all four methods, and the data are labeled as LC-APP, FIA-APP, LC-LLE, and FIA-LLE. The last method included liquid−liquid extraction with ethyl acetate followed by flow injection (FIA-LLE) into electrospray MS/MS, which is currently used in the pilot study carried out at the Department of Health (DOH) for newborn screening in Washington State and has been used to screen over 20 000 newborns (23 203 for GAA and GLA and 19 102 for IdA).[11] The FIA-LLE results were compared to those from a modified method where ethyl acetate extracts were injected onto the LC columns (LC-LLE). The direct infusion of acetonitrile-quenched assays into the mass spectrometer (FIA-APP) represents the most facile method for a Newborn Screening Laboratory.

The enzyme activities measured by the four methods are compared in Table S8 in the Supporting Information. Overall, all four methods showed a satisfactory performance for use in newborn screening, and the results were in agreement with long-term enzyme activity distributions reported by the pilot study and the Department of Health, Newborn Screening Laboratory. The respective mean and median activities in micromoles hour^{-1} liter^{-1} were 15.7, 14.5 for GAA; 9.4, 8.3 for GLA; and 3.5, 3.4 for IdA. For each method, blanks (filter paper punch lacking blood) have near zero activities and were significantly below the residual activities in samples from affected individuals; therefore, the measured activities were not blank subtracted. The activities measured in the QC samples showed consistent trends from the QC base through QC high (Figure 3 and Figures S5−S7 in the Supporting Information). For example, LC-APP (Figure 3) showed that the QC base can be used as an enzyme activity cutoff for GLA and IdA, and the QC low may serve as an enzyme activity cutoff for GAA. The correlation of activities measured by the four methods for GAA, GLA, and IdA is shown in Figure S8 (Supporting Information). The GAA and GLA activities closely correlate for all methods. The IdA activity measured by FIA-LLE is offset to lower values than those from other methods, although the lows and highs show a consistent trend for all four sets of data. Thus, any one of the combinations of the sample workup and instrumental method

has the potential of distinguishing between healthy individuals and patients, although the differences in the enzyme activities measured by different methods are sometimes significant. The LC-based instrumental method revealed somewhat greater differences between the normal and affected enzyme activities, regardless of the sample workup. This can be seen when distinguishing MPS-I patients from healthy individuals where the LC analysis yielded a 2.9-fold higher mean activity for healthy specimens than for MPS-I patients, compared to a 2.6-fold increase determined by FIA. In screening for Pompe and Fabry patients, the differences are smaller, as there is a 10−20 fold decrease in enzyme activities in patients compared to the mean of healthy individuals. Another favorable feature of LC was the higher reproducibility of internal standard peak areas, as demonstrated by Figure S9 in the Supporting Information. The CV data are summarized in Table S5 in the Supporting Information. Here, the liquid−liquid extraction can be identified as the main contributor to the variation, followed by matrix effects occurring in FIA-ESI.

CONCLUSIONS

An LC−ESI-MS/MS method has been developed and evaluated for the simultaneous (triplex) determination of enzyme activities in dried blood spots for the early detection of Fabry, Pompe, and MPS-I affected newborns. The method is rapid (1.5 min per a triplex analysis) and shows figures of merit that are comparable to those of currently used methods which involve liquid−liquid extraction and flow injection into the mass spectrometer. A simplified postincubation workup reduces the number of sample manipulation steps to a single liquid transfer, followed by centrifugation, thus minimizing the cost of disposable material and labor. This sample workup is applicable to both LC and FIA-based methods. The LC-based method has advantages for expanding the assays to include additional products and internal standards for multiplexing all nine currently available lysosomal enzyme assays in DBS (Gaucher, Niemann-Pick, Fabry, Krabbe, Pompe, MPS-I, MPS-II,[25a] MPS-IV,[25b] and MPS-VI[25c]) as well as allowing other metabolites to be quantified. Studies to extend this method to the full set of treatable lysosomal storage disorders are underway in this laboratory, and the results will be reported shortly. The disadvantage compared to FIA-MS/MS is the initial cost of equipment and column replacement, but these are minimal compared to the overall equipment costs. In summary, the LC−ESI/MSMS method provides an alternative approach to high-throughput multiplex screening for inborn errors of metabolism to be considered by newborn screening laboratories.

ASSOCIATED CONTENT

Supporting Information. Details of experimental procedures and instrument settings, Scheme S1 with chemical structures, Tables S1−S8, and Figures S1−S9. This material is available free of charge via the Internet at http://pubs.acs.org.

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