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Multiplex tandem mass spectrometry assay for newborn screening of X-linked adrenoleukodystrophy, biotinidase deficiency, and galactosemia with flexibility to assay other enzyme assays and biomarkers

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Abstract

All States screen for biotinidase deficiency and galactosemia, and X-linked adrenoleukodystrophy (X-ALD) has recently been added to the Recommended Uniform Screening Panel (RUSP). We sought to consolidate these tests by combining them into a single multiplex tandem mass spectrometry assay as well as to improve the current protocol for newborn screening of galactosemia. A 3 mm punch of a dried blood spot (DBS) was extracted with organic solvent for analysis of the C26:0-lysophosphatidylcholine biomarker for X-ALD. An additional punch was used to assay galactose-1-phosphate uridylyltransferase (GALT) and biotinidase. All assays were combined for a single injection for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (2.3 min per sample). The GALT LC-MS/MS assay does not give a false positive for galactosemia if glucose-6-phosphate dehydrogenase is deficient. The multiplex assay shows acceptable reproducibility and provides for rapid analysis of X-ALD, biotinidase deficiency, and galactosemia. The throughput and ease of sample preparation are acceptable for newborn screening laboratories. We also show that the LC-MS/MS assay is expandable to include several other diseases including Pompe and Hurler diseases (enzymatic activities and biomarkers). Because of consolidation of assays, less manpower is needed compared to running individual assays on separate platforms. The flexibility of the LC-MS/MS platform allows each newborn screening laboratory to analyze the set of diseases offered in their panel.

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Author contributions

M. H. Gelb conceived of the project and directed the laboratory where the studies were carried out.

X. Hong did most of the assay studies in this paper.

A. B. Kumar synthesized all the new compounds

C.R. Scott helped direct the studies along with Gelb

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Keywords

lysosomal storage disease; inborn errors of metabolism; enzyme deficiency

Introduction

X-ALD have recently been added to the Recommended Uniform Screening Panel (RUSP) in the USA. Several State newborn screening programs are either live for this disease or are in the validation phase. Newborn screening for X-ALD is also occurring outside of the USA. Most newborn screening laboratories also screen for biotinidase deficiency and galactosemia. It is thus of interest to consolidate all of these tests into a single multiplex panel assay. This 3-plex assay can also be combined with assays for lysosomal enzymes and biomarkers for lysosomal storage diseases.

Most newborn screening laboratories assay GALT in DBS using the quantitative Beutler fluorimetric assay [1], which is outlined in Figure 1. The DBS extract is mixed with buffer containing galactose-1-phosphate, UDP-glucose, and NADP⁺. GALT generates UDP-galactose and glucose-1-phosphate. Phosphoglucumutase converts glucose-1-phosphate to glucose-6-phosphate, which is then converted to 6-phospho-D-glucono-1,5-lactone by glucose-6-phosphate dehydrogenase and then to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase. These latter 3 enzymes are provided by the DBS, and the latter two enzymatic steps generate 2 equivalents of NADPH, which is detected by its fluorescence. It is important to note that this fluorimetric GALT assay may give rise to a false positive with patients who are deficient in glucose-6-phosphate dehydrogenase [2]. This deficiency is common in certain parts of Africa, Asia, the Mediterranean, and the Middle East. As part of our effort to develop a multiplex assay involving GALT, we sought to provide a new assay that is not affected by glucose-6-phosphate dehydrogenase deficiency.

Methods

Materials

All patient samples were obtained with IRB approval. The following reagents were synthesized as described in Supplemental Material: Biotinidase-substrate, biotinidase-internal standard, and GALT-substrate. MPS-I-substrate, MPS-I-internal standard, Pompe-substrate, and Pompe-internal standard were synthesized as described [3,4]. Sources of other reagents are given in Supplemental Material. The LgtC enzyme was purified as described [5], and its concentration was measured by protein assay using the BCA kit from ThermoFisher (Cat. 23252) using bovine serum albumin as a standard.

Preparation of reagent stock solutions and assay cocktails are given in Supplemental Material.

X-ALD, GALT, and biotinidase assays

The assay uses two 3 mm DBS punches, one for X-ALD (organic solvent extraction) and one for GALT and biotinidase (enzymatic assays in aqueous buffer). To each well of a 96-well, polypropylene, shallow-well plate was added a 3 mm DBS punch followed by 30 μ L of biotinidase/GALT assay cocktail (Supplemental Material), and then 10 μ L of LgtC working solution (Supplemental Material). The plate was sealed with film-type sealing film or with a silicone-sealing matt. To a second plate was added a second 3 mm DBS punch followed by 100 μ L of 18.75 nM d₄-C26-LPC in methanol, and the plate was sealed as above. Both plates were shaken on an orbital platform at 250 rpm at 37 °C for 3 hr.

After incubation, the biotinidase/GALT plate was quenched with 100 μ L of ethyl acetate/methanol (1/1), and the plate was centrifuged in a swinging bucket rotor at $\sim$ 900 $\times$ g for 5 min at room temperature. Thirty μ L of supernatant was transferred to a 96-well, shallow-well, black, fluorimeter plate, and to each well in this plate was added 200 μ L of water. The remaining liquid in the biotinidase/GALT plate was transferred to a polypropylene deep-well plate. To each well was added 400 μ L of ethyl acetate and 200 μ L of 0.5 M NaCl in water. The well contents were mixed by aspirating up and down with a pipetor $\sim$ 10 times, then the plate was centrifuged as above. A portion of the upper ethyl acetate layer (200 μ L) was transferred to a new 96-well, polypropylene, shallow-well plate. After 3 hr incubation, the plate containing the d₄-C26-LPC was centrifuged as above, and 50 μ L was transferred to the plate containing the 200 μ L ethyl acetate extract. Solvent was removed with a jet of nitrogen or oil-free air for $\sim$ 30 min at room temperature. To the residue in each well was added 100 μ L of methanol/water (65/35) with 5 mM ammonium acetate. This plate was wrapped with aluminum foil (to prevent solvent evaporation) and placed in the autosampler of the LC-MS/MS instrument (autosampler chamber at 8 °C to prevent solvent loss).

X-ALD, GALT, biotinidase Pompe, and Hurler assays

This 5-plex assay was carried out using a total of three 3 mm DBS punches or two punches. First, we give the 3-punch method. The X-ALD and GALT/biotinidase plates were setup as above. A third plate was setup with a third 3 mm DBS punch per well. To each well was added 30 μ L of MPS-I/Pompe assay cocktail (Supplemental Material), and the plate was sealed as above.

After 3 hr incubation, the biotinidase/GALT plate was quenched with 100 μ L of ethyl acetate/methanol (1/1), and the plate was centrifuged in a swinging bucket rotor at $\sim$ 900 $\times$ g for 5 min at room temperature. Thirty μ L of supernatant was transferred to a 96-well, shallow-well, black, fluorimeter plate, and to each well in this plate was added 200 μ L of water. A second portion (75 μ L) from the centrifuged plate was transferred to the plate containing the MPS-I/Pompe reactions; this transfer served to quench these reactions. All of the liquid in each well was transferred to a 96-well, polypropylene, deep-well plate. To each well was added 400 μ L of ethyl acetate and 200 μ L of 0.5 M NaCl in water. The well contents were mixed by aspirating up and down with a pipetor $\sim$ 10 times, then the plate was centrifuged as above. A portion of the upper ethyl acetate layer (200 μ L) was transferred to a new 96-well, polypropylene, shallow-well plate. After incubation, the plate containing the d₄-C26-LPC was centrifuged as above, and 50 μ L was transferred to the plate containing the

200 μ L ethyl acetate extract. Solvent was removed with a jet of nitrogen or oil-free air for ~30 min at room temperature. To the residue in each well was added 100 μ L of methanol/water (65/35) with 5 mM ammonium acetate. This plate was wrapped with aluminum foil (to prevent solvent evaporation) and placed in the autosampler of the LC-MS/MS instrument (autosampler chamber at 8 °C to prevent solvent loss).

The above 5-plex assay was also carried out using a total of two 3 mm DBS punches as follows. To a 96-well, polypropylene, shallow-well plate (plate A) containing a single 3 mm DBS punch per well was added 30 μ L of 0.9% NaCl in water. The plate was sealed as above and shaken at 37 °C and 250 rpm for 30 min. The plate seal was removed, and 15 μ L was transferred from each well to the well of a second plate (plate B). To plate A with the DBS punch was added 15 μ L of 2X-MPS-I/Pompe assay cocktail (Supplemental Material). To plate B (without the DBS punch) was added 15 μ L of 2X-biotinidase/GALT assay cocktail (Supplemental Material) and 10 μ L of LgtC working solution (Supplemental Material). The third plate was prepared with a second 3 mm DBS punch with d₄-C26-LPC as above. All 3 plates were sealed and incubated for 3 hr at 37 °C with shaking at 250 rpm. The workup of the 3 plates was exactly as described above for the 3-punch method.

Fluorimetry

The 96-well, black, fluorimetry plate was placed in a plate fluorimeter (PerkinElmer Victor 3V) with excitation at 355 nm and emission at 460 nm. Blank fluorimetric assays were carried out as described in Supplemental Materials.

LC-MS/MS

LC-MS/MS was carried out with a Waters Xevo-TQ MS/MS instrument coupled to a Waters Aquity binary solvent system for LC. The LC column and guard column were from Waters (XBridge C8 with guard, Cat. 186006041 and 186007781) and held at 40 °C in the column oven. The flow rate was 0.45 mL/min. Solvent A was water/methanol (1/1) with 5 mM ammonium acetate, and solvent B was methanol/acetonitrile (1/1) with 5 mM ammonium acetate. It was convenient to prepare a 5 M ammonium acetate stock in water and to use this to make the mobile phases. The solvent program was 25% solvent B from 0–0.8 min, then a linear gradient to 100% solvent B from 0.8–0.85 min, then hold at 100% solvent B from 0.85–1.75 min, then jump back to 25% solvent B at 1.75 min and hold at 25% solvent B until 1.85 min. The inject-to-inject was 2.3 min when using the “load ahead” function and a “loop offline time” of 0.5 min. The weak and strong needle wash solvents for the autosampler were water/acetonitrile (9/1) and methanol/isopropanol/water (47.5/47.5/5), respectively. Ten μ L were injected per sample using the full-loop method with a loop overflow of 1.5X. MS/MS parameters are given in Supplemental Tables 5 and 6. A standard diversion valve was programmed to divert the LC void volume and the post-C26-LPC LC eluant to waste rather than to the electrospray source so as to help maintain source cleanliness.

Calculations

Specific activities of enzymes (μ mol/hr/L of blood) were calculated by multiplying the ratio of ion counts for each enzymatic product to that of the corresponding internal standard by the μ moles of internal standard in the assay well, then dividing by the incubation time (3 hr)

and by the volume of blood in a 3 mm DBS punch (3.2 μ L for the 3-punch method and 1.6 μ L for the 2-punch method). The concentration of C26-LPC in blood in nanomolar was obtained by taking the ratio of ion counts for C26-LPC to that of d₄-C26-LPC and multiplying by the nmoles of d₄-C26-LPC in the assay (0.001875 nmole) and dividing by the volume of blood in a 3 mm DBS punch (3.2×10^{-6} liters). The specific activity of GALT for the fluorimetric assay (Units per g hemoglobin) was obtained by comparing the fluorimetry reading of the sample to a standard curve made with low, medium, and high quality control GALT DBS obtained from the CDC (Y-axis is fluorimeter reading, X-axis in Units per g hemoglobin for the standards as reported by the CDC (https://www.cdc.gov/labstandards/pdf/nsqap/GALT_Certification_Set_2_2017.pdf)).

Results

Description of the enzymatic assays

Figure 1 shows the enzymatic reactions including GALT and those that generate fluorescent NADPH that is detected in the standard fluorimetric GALT assay. In addition, we show a novel reaction in which the bacterial enzyme α -1,4-galactosyltransferase from *Neisseria meningitidis* (LgtC) transfers a galactosyl group from UDP-galactose to the lactosyl group of GALT-substrate to form the trisaccharide gal-GALT-product (Figure 2 shows the structures). Note that GALT-substrate is not a direct substrate for GALT but is used to measure GALT activity via the coupled assay with LgtC. LgtC displays no enzymatic activity with UDP-glucose as shown below, and thus gal-GALT-product is formed only when UDP-galactose is generated via the action of GALT on galactose-1-phosphate plus UDP-glucose. Detection of gal-GALT-product by LC-MS/MS constitutes an assay for GALT enzymatic activity that does not depend on glucose-6-phosphate dehydrogenase and is thus not affected by deficiency of this latter enzyme (Figure 1). Detection is quantitative by the use of the internal standard UDP-[¹³C]galactose. This is converted into [¹³C]gal-GALT-product by LgtC in the incubation mixture. The heavy isotopic trisaccharide is detected along with the non-isotopic trisaccharide gal-GALT-product by LC-MS/MS. Note, any incomplete conversion of UDP-galactose to gal-GALT-product by LgtC is accounted for by using UDP-[¹³C]galactose as the internal standard since the latter has to undergo the same LgtC-catalyzed step.

The new biotinidase assay is shown in Figure 3. Biotinidase acts on biotinidase-substrate to produce the substituted aniline biotinidase-product, which is quantified by LC-MS/MS with use of a structurally identical but isotopically substituted biotinidase-internal standard (Figure 3). All previously reported substrates used to detect biotinidase in DBS contain an aromatic-amine as the leaving group, presumably because substrates with aliphatic-amines are more sluggish substrates (poorer leaving group).

Testing for X-ALD is based on the method of Haynes et al. in which the elevated biomarker C26-LPC is quantified by LC-MS/MS with use of a structurally identical, but isotopically substituted, internal standard d₄-C26-LPC [6]. Haynes and colleagues detected C26-LPC in negative ion mode. In our multiplex, we used positive ionization mode since the other analytes are also detected in positive ion mode. In positive ion mode, other species in DBS that are isobaric with C26-LPC are detected, but they are fully resolved from C26-LPC by

LC (Supplemental Figure 1)[6]. It is also possible to switch from positive to negative ionization mode just prior to the elution of C26-LPC from the LC column, but this is not needed.

In some studies we also added assays for the lysosomal enzymes relevant to MPS-I and Pompe disease. Substrates and internal standards are as reported previously [4,7]. Again, use is made of structurally-identical, isotopically-substituted internal standards.

Assay procedure

The assay procedure is summarized in Figure 4. One 3 mm DBS punch is needed for methanol extraction for X-ALD. This solvent is denaturing to enzymes, and thus cannot be used for enzymatic assays. A second 3 mm DBS punch is used in a single buffer to assay GALT and biotinidase. After a 3 hr incubation, the samples are worked up and analyzed with a single injection into the LC-MS/MS instrument. A typical work schedule would consist of setting up the multiplex assay in the afternoon of day 1 (after receipt of the DBS in the morning). Setting up the plates for 3 hr incubation requires about 30 min. After incubation, the pre-LC-MS/MS sample processing requires about 1 hr. The autosampler plate is thus loaded on the LC-MS/MS instrument at the end of day 1, and the data is available in the morning of day 2. As an option, an aliquot of the biotinidase/GALT assay can be read on a standard fluorimetry plate reader in the afternoon of day 1, requiring ~30 min, if a same-day GALT result is required. Note that each multiplex involves one LC-MS/MS per newborn since all incubations are combined into a single well of the autosampler plate.

We also combined the above 3-plex with assays for two lysosomal storage diseases, Pompe and Hurler diseases. In this case a separate buffer at low pH is needed for the lysosomal enzymes. This can be carried out either with a third 3 mm DBS punch or by pre-extracting a 3 mm DBS into saline and using a portion of the extract for GALT/biotinidase and a portion for the lysosomal enzymes. All assays are combined for a single injection into the LC-MS/MS instrument. In this study we used a 3 hr incubation for all enzymes so that the LC-MS/MS instrument is loaded with samples at the end of day 1.

The liquid-liquid extraction with ethyl acetate is recommended since essentially all of the buffer components including the sodium taurocholate detergent remain in the aqueous phase and are thus not injected onto the LC column. Note that only 10–15% of the sample in the autosampler well is injected, and thus the same sample can be re-injected should a problem occur with the overnight LC-MS/MS run. Thus, an additional DBS punch and sample processing are not needed.

Supplemental Figure 1 shows the LC-MS/MS selective ion traces for the one variation of this method, a 5-plex in which 5 analytes (C26-LPC and products for GALT, biotinidase, Pompe and Hurler enzymes) are quantified along with their respective internal standards. The inject-to-inject time is 2.3 min. Note that two product peaks are seen in the case of Pompe disease. The earlier peak elutes at the retention time of the Pompe-substrate and is due to a small amount of substrate-to-product conversion in the heated electrospray ionization source. This peak is of no concern since it is well resolved from the later eluting product peak, which is due to the enzymatically-generated product. The MPS-I-substrate

also breaks down in the electrospray source, but no second product peak is observed since the substrate elutes in the void volume. Breakdown of GALT-substrate is of no concern since it cannot generate gal-GALT-product. Biotinidase-substrate breaks down to biotinidase-product in the heated electrospray source, but the breakdown is barely detectable, and the substrate elutes well before the enzymatically-generated product (Supplemental Figure 1). All enzymatically-generated products elute at the same retention time as their respective internal standards, as expected since internal standards and products are structurally identical. This is ideal since any loss of MS/MS signal due to ion suppression of the product in the electrospray source is accounted for by an identical degree of suppression of the internal standard.

Assay validation and performance

We carried out a series of control experiments to evaluate the new GALT assay. The product gal-GALT-product was not observed if any one of galactose-1-phosphate, UDP-glucose or LgtC was omitted from the assay, but was observed if NADP⁺ was omitted (not shown). This is the expected results since formation of gal-GALT-product requires only the formation of UDP-galactose, and NADP⁺ is required only for enzymes downstream of GALT (Figure 1). This result shows that GALT would read normal if glucose-6-phosphate dehydrogenase were deficient. The control results also shows that the LgtC enzyme does not utilize UDP-glucose since formation of the trisaccharide with glucose attached to GALT-substrate would have been seen by LC-MS/MS since it is isobaric with gal-GALT-product. GALT activity was measured as a function of the amount of LgtC added, and results indicate that addition of 0.6 µg of LgtC per assay is sufficient such that no further increase in activity is seen if additional LgtC is used (not shown). Thus, conversion of GALT-substrate to gal-GALT-product is limited by the GALT step rather than the LgtC step as desired.

Next, we carried out reproducibility studies using the 5-plex assay (GALT, biotinidase, X-ALD, Pompe and Hurler). Tables 1–5 show the values of coefficient of variation (CV) for 3–5 repeats of the 5-plex LC-MS/MS assay using multiple punches from a single DBS. These studies were carried out with filter paper only (no blood control), a DBS from a healthy adult, and a DBS from a healthy adult spiked with 0.5 µM C26-LPC (to mimic an X-ALD patient). Table 1 gives the X-ALD component of the 5-plex, and Table 2 gives the biotinidase component. Table 3 gives additional GALT data by LC-MS/MS for the CDC quality control low, medium, and high GALT DBS. Table 4 gives the MPS-I result, and Table 5 gives the Pompe result. Finally, Table 6 gives the GALT data measured with the fluorimetric assay. Assay reproducibility for all 5 tests was acceptable. For biotinidase and GALT, the specific activity of the enzymes (µmole/hr/L) were slightly lower for the 2-punch method compared to the 3-punch method suggesting that not all of the enzymes were extracted into saline for the 2-punch method. On the other hand, the X-ALD, MPS-I, and Pompe disease results were very similar for the 2- and 3-punch methods. Figures 5 and 5 show good linearity for the GALT assays (LC-MS/MS and fluorescence) versus the amount of GALT in the CDC quality control standards (in units of Units/g hemoglobin) as provided by the certification sheets on the CDC website (see Methods). Since the blank fluorescence for all the blank samples are similar (Table 6), one may choose to ignore the blank and read

the GALT activity in U/g hemoglobin from the fluorescence reading of the newborn DBS compared to those from the standard curve (Figure 6) without blank correction.

Also given in the data tables is the 5-plex data for DBS from 30 random newborns as well as three newborns previously diagnosed with classical galactosemia (GALT deficient) and one newborn previously diagnosed with biotinidase deficiency. The patients showed a clear deficit in the level of the relevant enzyme. We did not submit DBS from patients confirmed to have MPS-I or Pompe disease to the 5-plex study since we have extensively shown that such DBS give vanishingly low enzymatic activities using the same substrates [4,7], and these DBS are in short supply. For X-ALD, we used DBS from non-affected donors as well as DBS made from blood spiked with levels of C26-LPC found in typical X-ALD patients [6]. The C26-LPC method used in our 5-plex is essentially identical to the previous method [6], which has been validated with affected-patient DBS [6].

Discussion

The new multiplex reported in this study may be useful for newborn screening for galactosemia, biotinidase deficiency, and X-ALD. Further studies are needed to validate the assay in a newborn screening laboratory. One advantage of the new multiplex is that it allows assays to be consolidated with or without addition of lysosomal storage diseases thus reducing overall costs. The lack of interference from glucose-6-phosphate dehydrogenase deficiency is an important factor in regions where there are a relatively high incidence of this deficiency.

In our previous MS/MS assays of lysosomal enzymes we used an overnight incubation period, whereas in the current study we incubated for 3 hrs [3,7]. The overnight period is needed only if the Krabbe disease enzyme is included since this enzyme has a relatively low specific activity. If Krabbe disease is omitted, a short incubation period of 3 hr is more than sufficient.

The new multiplex assay is expected to be easily incorporated into previously developed MS/MS assays. For example, Supplemental Figure 2 shows an LC-MS/MS trace where X-ALD, biotinidase and GALT are multiplexed with 6 lysosomal enzymes (relevant to Gaucher, Fabry, Krabbe, and Niemann-Pick-A/B). This 9-plex requires ultra-high pressure LC (UPLC), and can be done on most modern LC systems (for example on the Waters Aquity system). More detailed studies are underway of this 9-plex to expand this assay to other lysosomal enzymes (for example, [8]) as well as biomarkers that cannot be analyzed in the standard MS/MS protocols used in newborn screening laboratories to analyze amino acids and acyl-carnitines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

C26-LPC	1-hexacosanoyl-2-hydroxy- <i>sn</i> -glycero-3-phosphocholine
DBS	dried blood spot on a newborn screening card
GALT	galactose-1-phosphate uridyltransferase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MPS-I	mucopolysaccharidosis-I
UDP-galactose	uridine diphosphate-galactose
UDP-glucose	uridine diphosphate-glucose
X-ALD	X-linked adrenoleukodystrophy

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Figure 1.

Shown is the biochemical pathway from galactose-1-phosphate plus UDP-glucose to ribulose-5-phosphate, where the first enzymatic step is catalyzed by GALT. Two equivalents of NADPH are formed, and this is detected via its fluorescence. Also shown is the LgtC-catalyzed transfer of the galactosyl group from GALT product UDP-galactose to GALT-substrate to form gal-GALT-product, which is detected by LC-MS/MS with the use of an internal standard (not shown).

Figure 2.
Structures of GALT-substrate and gal-GALT-product.

Figure 3.
Biotinidase catalyzed conversion of biotinidase-substrate (top) to biotinidase-product (middle). As shown is biotinidase-internal standard (bottom).

Figure 4. Panel A shows the assay using three punches from the DBS, and panel B is for the 2 punch method. See text for more information.

Figure 5.

LC-MS/MS GALT assay using CDC GALT Low, Medium, and High DBS standards and the 3-punch method (filled circles) or the 2-punch method (open circles). The Y-axis is the activity measured by LC-MS/MS, and the X-axis are the activities for the quality control standards determined by the CDC (https://www.cdc.gov/labstandards/pdf/nsqap/GALT_Certification_Set_2_2017.pdf).

Figure 6.
Same as Figure 5 but using the Fluorimetric GALT assay.

Table 1

LC-MS/MS assay. X-ALD analysis

DBS Sample	Method	C26-LPC (ion counts) ¹	d4-C26-LPC (ion counts) ¹	C26-LPC (nM) ²	C26-LPC (nM) Average (% CV)
filter paper (no blood)	3-punch	4.0	2069	essentially 0	
normal adult	3-punch	124	2,241	32.5, 40.1, 31.0, 30.4, 21.4	31.1 (19)
normal adult + 0.5 µM C26-LPC	3-punch	2,714	2,838	560, 470, 556, 527, 528	528 (6)
filter paper (no blood)	2-punch	4.8	1,450	essentially 0	
normal adult	2-punch	181	3,486	30.4, 24.0, 18.6, 23.0, 21.5	23.5 (16)
normal adult + 0.5 µM C26-LPC	2-punch	1,950	3,256	351, 343, 417, 374, 430	383 (9)

¹ Ion counts for a typical run are shown.

² Results from multiple punches from the same DBS are shown.

Table 2

LC-MS/MS assay. Biotinidase analysis

DBS Sample	Method	Product (ion counts) ¹	Internal Standard (ion counts) ¹	Enzymatic Activity (μM/hr/L) ²	Average (% CV)
filter paper (no blood)	3-punch	355	19,084	0.011, 0.013, 0.015, 0.011, 0.012	0.012 (11)
normal adult	3-punch	7,859	10,290	0.45, 0.50, 0.38, 0.40, 0.45	0.44 (9)
normal adult + 0.5 μM C26-LPC	3-punch	14,911	20,895	0.42, 0.61, 0.56, 0.54, 0.60	0.54 (13)
30 Random Newborns	3-punch			0.028 – 0.149	
Galactosemia #1	3-punch			0.15, 0.15, 0.14	0.14 (4)
Galactosemia #2	3-punch			0.32	
Galactosemia #3	3-punch			0.36, 0.27, 0.31	0.31 (12)
Biotinidase deficient #1	3-punch			0.0029	
filter paper (no blood)	2-punch	250	13,542	0.022, 0.021, 0.021, 0.019, 0.020	0.020 (4)
normal adult	2-punch	3,296	13,671	0.28, 0.29, 0.25, 0.28, 0.27	0.27 (6)
normal adult + 0.5 μM C26-LPC	2-punch	3,336	13,455	0.29, 0.38, 0.23, 0.24, 0.38	0.30 (21)
30 Random Newborns	2-punch			0.010 – 0.040	
Galactosemia #1	2-punch			0.063, 0.041, 0.062	0.055 (18)
Galactosemia #2	2-punch			0.12	
Galactosemia #3	2-punch			0.097, 0.059, 0.11	0.087 (23)
Biotinidase deficient #1	2-punch			0.0076	

¹ Ion counts for a typical run are shown.

² Results from multiple punches from the same DBS are shown.

Table 3

LC-MS/MS assay. GALT analysis

DBS Sample	Method	Product (ion counts) ¹	Internal Standard (ion counts) ¹	Enzymatic Activity (μM/hr/L) ²	Average (% CV)
filter paper (no blood)	3-punch	6,155	102,845	0.70, 0.41, 0.80	0.6 (31)
normal adult	3-punch	1,030,366	34,864	346, 279, 285, 286	292 (10)
normal adult + 0.5 μM C26-LPC	3-punch	917,160	29,142	323, 369, 362, 353, 345	350 (4)
CDC GALT low	3-punch	1,745	53,037	0.4, 0.4, 1.8	0.8 (77)
CDC GALT medium	3-punch	390,434	48,674	90, 94, 95	93 (2)
CDC GALT high	3-punch	1,167,212	37,649	309, 300, 313	307 (2)
30 Random Newborns	3-punch			92 – 300	
Galactosemia #1	3-punch			3.0, 2.4, 2.7	2.7 (10)
Galactosemia #2	3-punch			5.6	
Galactosemia #3	3-punch			16.1, 9.3, 14.3	12(22)
Biotinidase deficient #1	3-punch			112	
filter paper (no blood)	2-punch	3,616	69,122	0.4, 1.3, 1.2, 1.2, 1.1	1.0 (34)
normal adult	2-punch	404,961	39,386	241, 229, 201, 200, 187	212 (9)
normal adult + 0.5 μM C26-LPC	2-punch	383,507	39,122	230, 296, 206, 214, 275	244 (14)
CDC GALT low	2-punch	7,442	72,162	2.4, 2.6, 4	3 (23)
CDC GALT medium	2-punch	184,200	65,219	66, 63, 65	65 (2)
CDC GALT high	2-punch	523,826	64,508	190, 200, 238	210 (10)
30 Random Newborns	2-punch			62 – 257	
Galactosemia #1	2-punch			4.5, 3.1, 2.8	3.5 (21)
Galactosemia #2	2-punch			4.1	
Galactosemia #3	2-punch			4.6, 4.2, 4.6	4.4 (4)
Biotinidase deficient #1	2-punch			65	

¹ Ion counts for a typical run are shown.² Results from multiple punches from the same DBS are shown.

Table 4

5-Plex LC-MS/MS assay. MPS-I analysis.

DBS Sample	Method	Product (ion counts) ¹	Internal Standard (ion counts) ¹	Enzymatic Activity (μM/hr/L) ²	Average (% CV)
filter paper (no blood)	3-punch	5,218	242,571	0.019, 0.013, 0.009, 0.013, 0.022	0.016 (31)
normal adult	3-punch	67,140	134,412	0.44, 0.44, 0.50, 0.45, 0.41	0.45 (6)
normal adult + 0.5 μM C26-LPC	3-punch	66,511	113,734	0.51, 0.49, 0.47, 0.50, 0.57	0.51 (6)
filter paper (no blood)	2-punch	1,755	185,908	0.017, 0.015, 0.016, 0.014, 0.033	0.019 (38)
normal adult	2-punch	23,827	81,872	0.51, 0.45, 0.40, 0.46, 0.47	0.46 (8)
normal adult + 0.5 μM C26-LPC	2-punch	18,759	66,009	0.50, 0.56, 0.57, 0.58, 0.66	0.57 (9)
30 Random Newborns	2-punch			0.19 – 1.0	
Galactosemia #1	2-punch			0.21, 0.20, 0.22	0.21 (5)
Galactosemia #2	2-punch			0.31	
Galactosemia #3	2-punch			0.52, 0.46, 0.52, 0.50	0.50 (6)
Biotinidase deficient #1	2-punch			0.57	

¹ Ion counts for a typical run are shown.

² Results from multiple punches from the same DBS are shown.

Table 5

5-Plex LC-MS/MS assay. Pompe disease analysis.

DBS Sample	Method	Product (ion counts) ¹	Internal Standard (ion counts) ¹	Enzymatic Activity (μM/hr/L) ²	Average (% CV)
filter paper (no blood)	3-punch	3,756	401,890	0.014, 0.007, 0.005, 0.007, 0.016	0.010 (44)
normal adult	3-punch	123,659	282,614	0.64, 0.62, 0.64, 0.65, 0.73	0.66 (6)
normal adult + 0.5 μM C26-LPC	3-punch	106,605	248,268	0.63, 0.63, 0.64, 0.65, 0.73	0.66 (6)
30 Random Newborns	3-punch			0.42 – 1.72	
filter paper (no blood)	2-punch	781	244,997	0.009, 0.009, 0.014, 0.007, 0.028	0.013 (57)
normal adult	2-punch	44,457	187,432	0.67, 0.57, 0.62, 0.65	0.64 (7)
normal adult + 0.5 μM C26-LPC	2-punch	31,580	168,100	0.55, 0.65, 0.64, 0.65, 0.84	0.67 (14)
Galactosemia #1	2-punch			0.42 – 1.72	
Galactosemia #2	2-punch			0.11, 0.11, 0.11	0.11 (0)
Galactosemia #3	2-punch			0.36	
Galactosemia #4	2-punch			0.41, 0.39, 0.44	0.41 (4)
Biotinidase deficient #1	2-punch			1.16	

¹ Ion counts for a typical run are shown.

² Results from multiple punches from the same DBS are shown.

Table 6

Fluorescence assay of GALT.

DBS Sample	Method	Fluorimeter Reading	Average (% CV)	GALT Activity (U/g Hb)
blank (normal adult)	3-punch	283,310,276,281,302	290 (4)	
blank (CDC GALT low)	3-punch	292, 293		
blank (CDC GALT medium)	3-punch	370, 307		
blank (CDC GALT high)	3-punch	304, 269		
blank (Galactosemia #1)	3-punch	381, 351		
filter paper	3-punch	198,187,187,201,220	200 (7)	
normal adult	3-punch	10,430, 8,734, 5,920, 8,573,8,247	8381 (17)	21, 18, 12, 17, 17
normal adult + 0.5 μ M C26-LPC	3-punch	7,194, 11,080, 9,943, 10,456, 9550	9644 (14)	15, 22, 20, 21, 19
CDC GALT low	3-punch	374, 358, 447	393 (10)	1.4
CDC GALT medium	3-punch	805, 1,277, 1,429	1170 (23)	3.8
CDC GALT high	3-punch	4,704, 3,355, 4,900	4319 (16)	9.1
30 Random Newborns	3-punch	2,965 – 7,593		5.3 – 12.0
Galactosemia #1	3-punch	605, 545, 590	579 (5)	2.0, 1.9, 2.0
Galactosemia #2	3-punch	573		2.0
Galactosemia #3	3-punch	719, 711, 736	722 (1)	2.2, 2.2, 2.2
Biotinidase deficient #1	3-punch	2,306		4.4
blank (normal adult)	2-punch	284,352,243,309,334	304 (13)	
blank (CDC GALT low)	2-punch	278, 322	300	
blank (CDC GALT medium)	2-punch	362, 309	335	
blank (CDC GALT high)	2-punch	317, 413	365	
blank (Galactosemia #1)	2-punch	385, 566	475	
filter paper	2-punch	255,261,211,239,206	234 (10)	
normal adult	2-punch	3,252, 2,897, 2,446, 2,596, 1,863	2,611 (18)	19, 17, 14, 15, 11
normal adult + 0.5 μ M C26-LPC	2-punch	2,773, 3,666, 3,240, 2,689, 3,393	3152 (12)	16, 22, 19, 16, 20
CDC GALT low	2-punch	354, 362, 357	358 (1)	1.4
CDC GALT medium	2-punch	786, 701, 797	761 (6)	3.8
CDC GALT high	2-punch	1,430, 1,580, 1,806	1605 (2)	9.1
30 Random Newborns	2-punch	1,338 – 3,293		6.0 – 15.0
Galactosemia #1	2-punch	464, 438, 443	448 (2)	1.9, 1.8, 1.8
Galactosemia #2	2-punch	487		2.1
Galactosemia #3	2-punch	516, 547, 629	564 (8)	2.2, 2.3, 2.7
Biotinidase deficient #1	2-punch	1,209		5.5