

Validation of a Multiplex Tandem Mass Spectrometry Method for the Detection of Selected Lysosomal Storage Diseases in Dried Blood Spots

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Abstract

Background: Interest in screening methods for lysosomal storage diseases (LSDs) has increased in recent years, since early diagnosis and treatment are essential to prevent or attenuate the onset of symptoms and the complications of these diseases. In the current work, we evaluated the performance of tandem mass spectrometry (MS/MS) for the detection of some LSDs, aiming the future use of this methodology for the screening of these disorders. **Methods:** Standard curves and quality control dried blood spots were assayed to evaluate the precision, linearity, and accuracy. A total of 150 controls were grouped according to age and subjected to measurement of lysosomal enzymes deficient in Niemann-Pick A/B, Krabbe, Gaucher, Fabry, Pompe, and Mucopolysaccharidosis type I diseases. Samples from 59 affected patients with a diagnosis of LSDs previously confirmed by fluorimetric methods were analyzed. **Results:** Data from standard calibration demonstrated good linearity and accuracy and the intra- and interassay precisions varied from 1.17% to 11.60% and 5.39% to 31.24%, respectively. Except for galactocerebrosidase and α -L-iduronidase, enzyme activities were significantly higher in newborns compared to children and adult controls. Affected patients presented enzymatic activities significantly lower compared to all control participants. **Conclusion:** Our results show that MS/MS is a promising methodology, suitable for the screening of LSDs, but accurate diagnoses will depend on its correlation with other biochemical and/or molecular analyses.

Keywords

lysosomal storage diseases, multiplex enzyme assay, tandem mass spectrometry

Introduction

Lysosomal storage diseases (LSDs) comprise a heterogeneous group of more than 50 genetic disorders, which result in the accumulation of macromolecular substrates that would normally be degraded/processed by proteins/enzymes involved in lysosomal metabolism.^{1,2} Although individual LSDs are rare, their combined incidence has been estimated at 1 per 7700 live births.¹ The progressive accumulation of these molecules leads to cellular dysfunction, which may affect both somatic organs and the central nervous system. Clinical features suggestive of LSDs include developmental delay, progressive regression after a period of normal development, ataxia, seizures, weakness, and dementia.^{2,3}

Since substrate accumulation and its tissue distribution can be variable even among patients with identical genotypes, the

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symptoms of LSDs are not always recognized early in life.⁴ Lysosomal storage diseases are usually diagnosed through biochemical assays for the enzyme of interest by using an artificial substrate with a fluorescent tag such as 4-methylumbelliferone.⁵ However, the availability of suitable treatments for some of these disorders has resulted in increased efforts to develop new, reliable, and robust methods to perform high-throughput population screening. Thus, there is a growing consensus for the development of methods to detect these disorders before the onset of clinical symptoms, then allowing the early begin of therapeutic interventions.^{6,7}

In 2006, Gelb and colleagues⁸ designed a method to directly analyze the activity of lysosomal enzymes by using electrospray ionization tandem mass spectrometry (MS/MS). The method was first tested to detect galactocerebrosidase (GALC) activity, which is deficient in Krabbe disease, in human cell lysates. Later, this technique was adapted for dried blood spot (DBS) samples and new substrates (S) and internal standards (IS) were developed for Gaucher, Fabry, Pompe, and Niemann-Pick diseases, and more recently, for Mucopolysaccharidosis type I (MPS-I).⁹ Since then, screening for LSDs by MS/MS has been implemented in North America and European countries as pilot projects.¹⁰⁻¹²

The present study addresses the implementation of the detection of 6 LSDs by MS/MS (Fabry, Niemann-Pick A/B, Pompe, Gaucher, Krabbe, and MPS-I diseases) by measuring the activities of α -galactosidase (GLA), acid sphingomyelinase (ASM), α -glucosidase (GAA), β -glucocerebrosidase (ABG), GALC, and α -L-iduronidase (IDUA), respectively, in DBS samples from affected and control individuals with different ages. Analyses were performed at Medical Genetic Service of Hospital de Clínicas de Porto Alegre (SGM/HCPA), Brazil, which is a reference center for the diagnosis of inborn errors of metabolism in Latin America.

Materials and Methods

Reagents

Reagents were purchased from Sigma Chemical Co (St Louis, Missouri). Cocktails containing the enzyme substrates and ISs were kindly donated by the Newborn Screening Translation Research Initiative at the North American Center for Disease Control and Prevention (CDC, Atlanta, Georgia). For the instrument calibration, standards with known ratios between the product of the reaction and the IS for each enzyme of interest were injected, which were also provided by CDC.

Patients and Controls

All participants of this study were recruited from SGM/HCPA, Brazil. A total of 150 control individuals were divided into 3 groups according to age: 50 newborns (mean age: 7.12 days), 42 children (mean age: 4.15 years), and 58 adults (mean age: 37.0 years).

Sixty-two DBSs from affected patients diagnosed at our institution by classic fluorimetric or radioisotopic methods (8 with Niemann-Pick A/B, 18 with MPS-I, 7 with Krabbe disease, 10 with Gaucher disease, 6 with Pompe disease, 10 with Fabry disease, and 3 heterozygous women for Fabry disease) were used as positive controls. Informed consent was obtained from all patients. For method validation, DBSs of the CDC Quality Assurance Program were used as positive controls.¹³ This research was approved by the institutional ethics committee of HCPA, Brazil (project 13-0239).

Assay Cocktails Preparation

For preparing the GAA assay cocktail, 1.8 mL of a 100 g/L solution of CHAPS in water, 15.9 mL of a buffer 0.34 M sodium phosphate + 0.17 M sodium citrate, pH 4.0, and 0.3 mL of a 8 mM solution of acarbose in water were added to a vial containing GAA-S/IS. The GLA assay cocktail was prepared by adding 0.45 mL of a 120 g/L solution of sodium taurocholate in water, 14.67 mL of 0.174 M sodium acetate buffer pH 4.6, and 2.88 mL of 1 M solution of *N*-acetylgalactosamine in water to a vial containing GLA-S/IS. The ABG assay cocktail consisted of 2.4 mL of a 120 g/L solution of sodium taurocholate in water and 15.6 mL of 0.72 M phosphate–0.36 M citrate buffer, pH 5.1, in a vial containing ABG-S/IS. For preparing the ASM assay cocktail, 0.15 mL of a 120 g/L solution of sodium taurocholate in water and 17.85 mL of 0.93 M sodium acetate plus 0.604 mM zinc chloride buffer, pH 5.7, were added to a vial containing ASM-S/IS. The GALC assay cocktail was prepared by adding 1.8 mL of a solution containing 96 g/L sodium taurocholate with 12 g/L oleic acid in water and 16.2 mL of 0.2 M phosphate–0.1 M citrate buffer, pH 4.4, to a vial containing GALC-S/IS. Finally, for preparing the IDUA assay cocktail, 0.5 mL of inhibitor solution (3.0 mM *D*-saccharic acid 1,4 lactone monohydrate in water) and 17.5 mL of 0.11 M sodium formate–0.16 M formic acid buffer, pH 3.6, were added to a vial containing IDUA-S/IS.

Sample Preparation

All the procedures were conducted based on the methods described by Zhang et al.¹⁴ and Duffey et al.¹⁵ We punched out 2 DBS disks of 3.2-mm diameter from each card. One disk was placed in a 96-well plate for the GALC assay, and the other disk was placed on a plate containing 70 μ L of sodium phosphate elution buffer and then mixed on an orbital shaker for 1 hour at 250 rpm at 37°C. Each enzyme cocktail (15 μ L), except the GALC assay cocktail, was added to a separate plate, and 10 μ L of this DBS extract was added to it. The GALC assay cocktail (30 μ L) was added to the GALC assay plate. All the plates were incubated at 37°C for 22 hours. After the reactions were complete, they were quenched with 100 μ L of ethyl acetate–methanol (1:1) solution. Then, the 6 enzyme assays were mixed and the samples were extracted by adding 400 μ L of both ethyl acetate and water to the plate. Then, 300 μ L of the top organic layer was transferred to a deep-well plate, which was dried

under N₂ gas. The dried extract was resuspended in a 100 μ L of ethyl acetate–methanol (19:1) solution. Next, a solid-phase extraction was conducted by adding 100 μ L of the resuspended extract into a multi-well filter plate (AcroPrep 96 Filter Plate 0.45 μ m polytetrafluoroethylene) containing 100 mg of silica gel. This plate was washed 4 times with 400 μ L of ethyl acetate–methanol (19:1) solution by using a microplate vacuum filtration apparatus. After drying the plate under N₂ gas, the extract was resuspended in 100 μ L of the mobile phase (80% acetonitrile, 20% water containing 0.2% formic acid) for injection in the MS/MS equipment.

Tandem Mass Spectrometry Analysis

Electrospray ionization MS/MS was performed using a Waters Quattro Micro API tandem mass spectrometer (Waters, Massachusetts) in a positive-ion, multiple-reaction monitoring mode. A volume of 20 μ L for each resuspended sample was injected into a Binary HPLC Pump using a Waters 2777C Sample Manager via a flow injection of 0.1 mL/min for 0.10 minutes, then decreasing to 0.05 mL/min until 1.5 minutes and backing to 0.1 mL/min until 2 minutes. The mobile phase consisted of 80/20 acetonitrile/water with 0.2% formic acid. The amount of product was calculated by multiplying the ratio of ion abundance of the product to that of the IS with the amount of the added IS and then dividing the value by the ratio of the response factor for each enzyme, which was calculated from the calibration curves obtained for the standards containing known ratios of product and IS. The enzyme activities were expressed in μ mol/h/L and were calculated from the amount of product by assuming that a 3.2-mm DBS disk contained 3.2 μ L of blood.

Evaluation of Precision, Accuracy, and Linearity

Intra-assay precisions (intra-assay coefficients of variation [CVs]) were determined by performing 3 replicated assays for the CDC QC samples at concentrations that showed medium and high activity. To evaluate the interassay CVs, enzyme activities of the same CDC samples were measured on 7 consecutive assays. Linearity was evaluated by analyzing the slopes and the correlation coefficients R^2 obtained from calibration curves constructed by injection of 6 calibration solutions containing the substrate and IS for each enzyme in predefined ratios (P/IS: 0, 0.10, 0.50, 1.0, 2.0, and 5.0). The accuracy was evaluated by comparing the enzymatic activities obtained in quality control DBSs with the results predetermined by the CDC.

Statistical Analysis

Comparison between enzyme activities from different individuals was performed using the nonparametric Mann-Whitney U test. A P value lower than .05 was considered significant. For the determination of the intra-assay and interassay CVs, results were expressed as mean (standard deviation). All analyses were

Table 1. Conditions for Tandem Mass Spectrometry and Multiple Reaction Monitoring (MRM) Transitions of the Products and Internal Standards.

	Cone Voltage, V	Collision Energy, eV	MRM Transition, m/z
ASM-P	28	25	398.4-264.4
ASM-IS	25	19	370.4-264.4
ABG-P	20	28	482.4-264.4
ABG-IS	28	27	510.4-264.4
GAA-P	20	20	498.4-398.4
GAA-IS	20	18	503.4-403.4
GLA-P	20	15	484.4-384.4
GLA-IS	18	15	489.4-389.4
GALC-P	20	20	426.4-264.4
GALC-IS	30	28	454.4-264.4
IDUA-P	12	15	391.1-291.20
IDUA-IS	19	12	377.10-277.20

Abbreviations: ABG, β -glucocerebrosidase; ASM, acid sphingomyelinase; GAA, α -glucosidase; GALC, galactocerebrosidase; GLA, α -galactosidase; IDUA, α -L-iduronidase; IS, internal standards; P, products.

performed using Statistical Package for the Social Sciences (SPSS) software, version 19.0, on a PC-compatible computer.

Results

Parameters of MS/MS analysis and data for the calibration standards are listed in Tables 1 and 2, respectively. Data demonstrated good linearity, with correlation coefficient R^2 ranged from 0.975 to 0.9991. In order to evaluate the precision and accuracy of the technique, quality control materials provided by the CDC were assayed. These materials correspond to a pool of inactivated cord blood supplemented with 5% (low), 50% (medium), and 100% (high) unprocessed cord blood.¹³ As demonstrated in Table 3, the mean of the enzyme activities measured in 7 consecutive assays was comparable to those reported by the CDC. The intra-assay CVs for GAA were 11.60% and 4.97%, for GLA were 5.96% and 4.91%, for ABG were 4.15% and 5.19%, for ASM were 1.42% and 6.67%, for IDUA were 11.33% and 6.08%, and for GALC were 7.86% and 1.17%, in medium and high QC samples, respectively. Inter-assay CVs were higher compared to intra-assay CVs; the inter-assay CVs for GAA were 5.8% and 11.25%, for GLA were 30.08% and 31.24%, for ABG were 8.37% and 5.39%, for ASM were 13.70% and 14.22%, for IDUA were 17.20% and 13.07%, and for GALC were 18.22% and 19.91%, at medium and high levels of enzyme activity, respectively.

The enzyme activities in the DBS samples from normal participants (newborns, infants, and adults) and nonnewborn patients are shown in Figures 1–6. As expected, the enzyme activities in the DBSs of patients were consistently lower than those obtained for the controls. Interestingly, the median activities of the enzymes GAA, GLA, ABG, and ASM were higher in newborns in relation to older controls. α -L-Iduronidase activity in control infants was higher in relation to newborns and adults. However, GALC activity did not present significant

Table 2. Calibration Results and Linearity Parameters.

Ratio P/IS	GAA, Observed Ratio	GLA, Observed Ratio	ABG, Observed Ratio	ASM, Observed Ratio	IDUA, Observed Ratio	GALC, Observed Ratio
0.00	0.03	0.003	0.019	0.022	0.03	0.02
0.10	0.11	0.12	0.094	0.11	0.12	0.11
0.50	0.57	0.54	0.45	0.47	0.47	0.49
1.00	1.09	0.99	0.90	1.11	0.96	0.99
2.00	1.93	2.11	2.07	1.96	1.91	1.96
5.00	3.54	5.06	4.17	4.6	4.63	4.8
R ²	0.975	0.99	0.99	0.99	0.99	0.99
Slope	0.70	0.99	0.84	0.92	0.92	0.97

Abbreviations: ABG, β -glucocerebrosidase; ASM, acid sphingomyelinase; GAA, α -glucosidase; GALC, galactocerebrosidase; GLA, α -galactosidase; IDUA, α -L-iduronidase; IS, internal standards; P, products.

Table 3. Comparison Between the Enzyme Activities Measurements ($\mu\text{mol/h/L}$) in Quality Control DBS Samples Determined in SGM/HCPA and by the CDC.

	ASM	GALC	ABG	GAA	GLA	IDUA
Reference values determined in QC DBS samples by the CDC						
Base pool	(0.0-0.33)	(0.03-0.12)	(0.0-0.45)	(0.0-0.60)	(0.0-0.90)	(0.0-0.41)
Low	(0.10-0.40)	(0.26-0.43)	(0.19-0.90)	(0.47-1.02)	(0.19-1.23)	(0.0-0.99)
Medium	(1.25-1.96)	(2.36-3.48)	(3.93-6.29)	(5.42-7.83)	(4.60-6.88)	(3.54-5.46)
High	(2.33-3.70)	(4.95-6.83)	(7.03-12.35)	(10.14-15.63)	(8.01-14.09)	(7.04-12.42)
Enzyme activities obtained from 7 consecutive assays in SGM/HCPA. Results were expressed as mean \pm SD						
Base pool	0.18 \pm 0.07	0.07 \pm 0.02	0.28 \pm 0.14	0.48 \pm 0.21	0.79 \pm 0.45	0.44 \pm 0.12
Low	0.39 \pm 0.17	0.33 \pm 0.08	0.75 \pm 0.24	0.98 \pm 0.16	1.16 \pm 0.33	0.82 \pm 0.27
Medium	2.20 \pm 0.30	2.82 \pm 0.51	5.61 \pm 0.47	5.30 \pm 0.30	6.16 \pm 1.85	5.53 \pm 0.95
High	4.48 \pm 0.63	5.54 \pm 1.10	11.30 \pm 0.60	10.37 \pm 1.16	12.62 \pm 3.94	11.59 \pm 1.51

Abbreviations: ABG, β -glucocerebrosidase; ASM, acid sphingomyelinase; CDC, Centers for Diseases Control and Prevention; DBS, dried blood spots; GAA, α -glucosidase; GALC, galactocerebrosidase; GLA, α -galactosidase; IDUA, α -L-iduronidase; SGM/HCPA, Medical Genetic Service of Hospital de Clínicas de Porto Alegre; SD, standard deviation.

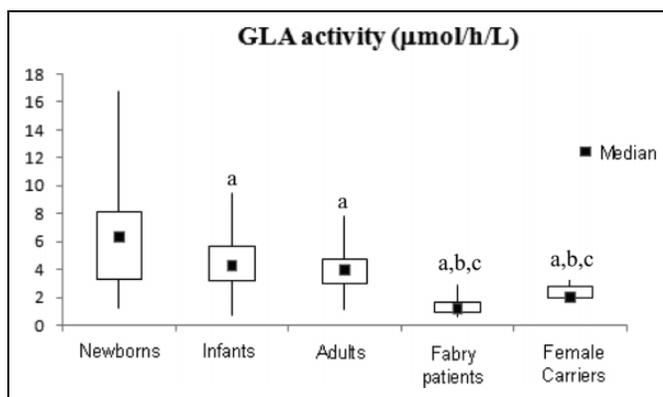


Figure 1. α -Galactosidase (GLA) activity in dried blood spot (DBS) samples from controls grouped according to age and from patients with Fabry disease. The error bars show the range of enzyme activity, the box represents those results within the 95th percentile, and the "■" symbol represents the median (Mann-Whitney *U* test, *P* < .05). ^aDifferent from newborns. ^bDifferent from infants. ^cDifferent from adult controls.

alterations between the different groups of controls. Using cutoff points of 30% of the median activity observed (0.51 $\mu\text{mol/h/L}$ for Krabbe disease, 4.0 $\mu\text{mol/h/L}$ for Gaucher disease, 1.91 $\mu\text{mol/h/L}$ for Fabry disease, 3.0 $\mu\text{mol/h/L}$ for Pompe disease, 2.15 $\mu\text{mol/h/L}$ for MPS-I, and 2.5 $\mu\text{mol/h/L}$ for Niemann-Pick A/B), we did not observe overlaps between the enzyme activities of the affected patients and healthy controls of any age group for the enzymes GALC, ASM, GAA, and ABG. For MPS-I, 3 patients presented IDUA activity higher than those observed in newborn controls but lower in relation to nonnewborn normal participants. For Fabry disease, some patients presented GLA activity similar to the values of normal individuals, which could be associated with the enzyme replacement therapy.

Discussion and Conclusion

The availability of therapies for many LSDs, the relatively high combined incidence, the delay between the onset of symptoms

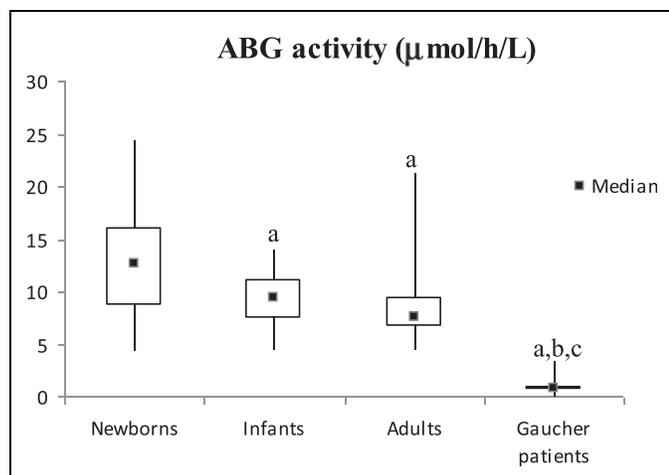


Figure 2. β -Glucocerebrosidase (ABG) activity in dried blood spot (DBS) samples from controls grouped according to age and from patients with Gaucher disease. The error bars show the range of enzyme activity, the box represents those results within the 95th percentile, and the “■” symbol represents the median (Mann-Whitney U test, $P < .05$). ^aDifferent from newborns. ^bDifferent from infants. ^cDifferent from adult controls.

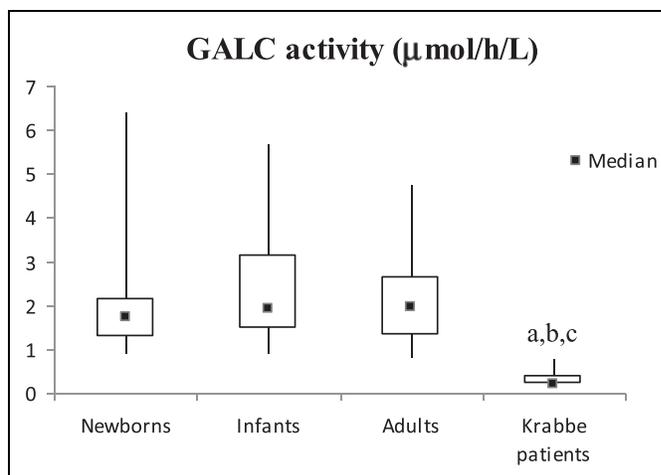


Figure 4. Galactocerebrosidase (GALC) activity in dried blood spot (DBS) samples from controls grouped according to age and from patients with Krabbe disease. The error bars show the range of enzyme activity, the box represents those results within the 95th percentile, and the “■” symbol represents the median (Mann-Whitney U test, $P < .05$). ^aDifferent from newborns. ^bDifferent from infants. ^cDifferent from adult controls.

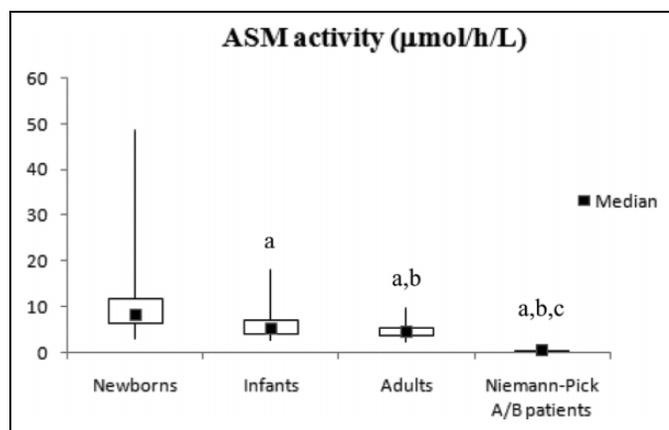


Figure 3. Acid sphingomyelinase (ASM) activity in dried blood spot (DBS) samples from controls grouped according to age and from patients with Niemann-Pick A/B disease. The error bars show the range of enzyme activity, the box represents those results within the 95th percentile, and the “■” symbol represents the median (Mann-Whitney U test, $P < .05$). ^aDifferent from newborns. ^bDifferent from infants. ^cDifferent from adult controls.

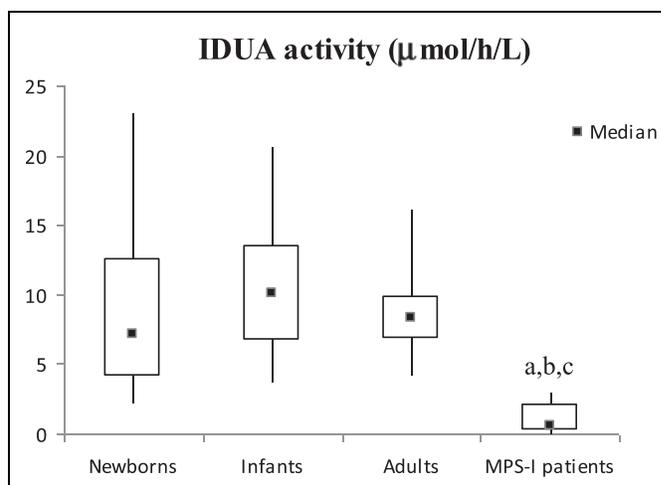


Figure 5. α -L-Iduronidase (IDUA) activity in dried blood spot (DBS) samples from controls grouped according to age and from patients with mucopolysaccharidosis type I (MPS-I). The error bars show the range of enzyme activity, the box represents those results within the 95th percentile, and the “■” symbol represents the median (Mann-Whitney U test, $P < .05$). ^aDifferent from newborns. ^bDifferent from infants. ^cDifferent from adult controls.

and diagnosis, and the benefits of early start of treatment justify the implementation of LSDs into routine newborn screening protocols.⁶ Studies carried out in Brazil indicated an average delay of 4.8 years between the onset of symptoms in patients with MPS and conclusive laboratory diagnosis,¹⁶ which may also occur for other LSDs. In Brazil, a study demonstrated that the median time between the onset of first symptoms of Fabry disease and diagnosis was 20.3 years in males and 14.3 years in females.¹⁷ This delay in diagnosis does not allow early initiation of appropriate treatment that could prevent many complications of the disease.

In 2001, Chamoles and colleagues¹⁸ reported the possibility of diagnosing lysosomal enzymes using DBSs, opening the door for the screening of LSDs. Considering that conventional methods of quantifying enzymatic activity, such as spectrophotometry and fluorometry, have the technical limitations of non-specificity and limited capacity for multiplexing, MS/MS has become an excellent option for this purpose, enabling the investigation of different LSDs simultaneously in a single patient's sample, with high specificity and sensitivity.⁷

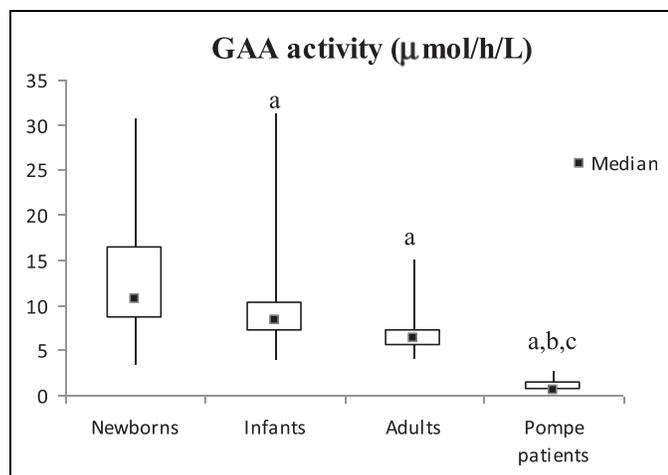


Figure 6. α -Glucosidase (GAA) activity in dried blood spot (DBS) samples from controls grouped according to age and from patients with Pompe disease. The error bars show the range of enzyme activity, the box represents those results within the 95th percentile, and the "■" symbol represents the median (Mann-Whitney U test, $P < .05$). ^aDifferent from newborns. ^bDifferent from infants. ^cDifferent from adult controls.

Although pilot projects for LSD screening by MS/MS are being reported since 2006 in North America, Europe, and Asia,⁹ there are few laboratories in Brazil investing in this area. Lysosomal storage diseases seem to be relatively frequent in Brazil. In 1997, a study published by Coelho et al¹⁹ demonstrated a higher proportion of LSDs (59.8%) diagnosed by selective screening at Medical Genetic Service of Hospital de Clínicas de Porto Alegre, Brazil, during the period from 1982 to 1995, compared to other groups of inborn errors of metabolism, such as aminoacidopathies and organic acidemias.

Therefore, aiming the future use of the MS/MS methodology for the screening of LSDs in our laboratory, in this work we validated the methodology for analysis of 6 lysosomal enzymes, GAA, GLA, ABG, ASM, IDUA and GALC, in DBS samples, evaluating the precision and accuracy of the method, as well as the ability in discriminating between positive and negative cases.

Results obtained from the calibration curves for method validation demonstrated linearity with the expected P/IS ratios and the enzyme activities determined in QC DBS samples correlated well with those reported by the CDC, demonstrating a good accuracy. Besides, CDC quality control specimens showed a good distinction between high and low activity levels for all analytes. In relation to the precision, the intra- and interassay CVs were comparable to those reported by other authors,^{20,21} except for GLA which showed higher rates of variation.

When analyzing the activities of GAA, GLA, ABG, ASM, IDUA, and GALC in DBS samples from healthy controls, a wide variation in the reference values has been reported by different laboratories, which can be explained by analytical and instrumental modifications used in the analysis. Although the number of normal samples analyzed in this work is small, our preliminary results showed that the median activities of these

6 enzymes for newborns and nonnewborn controls were comparable to those reported in the literature^{22,23} and the assays enabled unambiguous differentiation between samples obtained from healthy individuals and patients, suggesting that the method was effective in detecting Pompe, Fabry, Gaucher, Niemann-Pick A/B, MPS-I, and Krabbe diseases. Corroborating with findings published by Brand et al,²⁴ our results also demonstrated that some lysosomal enzymes present a higher activity in newborns when compared to older individuals.

In this study, GLA was the enzyme that showed less discrimination between samples from affected patients and healthy individuals, especially when compared with the activities found in adults. The median GLA activity in patients with Fabry disease was 71.36% lower than those observed in adult controls; however, the medians for GAA, ABG, ASM, IDUA, and GALC were 90%, 89.02%, 90.2%, 93%, and 89.44%, respectively, lower than those obtained for normal adult individuals. It is also important to emphasize that 5 patients with Fabry disease were under enzyme replacement therapy, which probably contributed to higher levels of enzyme activity. Only through the screening of a higher number of samples from untreated patients will be possible to accurately determine the difference in GLA activity in Fabry disease compared to normal participants. Even so, other biochemical assays may be interesting for Fabry disease investigation to confirm the diagnosis. Our results also agree with those obtained by other authors, showing that women heterozygous for Fabry disease present GLA activity close to normality.^{7,25}

In the current work, we were able to detect precision in patients with Pompe, Fabry, Gaucher, Niemann-Pick A/B, MPS-I, and Krabbe diseases by MS/MS. However, additional analyses with a larger number of DBS samples are in progress in order to better determine the normal range of these enzyme activities, as well as the specificity of the method by analyzing false-negative or false-positive cases. It should also be mentioned that for all cases of abnormal or nondiscriminative results, leukocytes isolation or a fibroblast skin culture should be requested for confirmation of the enzyme deficiency. It is also important to correlate the results of biochemical assays with clinical data of patients aiming to achieve an accurate diagnosis.

In conclusion, we evaluated the performance of MS/MS in detecting 6 LSDs, and the preliminary results of this study suggest that this methodology can be successfully employed in the screening of LSDs, which will allow faster diagnosis and treatment of patients, reducing the morbidity of the diseases and improving patient survival and quality of life.

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Declaration of Conflicting Interests

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