Clinical Chemistry 54:10
1725–1728 (2008)

Brief Communications

Multiplex Enzyme Assay Screening of Dried Blood Spots for Lysosomal Storage Disorders by Using Tandem Mass Spectrometry

X. Kate Zhang,* Carole S. Elbin, Wei-Lien Chuang, Samantha K. Cooper, Carla A. Marashio, Christa Beauregard, and Joan M. Keutzer

Genzyme Corporation, Framingham, MA; *address correspondence to this author at: Five Mountain Road, Framingham, MA 01701. Fax 508-872-9080; e-mail kate.zhang@genzyme.com.

BACKGROUND: Reports of the use of multiplex enzyme assay screening for Pompe disease, Fabry disease, Gaucher disease, Niemann-Pick disease types A and B, and Krabbe disease have engendered interest in the use of this assay in newborn screening. We modified the assay for high-throughput use in screening laboratories.

METHODS: We optimized enzyme reaction conditions and procedures for the assay, including the concentrations of substrate (S) and internal standard (IS), assay cocktail compositions, sample clean-up procedures, and mass spectrometer operation. The S and IS for each enzyme were premixed and bottled at an optimized molar ratio to simplify assay cocktail preparation. Using the new S:IS ratio, we validated the modified assay according to CLSI guidelines. Stability of the S, IS, and assay cocktails were investigated. Dried blood spots from 149 healthy adults, 100 newborns, and 60 patients with a lysosomal storage disorder (LSD) were tested using the modified assay.

RESULTS: In our study, the median enzyme activity measured in adults was generally increased 2–3–fold compared to the original method, results indicating higher precision. In the multiplex format, each of the 5 modified enzyme assays enabled unambiguous differentiation between samples from healthy individuals (adults and newborns) and the corresponding disease-specific samples.

CONCLUSIONS: The modified multiplex enzyme assay with premixed S and IS is appropriate for use in high-throughput screening laboratories.

Pompe disease, Fabry disease, Gaucher disease, Niemann-Pick disease types A and B (NP A/B),[1] and Krabbe disease are lysosomal storage disorders (LSDs) caused by deficiencies of acid α-glucosidase (GAA, EC 3.2.1.20), acid α-galactosidase (GLA, EC 3.2.1.22), acid β-glucocerebrosidase (GBA, EC 3.2.1.45), acid sphingomyelinase (ASM, EC 3.1.4.12), and galactocerebrosidase (GALC, EC 3.2.1.46), respectively *(1)*. The availability of disease-specific therapies and the possibility that early diagnosis may lead to improved patient outcomes have prompted several groups to develop methods to screen newborns for LSDs *(2, 3)*. Li et al. *(3)* developed a novel approach to determine GAA, GLA, GBA, ASM, and GALC activities in dried blood spots (DBS). The extractant from one 5-mm DBS was used in 5 concurrent individual enzymatic reactions, and the activities were measured simultaneously by tandem mass spectrometry (MS/MS). We optimized the assay method reported by Li et al., modifying the procedures for sample extraction, assay cocktail composition, sample clean-up, and MS/MS operation. We validated the assay by using samples from healthy individuals (adult and newborn) and samples from patients with LSDs.

The structures of the substrate (S) and internal standard (IS) for GAA, GLA, GBA, ASM, and GALC have been published *(3)*. S and IS, individually and mixed at a fixed S:IS ratio, were manufactured by Genzyme Pharmaceuticals. The molar ratios of S:IS for GAA, GLA, GBA, ASM and GALC were 100, 500, 50, 50 and 150, respectively. The sources of chemicals and materials are in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol54/issue10.

Specimens from previously diagnosed patients (14 Pompe disease, 10 Krabbe disease, 13 Fabry disease, 13 Gaucher disease, and 10 NP A/B patients) were collected with the institutional research board–approved protocol after patients gave written informed consent. Specimens from 149 apparently healthy adults were purchased from ProMedDx LLC. The preparation and storage of DBS are summarized in the online Data Supplement. One hundred deidentified newborn DBS samples were obtained from the University Children's Hospital Vienna, Vienna, Austria.

During assay optimization, the S/IS stock solutions, assay buffers, and cocktails were prepared as previously reported *(3)*. After optimization, we mixed the S and IS for each enzyme in a fixed molar ratio to simplify and standardize assay cocktail preparation. For the GAA assay cocktail, 1.8 mL of 100 g/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, CAS No. 75621-03-3) in water was added to a vial of GAA S/IS and vortex-mixed briefly. Next

[1] Nonstandard abbreviations: NP A/B, Niemann-Pick disease types A and B; LSD, lysosomal storage disorder; GAA, acid α-glucosidase; GLA, acid α-galactosidase; GBA, acid β-glucocerebrosidase; ASM, acid sphingomyelinase; GALC, galactocerebrosidase; DBS, dried blood spots; MS/MS, tandem mass spectrometry; S, substrate; IS, internal standard; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Table 1. Assay cocktail compositions used in the multiplex assay.

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

[a] 10 of 70 μL of DBS extractant was used in the assay, equivalent to 1/7 of 1 whole DBS.
[b] One whole DBS was used in the assay.

15.9 mL of 0.3 mol/L of phosphate citrate buffer and 0.3 mL of 0.8 mmol/L acarbose (Toronto Research Chemicals) in water were added, and the vial was vortex-mixed. The final GAA assay cocktail contained 0.67 mmol/L of GAA-S, 6.67 μmol/L of GAA-IS, 10 g/L CHAPS, 13.3 μmol/L acarbose, 0.3 mol/L phosphate, and 0.15 mol/L citrate, pH 4.0. Preparation of the other 4 assay cocktails is described in the online Data Supplement.

The multiplex enzyme assay was adapted to a 96-well plate. Chloroform was replaced with ethyl acetate. The composition and volume of solvents used in liquid/liquid- and solid-phase extractions were modified. We used two 3.2-mm DBS punches instead of one 5-mm punch. One punch was incubated directly in the GALC assay cocktail, thereby eliminating the enzyme elution step. Complete details of the modified assay protocol are in the online Data Supplement. We monitored all analytes by using selected-reaction monitoring, rather than constant neutral loss or parent-ion scanning *(3)*, with an API 4000 triple-quadrupole MS/MS (MDS Sciex). The enzyme activity of each sample was calculated from the ion abundance ratio of product to IS measured by MS. All enzyme activities were blank subtracted (details in the online Data Supplement). Ion suppression was effectively controlled, as demonstrated by the regression analysis of calibration curves collected during the validation study (Table 1 in the online Data Supplement).

With use of the previously reported method *(3)*, ASM activities measured in samples from adults and patients with NP A/B overlapped. Zn^{2+} is required for optimal activity of both secreted and lysosomal ASMs *(4–6)*, which are deficient in NP A/B patients. We chose the 2 highest-activity NP A/B patient samples and 3 low-activity adult samples and monitored their activities with increasing concentrations of $ZnCl_2$. ASM activities in the adult samples increased with $ZnCl_2$, with maximal distinction between the normal and disease samples at 0.5–1.5 nmol/L $ZnCl_2$.

The previously reported GALC assay gave high CVs attributable to low activity values, i.e., the minimum activity in an adult sample was 0.6 μmol/h/L in whole blood. When we varied pH, S, taurocholate, and oleate concentrations, GALC activity increased in adult samples. The S concentration was increased 5-fold to 1 mmol/L (Supplemental Fig. 1 in the online Data Supplement). We determined that GALC activity was preserved by eluting samples directly into the GALC assay cocktail.

With the use of maltose or acarbose as an inhibitor of maltase glucoamylase, DBS can be used in the diagnosis of Pompe disease *(3, 7)*. With the fluorogenic substrate 4-methylumbelliferyl-α-glucoside, acarbose concentrations of 3–9 μmol/L are sufficient to inhibit maltase glucoamylase activity in the assay *(8, 9)*. We found that with the synthetic substrate in this assay, 8 μmol/L acarbose provided sufficient inhibition of maltase glucoamylase and maintenance of GAA activity (Supplemental Fig. 2 in the online Data Supplement). To minimize the effect of detergent on the MS/MS, Triton X-100 was replaced by CHAPS, which is more effectively removed by solid-phase extraction and results in an increase in GAA activity.

To improve assay precision and facilitate assay automation, the GLA assay was modified to use a 15-μL instead of a 2.5-μL assay cocktail. Optimization of N-acetyl galactosamine, buffer, and detergent concentrations increased GLA activity and decreased assay variation. Changes to the GBA assay were limited to increasing the S concentration. The modifications of

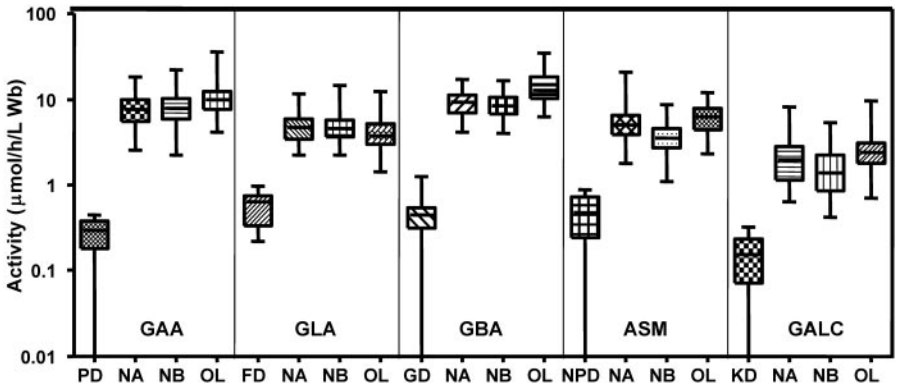

Fig. 1. Comparison of enzyme activities in DBS samples from healthy adults and newborns, patients with LSDs (with corresponding enzyme deficiencies), and the remaining 4 disease samples in each enzyme assay.

NA, 149 normal adults; NB, 100 newborns; PD, 14 Pompe disease patients; GD, 13 Gaucher disease patients; NPD, 10 Niemann-Pick disease patients; FD, 13 Fabry disease patients; KD, 10 Krabbe disease patients; OL, patients with the other 4 LSDs; Wb, whole blood.

the 5 assays are summarized in Table 1, and their comparison with the previous assay are summarized in Supplemental Table 2 in the online Data Supplement.

The assay was validated according to the CLSI standard protocol *(10)*. For each enzyme assay, within-plate imprecision was calculated using 20 punches from 1 healthy adult sample and 1 corresponding patient sample. The within-plate CVs for the adult sample in the GAA, GLA, GBA, ASM, and GALC assays were 8.3%, 9.8%, 11.9%, 12.5%, and 5.8%, respectively, and for the disease sample were 10.1%, 24.8%, 24.8%, 23.1%, and 12.3%, respectively. Overall CVs in the GAA, GLA, GBA, ASM, and GALC assays were 7.5%, 14.0%, 11.7%, 13.7%, and 8.2%, respectively. The limits of the blank for the GAA, GLA, GBA, ASM, and GALC assay were 0.02, 0.09, 0.32, 0.25, and 0.02 μmol/h/L in whole blood, respectively. The limits of detection (defined as the 1.65 times the SD above the limits of the blank) of the GAA, GLA, GBA, ASM, and GALC assays were 0.15, 0.29, 0.50, 0.66, and 0.07 μmol/h/L in whole blood, respectively. We verified that the GAA, GLA, GBA, ASM, and GALC assay results were linear in the range between the limits of detection and 7.2, 4.9, 5.3, 7.6 and 4.7 μmol/h/L in whole blood, respectively. Analysis performed with assay cocktails stored for 14 days at 37 °C, 30 days at 25 °C and 45 days at 2–8 °C resulted in measured activities that were within 20% of those for once-frozen assay solutions (Supplemental Figs. 2–5 in the online Data Supplement). The use of assay cocktails after 1, 2, and 3 freeze-thaw cycles resulted in enzyme activities within 20% of those measured in freshly prepared assay solutions (Supplemental Fig. 6 in the online Data Supplement). There was no detectable MS/MS carryover in the assay.

Each of the 5 modified enzyme assays in the multiplex format showed an unambiguous distinction between samples from healthy individuals (adult and newborn) and corresponding samples from patients with LSDs (Fig. 1 and Supplemental Table 3 in the online Data Supplement). The median activities in samples from healthy adults (n = 149) were increased 2–3-fold relative to the original method. In the GAA and GALC assays, the separation between normal and disease samples became even more pronounced, and the disease sample with the highest percentage of mean normal activity decreased from 15% to 5% and 19% to 15%, respectively. The limits of detection of each assay were at least 2-fold below the maximum observed disease activity.

The availability of S and IS premixed for each enzyme assay has simplified cocktail preparation. The modified assay proved to be reproducible and reliable for the differentiation of normal and disease-specific samples. Transfer of the high-throughput multiplex enzyme assay to newborn screening laboratories should be a straightforward process.

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

or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures of Potential Conflicts of Interest: *Upon submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: Christa Beauregard, Genzyme; Joan M Keutzer, Genzyme.
Consultant or Advisory Role: None declared.
Stock Ownership: Christa Beauregard, Genzyme
Honoraria: None declared.

Research Funding: None declared.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We are grateful to the patients and physicians who contributed samples and to Dr. Olaf Bodamer, University Children's Hospital Vienna, Vienna, Austria, for contributing newborn DBS for our study.

References

1. Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. 8th ed. New York (NY): McGraw-Hill; 2001.
2. Kemper AR, Hwu W-L, Lloyd-Puryear M, Kishnani PS. Newborn screening for Pompe disease: synthesis of the evidence and development of screening recommendations. Pediatrics 2007;120: e1327–e34.
3. Li Y, Scott CR, Chamoles NA, Ghavami A, Pinto BM, Turecek F, Gelb MH. Direct multiplex assay of lysosomal enzymes in dried blood spots for newborn screening. Clin Chem 2004;50: 1785–96.
4. Schuchman EH, Desnick RJ. Niemann-Pick disease types A and B: sphingomyelinase deficiencies. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic and molecular bases of inherited disease. 8th ed. New York (NY): McGraw-Hill; 2000:3589–610.
5. He X, Chen F, McGovern MM, Schuchman EH. A fluorescence-based, high-throughput sphingomyelin assay for the analysis of Niemann-Pick disease and other disorders of sphingomyelin metabolism. Anal Biochem 2002;306: 115–23.
6. He X, Chen F, Gatt S, Schuchman EH. An enzymatic assay for quantifying sphingomyelin in tissue and plasma from humans and mice with Niemann-Pick disease. Anal Biochem 2001;293: 204–11.
7. Chamoles NA, Niizawa G, Blanco M, Gaggioli D, Casentini C. Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper. Clin Chim Acta. 2004;347: 97–102.
8. Kallwass H, Carr C, Gerrein J, Titlow M, Pomponio R, Deeksha B, et al. Rapid diagnosis of late-onset Pompe disease by fluorometric assay of alpha-glucosidase activities in dried blood spots. Mol Genet Metab 2007;90:449–52.
9. Zhang H, Kallwass H, Young SP, Carr C, Dai J, Kishnani PS, Millington DS, et al. Comparison of maltose and acarbose as inhibitors of maltase-glucoamylase activity in assaying acid alpha-glucosidase activity in dried blood spots for the diagnosis of infantile Pompe disease. Genet Med 2006;8:302–6.
10. Clinical and Laboratory Standards Institute. Tentative guideline EP5-T: user evaluation of precision performance of clinical chemistry devices. Wayne (PA): Clinical and Laboratory Standards Institute (formerly NCCLS); 1984.

DOI: 10.1373/clinchem.2008.104711