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Multiplex Lysosomal Enzyme Activity Assay on Dried Blood Spots Using Tandem Mass Spectrometry

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Abstract

Deficiencies in any of the 50 degradative enzymes found in lysosomes results in the accumulation of undegraded material and subsequently cellular dysfunction. Early identification of deficiencies before irreversible organ and tissue damages occur leads to better clinical outcomes. In the method which follows, lysosomal α -glucosidase, α -galactosidase, β -glucocerebrosidase, acid sphingomyelinase, and galactocerebrosidase are extracted from dried blood spots and incubated individually with an enzyme-specific cocktail containing the corresponding substrate and internal standard. Each enzyme cocktail is prepared using commercially available mixture of substrate and internal standard at the predetermined optimized molar ratio. After incubation, the enzymatic reactions are quenched using an ethyl acetate/methanol solution and all five enzyme solutions are combined. The mixtures of the reaction products are prepared using liquid–liquid and solid-phase extractions and quantified simultaneously using selected ion monitoring on LC-MS-MS system.

Keywords

Dried blood spot; β -glucocerebrosidase; acid sphingomyelinase; galactocerebrosidase; lysosomal α -glucosidase; α -galactosidase; lysosomal storage disorders; mass spectrometry; solid phase extraction; enzyme activity assay

1. Introduction

Lysosomal storage disorders (LSDs) are genetic diseases caused by a deficiency in one of the 50 degradative enzymes that hydrolyze proteins, DNA, RNA, polysaccharides, and lipids in lysosomes. In LSDs, undegraded material accumulates in the lysosomes of affected individuals, causing an increase in the size and number of lysosomes within cells, cellular dysfunction, and progressive clinical manifestations (1). Most LSDs result from deficiencies in a single lysosomal enzyme. The prevalence of lysosomal storage disorders as a group is $\sim 1/5,000$ (2). Enzyme replacement therapy and umbilical stem cell transplantation are available for some of LSDs (3, 4). Better clinical outcomes are achieved if patients are treated early, before irreversible organ and tissue damages occur (3–5). Given these realizations, there is now widespread interest in newborn screening or high-risk population screening for treatable LSDs.

This chapter focuses on the use of tandem mass spectrometry (MS) to measure the activity of five lysosomal enzymes, lysosomal α -glucosidase (GAA), α -galactosidase (GLA), β -glucocerebrosidase (GBA), acid sphingomyelinase (ASM), and galactocerebrosidase (GALC). These enzymes are deficient in Pompe disease, Fabry disease, Gaucher disease, Niemann-Pick type A/B, and Krabbe disease, respectively (6–10). The enzymes are

extracted from dried blood spots on filter paper and incubated individually and concurrently with the enzyme-specific substrate and internal standard. The substrates are the structural analogues to the natural substrates. This avoids the problem that some mutations may render the enzyme less active on an artificial substrate while activity on the natural substrate is maintained or vice versa.

The enzymatic reactions are shown in Fig. 32.1. Each enzyme cocktail is prepared directly from the commercially available mixture of substrate and internal standard at the predetermined and optimized molar ratio. The enzymatic reactions are quenched using an ethyl acetate/methanol solution and subsequently the five reaction solutions are combined. The mixtures of the reaction products are prepared using liquid–liquid and solid-phase extraction and quantified simultaneously using selected ion monitoring on a LC-MS-MS system. Recently the enzyme activity assay for mucopolysaccharidosis I (11) is being incorporated into the multiplex assay; and efforts are underway to develop tandem MS assays for other lysosomal enzymes and other diseases caused by deficiency in specific enzymes.

2. Materials

2.1. Samples: Dried Blood Spot (DBS) Preparation from Venous Whole Blood

1. Collect venous blood in EDTA tube(s). If blood is collected the blood samples, ship at a remote location on cold packs for overnight delivery and hold at 4°C upon arrival.
2. Mix the blood by inverting the tube several times.
3. Pipet 75 µL of well-mixed whole blood onto the filter paper. The five LSD enzymes tested in this assay are stable in whole blood held at 4°C for up to 72 h.
4. Dry the spotted cards horizontally without touching each other for 4–8 h at ambient room temperature.
5. Store the dried cards in sealed plastic bags containing desiccant and a humidity indicator card at 4°C for up to 1 week or at –20°C for extended periods.

2.2. Reagents and Buffers (see Note 1)

1. 0.1 M zinc chloride solution
2. N-Acetylgalactosamine (GALNAc), and acarbose (Toronto Research Chemicals, Toronto, Ontario, Canada).
3. Analyte specific reagent (ASR) of each of the following enzyme substrates (S) and internal standards (IS) mixtures, (CDC Foundation, Atlanta, GA, USA) (see Note 2):

¹All solutions and buffers should be prepared with HPLC-grade water. Sterile filter buffers to remove particulates and debris. Assay buffers do not need to be handled as sterile solutions. Discard any solutions showing evidence of contamination.

²Obtain ASRs and QC samples from the CDC Foundation:

Dr. Hui Zhou/Dr. Victor De Jesus
CDC Foundation
Newborn Screening and Molecular Biology Branch
Centers for Disease Control and Prevention
4770 Buford Hwy NE, Mail stop F19
Atlanta, GA 30341

- i. Acid sphingomyelinase (ASM) ASR: Each vial typically contains a mixture of ASM-S and ASM-IS at a molar ratio of 50:1 with the total weight of 3.41 mg.
 - ii. β -glucocerebrosidase (GBA) ASR: Each vial typically contains a mixture of GBA-S and GBA-IS at a molar ratio of 50:1 with the total weight of 7.84 mg.
 - iii. Lysosomal α -glucosidase (GAA) ASR: Each vial typically contains a mixture of GAA-S and GAA-IS at a molar ratio of 100:1 with the total weight of 7.97 mg.
 - iv. α -galactosidase (GLA) ASR: Each vial typically contains a mixture of GLA-S and GLA-IS at a molar ratio of 500:1 with the total weight of 38.78 mg.
4. Galactocerebrosidase (GALC) substrate (S) and internal standard (IS) (Genzyme Pharmaceutical, Liestal, Switzerland). Each vial contains a mixture of GALC-S and GALC-IS at a molar ratio of 150:1 with the total weight of 10.6 mg.
5. Quench solution: Mix equal volumes of ethyl acetate and methanol (both analytical grade). Stable when stored at room temperature for 1 month.
6. Wash solution: Mix 190 mL of ethyl acetate and 10 mL of methanol (analytical grade). Stable when stored at room temperature for 1 month.
7. Mobile phase: 80% acetonitrile and 20% water with 0.2% formic acid.
8. Extraction buffer solution: 20 mM sodium phosphate, pH 7.1. Add 2.40 g of sodium phosphate monobasic (NaH_2PO_4) to a beaker containing about 900 mL of water and stir to dissolve the powder. Adjust the pH to 7.1 with sodium hydroxide. Bring the solution to 1000 mL with water. Sterile filter and store at room temperature for up to 6 months.
9. Acid sphingomyelinase (ASM) buffer solution: Add 28.45 g of sodium acetate trihydrate to a beaker containing about 200 mL of water and stir to dissolve the powder. Add 1.34 mL of glacial acetic acid, and 1.51 mL of 0.1 M zinc chloride solution to the beaker. Adjust pH to 5.7 with sodium hydroxide or acetic acid (*do not use hydrochloric acid*). Bring the solution to 250 mL with water. The final acetate concentration is 0.92 M (pH 5.7). Sterile filter and store at 2–8°C for up to 6 months.
10. GAA, GBA, and GALC buffer solutions: Add 10.20, 21.45, or 5.99 g of sodium phosphate monobasic, respectively, to beakers containing about 200 mL of water. Add 12.5, 26.29, or 7.35 g of sodium citrate tribasic dehydrate, respectively, to the above solutions and stir until dissolved. Adjust the pH of the buffers to 4.0, 5.1, or 4.4, respectively, with hydrochloric acid or sodium hydroxide. Bring each solution to 250 mL with water. The final citrate–phosphate concentration is 0.3 M (pH 4.0) for GAA, 0.62 M (pH 5.1) for GBA, and 0.18 M (pH 4.4) for GALC buffer solution. Sterile filter and store at 2–8°C for up to 6 months.
11. GLA buffer solution: Add 2.45 g of sodium acetate trihydrate to a beaker containing about 200 mL of water and stir to dissolve the powder. Add 1.46 mL of glacial acetic acid. Adjust pH to 4.6 with sodium hydroxide or acetic acid (*do not use hydrochloric acid*). Bring the solution to 250 mL with water. The final acetate concentration is 0.174 M (pH 4.6). Sterile filter and store at 2–8°C for up to 6 months.

2.3. Detergent and Inhibitor Solutions

1. 120 g/L sodium taurocholate in water. Store at -20°C for up to 6 months.
2. 96 g/L sodium taurocholate plus 12 g/L oleic acid in water. Store at -20°C for up to 3 months.
3. 100 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in water. Store at -20°C for up to 3 months.
4. 0.8 mM acarbose in water. Make fresh on the day that assay cocktails are prepared.
5. 1.0 M N-Acetylgalactosamine (GALNAc) in water. Make fresh on the day that assay cocktails are prepared.

2.4. Assay Cocktails (see Notes 3, 4, and 5)

1. ASM assay cocktail: Add 0.15 mL of 120 g/L sodium taurocholate in water to one vial of ASM ASR and vortex briefly. Add 17.85 mL of ASM buffer solution to the vial and vortex again. The final ASM assay solution contains 0.33 mM of ASM-S, 6.67 μM of ASM-IS, 1.0 g/L sodium taurocholate, 0.6 mM of zinc chloride, and 0.92 M sodium acetate, pH 5.7.
2. GBA assay cocktail: Add 2.40 mL of 120 g/L sodium taurocholate in water to one vial of GBA ASR and vortex briefly. Add 15.6 mL of GBA buffer to the vial and vortex again. The final GBA assay solution contains 0.67 mM of GBA-S, 13.33 μM of GBA-IS, 16.0 g/L sodium taurocholate, and 0.62 M of phosphate plus 0.31 M of citrate, pH 5.1.
3. GALC assay cocktail: Add 1.8 mL of 96 g/L sodium taurocholate plus 12 g/L oleic acid solution in water to one vial of GALC ASR and vortex briefly. Add 16.2 mL of GALC buffer to the vial and vortex again. The final GALC assay solution contains 1 mM of GALC-S, 6.67 μM of GALC-IS, 9.6 g/L sodium taurocholate, 1.2 mM of oleic acid, and 0.18 M phosphate plus 0.09 M citrate, pH 4.4.
4. GAA assay cocktail: Add 1.8 mL of 100 g/L CHAPS in water to one vial of GAA ASR and vortex briefly. Add 15.9 mL of GAA buffer followed by 0.3 mL of 0.8 mM acarbose in water to the vial and vortex again. The final GAA assay solution contains 0.67 mM of GAA-S, 6.67 μM of GAA-IS, 10 g/L CHAPS, 13.3 μM acarbose, and 0.3 M phosphate plus 0.15 M citrate, pH 4.0.
5. GLA assay cocktail: Add 0.45 mL of 120 g/L sodium taurocholate in water to one vial of GLA ASR and vortex briefly. Add 14.67 mL of GLA buffer followed by 2.88 mL of 1.0 M GALNAc in water to the vial and vortex again. The final GLA assay solution contains 3.33 mM of GLA-S, 6.67 μM of GLA-IS, 3 g/L sodium taurocholate, 160 mM GALNAc, and 0.142 M sodium acetate, pH 4.6.
6. Assay cocktails are stable for up to 24 weeks at 4°C or up to 3 month at -20°C . The stability may be extended after further testing.

³All assay cocktail solutions prepared to 18 mL are sufficient for approximately 600 tests (wells) for GALC or 1200 tests for the other four enzymes. It is important to add the detergent first when making assay cocktails. Aliquots sufficient for a single day's use may be prepared and stored frozen to avoid repeated freeze thaw cycles. If more than one vial of an assay cocktail is prepared, pool reconstituted vials of the same assay cocktail together before making aliquots.

⁴If an assay cocktail looks hazy or particulates are visible, heat it briefly in warm tap water and/or sonicate it at room temperature for 5 min.

⁵Assay cocktails are stable for up to three freeze-thaw cycles.

2.5. Calibration Solutions

1. P and IS calibration solutions for each enzyme (CDC Foundation, Atlanta, GA) (*see* Note 2).
2. Prepared solutions with P/IS ratios of 0, 0.05, 0.1, 0.5, 1.0, and 2.0. Verify ratios using mass spectrometry. The amount of IS is fixed at 0.1 μM for ASM, GAA, GLA, and GALC assays and 0.2 μM for GBA assay.
3. Example of regression analysis of the calibration curves is illustrated in Table 32.1.

2.6. Quality Control Samples

Quality control samples with low, medium, and high enzyme activities (CDC foundation, Atlanta, GA) (10) (*see* Note 2).

2.7. Equipment and Supplies

1. Protein Saver 903 specimen collection filter paper card (Whatman, Kent, UK or equivalent)
2. Deep 96-well polypropylene plates
3. Polypropylene 96-well plates
4. Aluminum plate sealer
5. Shaking microplate incubator capable of ~ 875 rpm
6. Temperature controlled orbital shaker capable of 225 ± 25 rpm
7. 96-well plate evaporator
8. 0.45 μm 96-well filter plates (Seahorse Labware, Chicopee, MA)
9. 96-well PVC plates (Falcon 353912, BD Biosciences, Bedford, MA)
10. Silica gel (230–400 mesh, 6000C5, Merck, Grade 9385) (Sigma Aldrich, St Louis, MO)
11. Solid-phase extraction MultiScreen_{HTS} vacuum manifold and plate adaptors (Millipore, Billerica, MA)
12. Leap HTC PAL autosampler (Leap Technology, Carrboro, NC)
13. Agilent 1100 binary HPLC system (Agilent, Santa Clara, CA)
14. API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada)

3. Methods

3.1. Extraction of Dried Blood Spots

1. Punch two spots from each DBS sample using a 1/8th inch (3.2 mm) hole puncher and place one spot in one polypropylene 96-well plate and the other spot into the corresponding well of the second plate. When all samples have been punched, cover but do not seal the second plate and set it aside at room temperature to be used for the GALC assay. Do not use a plate sealer to cover the second plate as static electricity may cause the spots to stick to the sealer.
2. To the first plate, add 70 μL of extraction buffer to each well containing spots and seal the plate with an aluminum plate sealer.

3. Centrifuge the plate at $2,000 \times g$ for 1 min (optional). Incubate the plate at 37°C for 60 min while shaking at about 875 RPM.
4. Remove the plate from the incubator after 1 h and centrifuge at $2,000 \times g$ for 1 min to collect the extract to the bottom of the wells. Proceed immediately to the enzymatic reactions step.

3.2. Enzymatic Reactions

1. Thaw the assay cocktails at room temperature. The step needs to be done prior to completion of the DBS extraction. Sonicate all five thawed assay cocktails.
2. Label four 96-well plates as ASM, GBA, GAA, or GLA. To each plate add 15 μL of ASM, GBA, GAA, or GLA assay cocktail into each well. To limit evaporation, set up assay cocktail plates no more than 10 min prior to the completion of the DBS extraction step.
3. Add 10 μL of the DBS extract to the corresponding well of each of the four plates containing assay cocktail. Cover each plate with an aluminum plate sealer.
4. To the plate previously set aside containing one whole punch per sample, add 30 μL of GALC assay cocktail. Cover the plate with an aluminum plate sealer.
5. The compositions of each enzymatic reaction mixtures are summarized in Table 32.2.
6. Centrifuge all five plates at $2,000 \times g$ for 1 min to ensure sample and assay cocktail mix together at the bottom of each well. Incubate at 37°C for 20–24 h with orbital shaking at 225 ± 25 RPM.
7. When incubation is complete add 100 μL of quenching solution into each well of all five plates. Work with one assay plate at a time.
8. Transfer the contents of the wells associated with the same sample on all five plates into one corresponding well of a deep-well polypropylene plate.

3.3. Sample Clean-Up

1. To limit dripping, pre-wet pipette tips with EA. Add 400 μL of EA followed by 400 μL of water into each well of the deep-well plate. Aspirate and dispense the solutions three times. Seal the plate with an aluminum plate sealer.
2. Centrifuge the plate for 5 min at $2,000 \times g$ to form the two-phase separation.
3. Transfer 300 μL of the top organic layer of each sample to a new deep-well plate. Bring to complete dryness under a stream of nitrogen on a 96-well plate evaporator. To speed the drying process, the plate may be gently heated to 25°C .
4. Reconstitute the plate by adding 100 μL of wash solution into each well.
5. Cover the plate with an aluminum plate sealer and shake it at about 200 RPM for 5 min on a plate shaker.
6. Prepare a silica gel filter plate. Load a 96-well PVC plate with silica to the top of each well (contains ~ 100 mg of silica gel per well). Scrape off excess silica with a glass slide. Invert an empty filter plate onto the silica-filled plate, taking care to align all the wells. With gloved hands hold the two plates tightly together and flip them over to transfer the silica into the wells of the filter plate.
7. Place a deep-well plate in the lower chamber of a vacuum manifold.

8. Condition the silica gel in the filter plate by adding 250 μL of wash solution. Apply gentle vacuum to draw the solvent through the silica gel (*see* Note 6). Discard the wash solution collected in the lower chamber.
9. After conditioning the silica gel, place a clean deep-well plate in the lower chamber of the vacuum manifold.
10. Transfer the reconstituted and mixed samples from step 5 to the corresponding well of the filter plate. Apply gentle vacuum to draw the samples through the silica gel and collect each sample in the deep-well plate in the lower chamber of the vacuum manifold.
11. Wash each well of the filter plate four times with 400 μL of wash solution, collecting each wash in the deep-well plate for a total wash volume of 1,600 μL .
12. Dry the deep well plate under a stream of nitrogen.
13. When dry, cover the plate with an aluminum plate sealer and store it at -20°C for future mass spectrometry analysis.
14. Prior to the mass spectrometry analysis, reconstitute the plate with 200 μL of mobile phase into each well.
15. Cover the plate with an aluminum plate sealer and shake it at about 200 RPM for 5 min on a plate shaker.
16. Remove the plate sealer and cover the plate with aluminum foil for mass spectrometry analysis. Do not use heavy weight aluminum foil.

3.4. MS-MS Analysis

1. MS-MS analysis is performed on an API 4000 triple quadrupole mass spectrometer (*see* Note 7). The instrument is operated in positive ion mode. All analytes are monitored by selected reaction monitoring (SRM). The typical experimental set-up on the API 4000 is listed in Table 32.3.
2. Thirty μL of sample solution is injected by an autosampler and delivered at a flow rate of 200 $\mu\text{L}/\text{min}$ with the HPLC system. The total run time on the LC-MS system is 3 min per sample.

3.5. Data Analysis

1. The enzyme activity is calculated using the formula below:

$$\text{Activity } (\mu\text{mol}/\text{hr}/\text{L of whole blood}) = 1000 * (\text{P}/\text{IS}) * [\text{IS}] / (\text{RF} * \text{V} * \text{T})$$

Where,

P/IS = measured product to internal standard ratio

[IS] = amount of internal standard injected (nmol);

0.1 nmol for GAA-IS, GLA-IS, and ASM-IS;

0.2 nmol for GALC-IS and GBA-IS.

RF = response factor ratio of product to internal standard

⁶Keep the vacuum gentle enough to avoid forming channels through the silica beds of the filter plate.

⁷The assay has been performed on other mass spectrometers, including, Sciex 3200, Quattro Micro, and Quattro Premier as a part of an investigation for assay comparability in different laboratories. Comparable results have been obtained on all instruments.

V = volume of whole blood used in the assay (μL)

T = incubation time (hour)

2. RF is the slope of the linearity curve of P and IS using P/IS calibration solutions.
3. The volume of blood used in the assay is 0.457 (one-seventh of 3.2) μL whole blood in GAA, GLA, GBA, and ASM assays and 3.2 μL in GALC assay.
4. The blank activity is subtracted from the measured activity to obtain the final activity of each sample. The blank activity is the average activity of blank filter paper samples ($n \geq 4$) on the same plate with samples.

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Fig. 32.1.

Substrates, products, and internal standards for the five lysosomal enzyme activity assays.

The masses of the products and internal standards are indicated. The ceramide products and internal standards ASM-P, ASM-IS, GALC-P, GALC-IS, GBA-P, and GBA-IS undergo CID to give the common ammonium ion shown (m/z = 264). GLA-P, GLA-IS, GAA-P, and GAA-IS undergo neutral-loss CID to give four different secondary ammonium ions.

Enzymatic reactions with DBS are shown by solid arrows, and CID is shown by dashed arrows. Reproduced from Ref. (7) with permission from the American Association for Clinical Chemistry (AACC).

Table 32.1

Regression analysis of the calibration curves

	N	Slope	Intercept	r ²	Sdy	Sdslope	Sdintercept
ASM	150	1.019	-0.0079	0.9988	0.0255	0.0029	0.0028
GBA	150	1.058	-0.0033	0.9994	0.0178	0.0021	0.0019
GALC	150	0.959	0.0008	0.9994	0.0164	0.0019	0.0018
GLA	150	0.941	-0.0001	0.998	0.0301	0.0035	0.0033
GAA	84	0.745	0.0044	0.9965	0.0653	0.005	0.0091

r²: correlation coefficient

Sdy: standard deviation of measured product/internal standard ratio

Sdslope: standard deviation of slope

Sdintercept: standard deviation of intercept

Table 32.2
Summary of the assay cocktail compositions used in the multiplex assay reaction mixtures

Assay	DBS punch	Assay volume μ L	[S] mM	[IS] μ M	Buffer	Detergent	Additional components
GAA	1/7 ^a	25	0.4	4	0.18 M citrate-phosphate, pH 4.0	6 g/L CHAPS	8 μ M acarbose
GLA	1/7 ^a	25	2	4	0.09 M sodium acetate, pH 4.6	1.8 g/L sodium taurocholate	96 mM GALNAc
GBA	1/7 ^a	25	0.4	8	0.37 M citrate-phosphate, pH 5.1	9.6 g/L sodium taurocholate	none
ASM	1/7 ^a	25	0.2	4	0.55 M sodium acetate, pH 5.7	0.6 g/L sodium taurocholate	0.36 mM zinc chloride
GALC	1 ^b	30	1	6.7	0.18 M citrate-phosphate, pH 4.4	9.6 g/L sodium taurocholate	1.2 g/L oleic acid

^a10 out of 70 μ L of DBS extractant was used in the assay, equivalent to 1/7 of DBS.

^bOne whole DBS was used in the assay.

Reproduced from Ref. (7) with permission from AACC.

Table 32.3

Typical parameters used on the API 4000 triple quadrupole mass spectrometer

LC Conditions			
Mobile Phase:	80/20 acetonitrile/water with 0.2% formic acid		
Flow Rate:	0.2 ml/min		
Gradient:	Isocratic		
Washes:	Methanol with 0.1% formic acid and 50/50 isopropanol/methanol		
Filter:	HAIFILTER from Higgins Analytical Part #: HF-FKIT		
Injection Volume:	20 µl		
MS/MS Conditions			
Instrument:	MDS SCIEX API 4000 triple quadrupole		
ES Source:	Turbo spray ES+		
Dwell Time:	100 msec		
Source Temp.:	200°C		
CAD Gas:	4		
Curtain Gas:	23 psi		
Gas 1:	23 psi		
Gas 2:	32 psi		
IonSpray Voltage:	4500 V		
Declustering Potential:	20 V		
Collision Cell Exit Potential:	20 V		
Name	MRM	CE (eV)	EP (V)
ASM-IS	370.32>264.27	23	2
ASM-P	398.36>264.27	23	2
GALC-IS	454.42>264.27	29	2
GALC-P	426.39>264.27	29	2
GBA-IS	510.48>264.27	33	2
GBA-P	482.45>264.27	31	2
GLA-IS	489.30>389.30	24	7
GLA-P	484.27>384.27	18	7
GAA-IS	503.32>403.32	21	7
GAA-P	498.29>398.29	27	7