

Newborn Screening for Lysosomal Storage Disorders in Belgium: The Importance of Sex- and Age-Dependent Reference Ranges

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Abstract

Lysosomal storage disorders (LSDs) are a group of metabolic disorders with various clinical presentations, which complicate diagnosis. A pilot study was performed to test the appropriateness and effectiveness of the newborn screening method for Pompe disease, Fabry disease and mucopolysaccharidosis (MPS) I in dried blood spots using liquid chromatography–tandem mass spectrometry. Around 20 000 newborn samples were analyzed for 3 lysosomal enzyme activities: α -glucosidase (deficient in Pompe disease), α -galactosidase (deficient in Fabry disease) and α -iduronidase (IDUA, deficient in MPS I). Data were used for statistical analysis and to establish sex- and age-dependent reference ranges. Statistically significant higher α -glucosidase, α -galactosidase, and IDUA enzyme activities were observed in female newborns compared to male newborns. Newborns with a higher gestational age have a statistically significant lower α -glucosidase, α -galactosidase, and IDUA enzyme activities compared to newborns with a lower gestational age. For the first time, the data of a large-scale LSD study were used to assess statistical differences in enzyme activity in the newborn population, and these data highlight the importance of using reference intervals for lysosomal enzyme activities in function of sex and gestational age.

Keywords

neonatal screening, lysosomal storage diseases, tandem mass spectrometry, cutoff, dried blood spots

Introduction

Lysosomal storage disorders (LSDs) are a group of metabolic disorders with various clinical presentations, which complicate diagnosis.^{1–3} Since effective LSD treatments, including enzyme replacement therapy, substrate reduction therapy, and stem cell transplantation, have become available and timely initiation of these treatments is necessary, early diagnosis has become essential and could be achieved by newborn screening.^{4–7}

Tandem mass spectrometry (MS/MS) has become an established tool for the detection of rare congenital metabolic disorders in newborn screening laboratories. An additional advantage of MS/MS-based enzyme assays is the capacity for multiplexing the analytical process by simultaneous introduction of products and internal standards (IS) into the mass spectrometer. To achieve this, synthetic enzyme substrates (S), products, and IS are designed so as to have mutually exclusive molecular masses.⁸ The establishment of enzyme assay screening in separate buffers, but by multiplex analysis on MS/MS for Pompe disease, Fabry disease, Gaucher disease, Niemann-Pick disease types A and B, and Krabbe disease, has prompted interest in the use of this assay in newborn screening.^{9,10}

In 2010, Duffey and coworkers reported on analyzing the enzyme activity of α -glucosidase (GAA), α -galactosidase A (GLA), and α -l-iduronidase (IDUA) in a single buffer. By assaying 3 enzymes at once, problematic sample preparation is spotted for reanalysis if enzyme activity values are low for all enzymes.¹¹ More recently, enzyme activities (acid GAA, galactocerebrosidase, glucocerebrosidase, GLA, IDUA, and sphingomyelin phosphodiesterase-1) were measured in a single buffer with very low number of screen positives.¹²

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The results of previous studies have identified sample handling as the major bottleneck in the screening procedure. These involved sample purification by solid-phase or liquid–liquid extraction to remove the S, buffer salts, and other assay additives that could impede electrospray ionization or cause interferences. Recently, research groups reported developments of liquid chromatography (LC)-based methods to introduce samples from lysosomal enzyme assays into the tandem mass spectrometer.^{13–15} When comparing the LC-based method with the method based on liquid–liquid extraction into ethyl acetate and flow injection for the triplex analysis of Pompe, Fabry, and Hurler diseases, the LC method was found to be superior in robustness and good quality data while requiring fewer liquid transfer steps and less disposable material and labor. Also obviating the offline sample preparation by including LC columns led to substantial savings in analytical time (approximately 70%) and reagent costs (approximately 50%).¹⁶ The LC-based method has advantages for expanding the assays to include additional products and IS for multiplexing all 9 currently available lysosomal enzyme assays in dried blood spot (DBS; Gaucher, Niemann-Pick, Fabry, Krabbe, Pompe, mucopolysaccharidosis [MPS] I, MPS II, MPS IV, and MPS VI) as well as allowing other metabolites to be quantified.¹⁷

Due to these technological advances, the worldwide interest for newborn LSD screening increased substantially. The results of pilot LSD screening studies show that the current clinical prevalence is underestimated. In a study performed by Scott et al in the United States, where more than 100 000 newborn DBS were evaluated, the prevalence of Fabry disease, Pompe disease, and MPS I combined is 1 in 7500. This is 2 to 4 times higher than the clinical diagnosis of these disorders.¹⁸ The practicality and appropriateness of including Gaucher, Niemann-Pick A/B, Pompe, Fabry, and MPS I in newborn screening panels were assessed in Austria by analyzing over 34 000 newborn babies. The combined overall proportion of LSD mutations was higher than expected, with most frequent mutations for Fabry disease (1 per 3859 births), followed by Pompe disease (1 per 8684) and Gaucher disease (1 per 17 368).¹⁵ Several LSD screening programs were initiated in various countries worldwide, such as the United States (Illinois), Italy, Korea, Brazil, and Hungary, all showing that the method using MS/MS was proven to be a robust, easy, valid, and feasible technology in newborn screening programs.^{18–22}

In summary, the LC-MS/MS method provides a non-laborious and effective approach to high-throughput multiplex screening for inborn errors of metabolism to be considered by newborn and high-risk population screening laboratories. Therefore, the “Provinciaal Centrum voor de Opsporing van Metabole Aandoeningen” (PCMA), responsible for the newborn screening program in the Flemish region of Belgium, has performed a pilot study to test the appropriateness and effectiveness of the LC-MS/MS screening method for Pompe disease, Fabry disease, and MPS I. Moreover, the need for age- and sex-dependent

reference ranges in the newborn population was evaluated and established.

Materials and Methods

Study Design and Population

An anonymous pilot project was conducted in DBS samples from 20 000 newborns, collected in the period from June 2015 to February 2016, in addition to the routine Flemish screening program that runs in the PCMA (around 40 000 newborn screenings yearly, with a coverage of 99.9%). Three lysosomal enzyme activities: GAA (deficient in Pompe disease), GLA (deficient in Fabry disease) and IDUA (deficient in MPS I) were analyzed. Study statistics was performed with MedCalc (MedCalc Software v 17.5.5). Kolmogorov-Smirnov test was used for data distribution. Nonparametric Mann-Whitney *U* test was performed for evaluating gender and gestation age parameters in enzymatic activity. Correlation coefficients were determined, and correlation between gestation age or weight and enzyme activity was graphically evaluated. Analysis of covariance was performed on log-transformed data to test the combined differences between gender and gestation. The ethics committee of the Antwerp University Hospital (UZA) in Belgium approved the study (B300201110577).

We used base, low, medium, and high control materials for the validation of the method and blanco (filter paper only), dummy (no paper), medium, and high controls for the pilot study. The control materials were provided by the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia).²² Pilot study plates were evaluated as valid when the enzyme activity of the quality controls met the internal quality control ranges. If the quality standards were not met, the study plate was repeated. All retests were performed within 3 weeks upon sample receipt. Dried blood spots from potentially enzyme-deficient newborns were retested. We tested LSD enzyme activity stability on DBS of healthy volunteers stored airtight with desiccant at 4 temperatures (room temperature, 4°C, –20°C and –80°C) for 0, 7, 13, 20, 69, and 97 days of storage.

Standards, Chemicals, and Reagents

The S and IS for GAA, GLA, and IDUA were manufactured at Genzyme Pharmaceutical (Liestal, Switzerland) and are available through the Newborn Screening Branch at the CDC.²² Ammonium formate (VWR International, PA, USA), sodium taurocholate (Sigma-Aldrich, MO, USA), *N*-acetyl-D-galactosamine (Sigma-Aldrich, MO, USA), acarbose (Sigma-Aldrich, MO, USA), acetonitrile (Biosolve, the Netherlands), water (Biosolve, the Netherlands), methanol (Biosolve, the Netherlands), and formic acid (VWR International, PA, USA) were purchased. All chemicals and solvents were of the highest purity available and were used without any further purification.

Table 1. List of All Monitored SRM Transitions Corresponding to Products and Internal Standards With Cone Voltage and Collision Energy.

Analyte	Function (Time)	Parent (Da)	Daughter (Da)	Dwell (seconds)	Cone (V)	Collision (eV)
IDUA-IS	1 (0.00-0.48)	377.10	277.10	0.015	25	16
IDUA-P	1 (0.00-0.48)	391.19	291.13	0.015	25	16
GLA-P	2 (0.49-1.00)	484.25	384.20	0.015	16	15
GLA-IS	2 (0.49-1.00)	489.31	389.21	0.015	16	15
GAA-P	2 (0.49-1.00)	498.25	398.24	0.015	18	15
GAA-IS	2 (0.49-1.00)	503.33	403.28	0.015	18	15

Abbreviations: GAA, acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase; IS, internal standards; P, product; SRM, selected reaction monitoring.

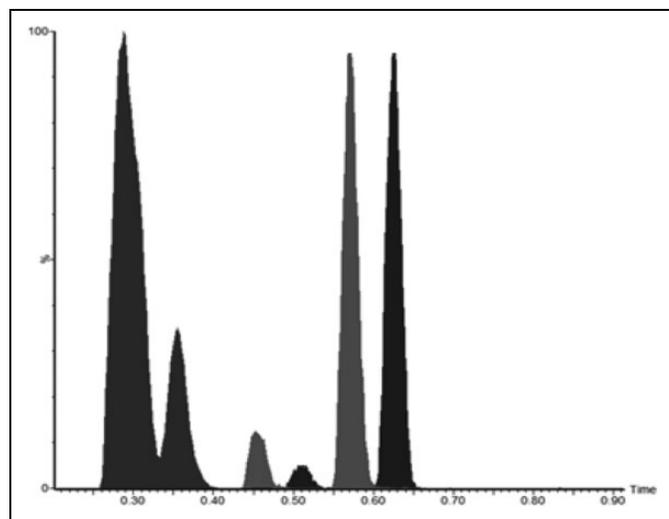


Figure 1. Substrate chromatographically separated from product. Relative abundance of IDUA substrate (S), IDUA enzymatic product (P), GLA-S, GAA-S, GLA-P, and GAA-P in function of retention time. All substrate that is broken down to product in the MS/MS source is separated from the enzymatically generated product. GAA denotes acid α -glucosidase; GBA, glucocerebrosidase; GLA, α -galactosidase A; IDUA, α -iduronidase.

Sample Preparation and Workflow

Here follows a summary and modifications of the sample preparation and workflow that was described by Spacil et al¹⁷ and used for the pilot study in our laboratory. For the GAA, GLA, and IDUA enzyme activity analysis, we used one 3.2 mm DBS punch in 96-well plates (0.5 mL, round bottom; VWR International, PA, USA). The DBS punch was dissolved in 30 μ L GAA, GLA, and IDUA buffer cocktail for GAA, GLA, and IDUA enzyme reaction. The plate was sealed with thermal adhesive sealing film (Simport Scientific Inc, Canada) and incubated for 3 hours at 37°C with orbital shaking (650 rpm, iEMS Incubator Shaker; Thermo Scientific, MA, USA). Acetonitrile (60 μ L) was added to quench the reaction and to precipitate proteins. The plates were covered and centrifuged at 2800g for 15 minutes (Megafuge 1.0 R; Heraeus Kendro Laboratory Products, Germany) to pellet the precipitate. The supernatant aliquots (45 μ L) were removed from the plate, avoiding dislodgement of the pellet, and transferred into a new 96-well plate and 15 μ L acetonitrile and 60

Table 2. General Aspects of Assay Performance and Pre- and Post-analytic Enzyme Stability.

	GAA; QC Low to High	GLA; QC Low to High	IDUA; QC Low to High
Intraday variation, %, n = 10	6.9-6.3	3.9-2.31	13.1-9.1
Interday variation, %, n = 58	15.5-8.1	14.5-8.5	15.1-12.2
Accuracy	in between 1 and 6-plex		
LOD, μ M/h	0.034	0.057	0.30
LOQ, μ M/h	0.1	0.189	0.99
Carryover, %	<LOD	<LOD	negative
Linearity	Linear from base to high level		
Preanalytical stability, days	21		
Postanalytical stability, days	12		

Abbreviations: GAA, acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase; LOD, limit of detection; LOQ, limit of quantification; QC, quality control.

μ L water were added per well. The plate was covered with pierceable sealing foil (Thermo Scientific, MA, USA) and subjected to LC-MS/MS (Waters, Milford, Massachusetts) analysis.

Liquid Chromatography–MS/MS Analysis

The chromatographic separation was performed with a linear (non-parallel) LC system (Acquity ultra performance [UP] with 2 dimensional technology; Waters), as previously described.¹⁶ The mobile phase consists of solvent A (75% water, 25% methanol, and 0.1% formic acid vol/vol/vol) and solvent B (80% acetonitrile, 20% methanol, and 0.1% formic acid vol/vol/vol) at a flow rate of 0.8 mL/min according to a linear gradient elution program: initial 30% B; 0.99 minutes, 100% B; 2.09 minutes, 100% B; 2.10 minutes, 30% B; 3.20 minutes, 30% B. Run time per analysis was 3.2 minutes.

Selected reaction monitoring–based MS/MS in the positive ion mode on a triple quadrupole mass spectrometer (Xevo TQ MS; Waters) with MassLynx software version 4.1 was performed as previously described.¹⁷ Selected reaction monitoring was performed for 2 functions (Table 1) to optimize dwell time and duty cycle in electrospray positive mode.

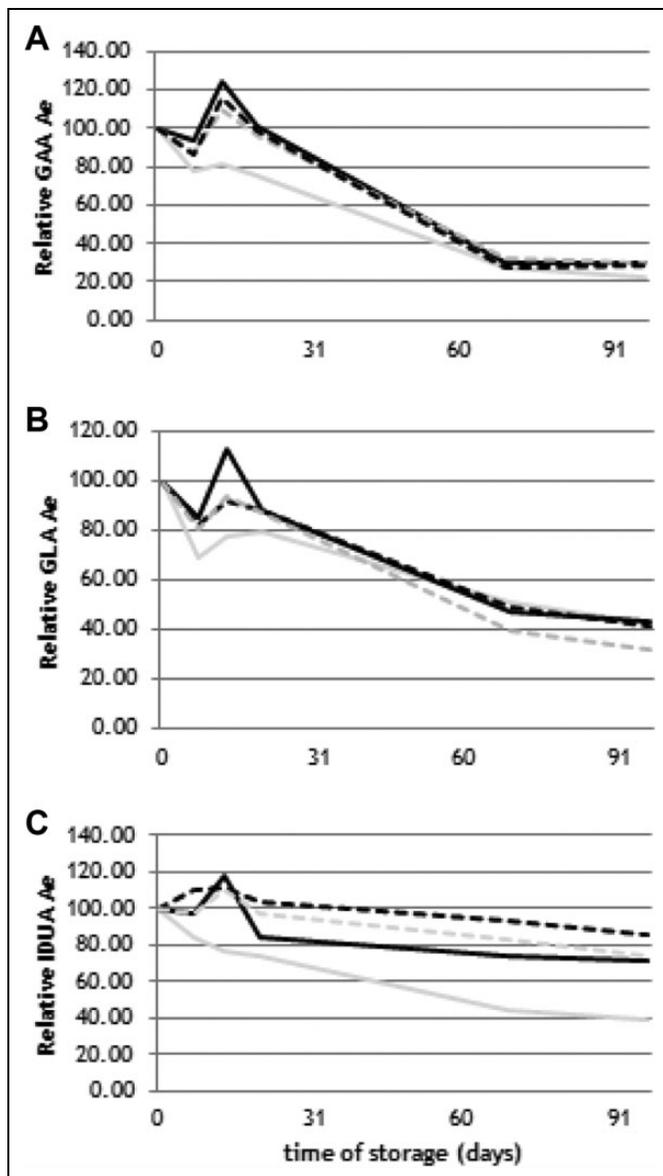


Figure 2. Preanalytical enzyme stability. Presentation of LSD enzyme activity stability on DBS of healthy volunteers stored at 4 temperatures (full gray line = room temperature, full black line = 4°C, dotted black line = -20°C, and dotted gray line = -80°C) for (A) GAA, (B) GLA, and (C) IDUA. GAA denotes acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase; LSD, lysosomal storage disorders.

Results

Method Validation and Enzyme Stability

The method described by Spacil et al¹⁷ was validated in our laboratory to analyze GAA, GLA, and IDUA enzyme activities as a 3-plex assay with 1 DBS in 1 buffer cocktail. After incubation, the quenched supernatant was analyzed by LC-MS/MS in 1 run of 3.2 minutes. Chromatographic separation of in-source breakdown of S to product with enzymatically generated product was adequate for all analytes (see Figure 1).

Table 2 presents the general aspects of assay performance and pre- and postanalytic enzyme stability. Intraday variation in DBS was 6.9% and 6.3% for GAA quality control (QC) low and high, 3.9% and 2.31% for GLA QC low and high, and 13.1% and 9.1% for IDUA QC low and high, respectively. Interday variation was 15.5% and 8.1% for GAA QC low and high, 14.5% and 8.5% for GLA QC low and high, and 15.1% and 12.2% for IDUA QC low and high, respectively.

As no specific reference values for 3-plex analysis (GAA, GLA, and IDUA) with the current method exist, the accuracy data were compared with the values of the CDC (1-plex analysis) and Spacil et al¹⁷ (6-plex analysis). For all analytes, the average values were as expected below the CDC average values and above the values presented by Spacil et al.¹⁷ A relative response factor was introduced to correct the data to better represent the values reported by Spacil et al.¹⁷

Column carryover was negligible for all analytes and linearity was demonstrated in the proper range (between CDC QC base level until CDC QC high level²³). The preanalytical enzyme stability of GAA, GLA, and IDUA was tested on DBS of healthy volunteers stored at 4 different temperatures, room temperature, 4°C, -20°C, and -80°C (see Figure 2). The GAA enzyme activity remains at around 100% after 3 weeks when stored cooled (4°C, -20°C, and -80°C). When stored at room temperature, a loss of around 25% enzyme activity was noticed. After 69 days of storage, the enzyme activity was only around 30% of the enzyme activity of freshly collected DBS regardless of the storage temperature. The GLA enzyme activity was 20% lower when the DBS was stored for 3 weeks at room temperature, compared to freshly collected DBS. In cooled samples (4°C, -20°C, and -80°C), the decline in enzyme activity was only around 10% after 3 weeks of storage. A 50% GLA enzyme activity loss was noticed after 69

Table 3. Presentation of the Number of Samples, Mean Enzyme Activity With Standard Deviations, Amount of Retests, and Recalls.^a

Analyte	Amount (n)	Mean (SD), μ M/h				N retest (%)	N recall (%)
		1	2	3	4		
GAA	19929	1.87 (0.93)	1.80 (0.90)	1.77 (1.08)	1.75 (0.94)	54 (0.271)	10 (0.050)
GLA	19933	2.69 (1.82)	1.66 (0.71)	2.41 (1.75)	1.57 (0.90)	6 (0.03)	1 (0.005)
IDUA	19922	6.8 (2.81)	6.6 (2.60)	6.4 (2.62)	6.4 (2.47)	54 (0.271)	3 (0.015)

Abbreviations: GAA, acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase; SD, standard deviation.

^a“1” represents female premature, “2” represents female mature, “3” represents male premature, and “4” represents male mature neonates.

days of storage on all temperatures compared to the activity detected immediately after blood collection. Enzyme activity of IDUA is stable when the DBS are stored on -20°C and -80°C for around 3 months (85% of original enzyme activity). When stored for 3 months at room temperature and at 4°C , around 45% and 75%, respectively, of the original IDUA enzyme activity remains present.

Postanalytically, a 10% difference in GAA and IDUA enzyme activities was detected when the samples were measured on the day of sample preparation or after 12 days of storage at 4°C . No difference was seen in GLA enzyme activity after 12 days of storage period.

Pilot Study

We conducted a pilot project to test the appropriateness and effectiveness of the method and to determine the reference ranges in our laboratory. In total, we analyzed around 20 000 newborn screening samples for each GAA, GLA, and IDUA enzyme activities (Table 3). A preliminary cutoff of the 0.1 percentile value on 3000 measurements was used to determine low enzyme activity. We found low enzyme activities for GAA in 54 (0.271%), for GLA in 6 (0.030%), and for IDUA in 54 (0.271%) samples. These samples were retested. Of the samples that were retested, 0.05%, 0.005%, and 0.015% for GAA, GLA and IDUA, respectively, of the initially analyzed samples had low enzyme activity and were considered suspicious for the respective disorder and would need a recall sampling. Further investigations of the positive samples could not be performed while this study was completely anonymized.

Lysosomal Storage Disorder Enzyme Activity in the Newborn Population

The enzyme activity of all evaluated enzymes was not normally distributed (Kolmogorov-Smirnov test) in the Flemish newborn population. Therefore, nonparametric statistics, Mann-Whitney U test, was used to compare the enzyme activity between the sexes and investigate correlation between enzyme activity and 1/weight at birth and 2/gestational age. We noticed statistically significantly higher GAA, GLA, and IDUA ($P < .0001$) enzyme activities in female newborns compared to male newborns. Our results show a trend that newborns with a higher birth weight had a lower GLA (significant level of $P < .0001$ correlation coefficient) enzyme activity compared to newborns with a lower birth weight. There was no trend between GAA and IDUA enzyme activities and birth weight. Regarding gestational age, there is a negative trend (significant level of $P < .0001$ correlation coefficient) between the GAA, GLA, and IDUA enzyme activities and the gestational age (Figure 3). The GAA ($P = .0026$), GLA ($P < .0001$), and IDUA ($P = .0004$) enzyme activities are statistically significantly higher in premature newborns (gestational age < 37 weeks) compared to in-term newborns. These data are presented in Table 4.

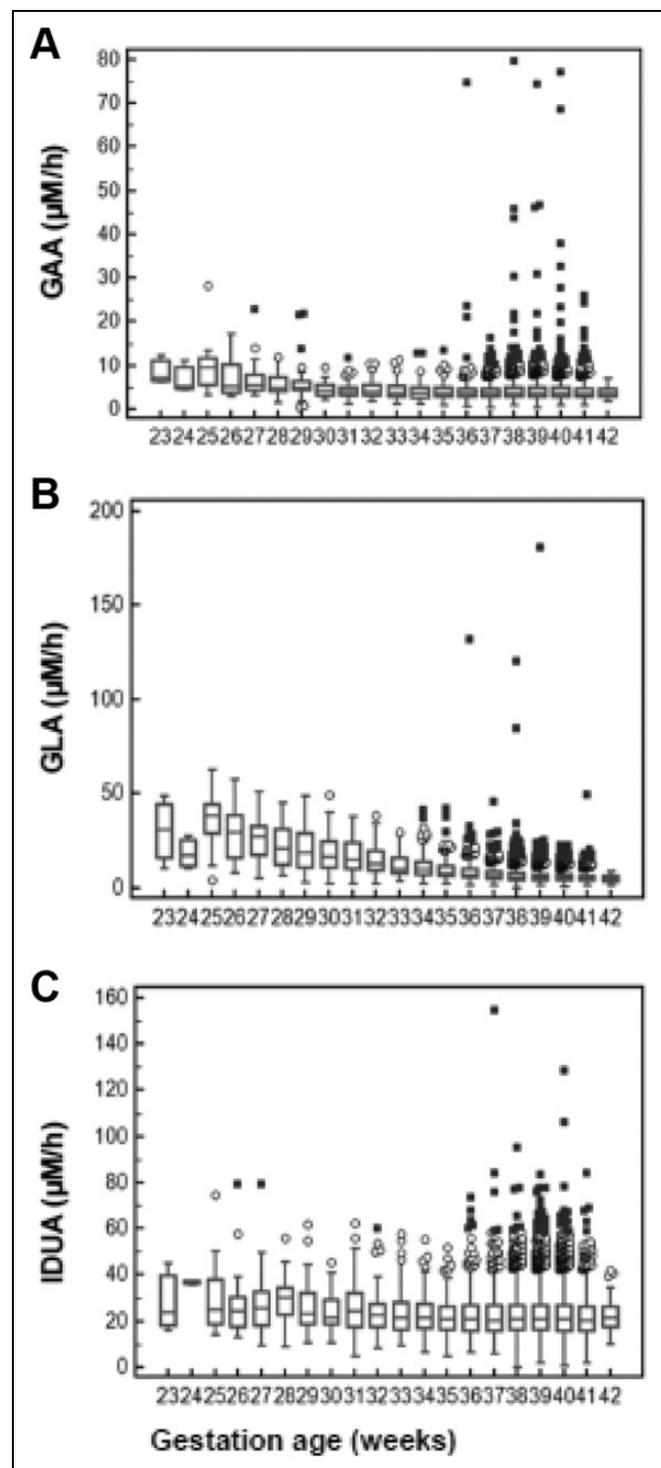


Figure 3. Correlation between enzyme activity and gestational age. Negative correlation between A/GAA, B/GLA and C/IDUA enzyme activities and gestational age using box plot graph per week of gestation age. GAA denotes acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase.

Sex- and Gestational Age-Dependent Cutoff Values

In view of the newborn population differences, the cutoff values, based on the 0.1 percentile, for GAA, GLA, and IDUA

Table 4. Statistical Analysis of LSD Enzyme Activity in the Newborn Population.^a

Analyte	Male Median, $\mu\text{M/h}$	Female Median, $\mu\text{M/h}$	Regression Birth Weight (Corr Coeff)
GAA	0.90 (0.895 to 0.912)	0.93 (0.9260 to 0.943)	-0.1151 (-0.0265 to 0.00343)
GLA	2.47 (2.448 to 2.492)	2.62 (2.590 to 2.645)	-0.3071 (-0.3206 to -0.2935)
IDUA	6.0 (5.918 to 6.027)	6.1 (6.064 to 6.171)	0.01311 (-0.001832 to 0.02805)

Abbreviations: corr coeff, correction coefficient; GAA, acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase.

^aMale and female median enzyme activity values and regression analysis for birth weight and gestation age with 95% confidence intervals in brackets. In bold statistically significant.

Table 5. Sex and Gestational Age-Dependent Cutoff Values and the Amount of Retests With General Cutoff (1) Compared to Sex- and Gestational Age-Related Cutoff (2).

Analyte	Female	Female	Male	Male >	# Retest 1 (%)	# Retests 2 (%)
	≤ 37 w (n = 1276)	> 37 w (n = 7903)	≤ 37 w (n = 1623)	37 w (n = 8771)		
GAA	≥ 0.50	≥ 0.50	≥ 0.44	≥ 0.44	54 (0.27)	35 (0.18)
GLA	≥ 0.49	≥ 0.57	≥ 0.52	≥ 0.43	6 (0.03)	6 (0.03)
IDUA	≥ 1.76	≥ 2.04	≥ 1.92	≥ 1.92	54 (0.27)	40 (0.2)

Abbreviations: GAA, acid α -glucosidase; GLA, α -galactosidase A; IDUA, α -iduronidase; w, weeks.

enzyme activities are calculated on the sex and the gestational age (see Table 5). Analysis of covariance was performed, and in female term newborns, we noticed significantly higher cutoff ranges compared to their male peers. The male preterm newborns showed higher cutoff values than the male term newborns. In female preterm newborns, we detected more variation in the enzyme activities of GLA and IDUA compared to the term female newborns, which resulted in higher cutoff values for the female term newborns. If the differences between the mean values of sex and gestation age showed overlapping 95% confidence intervals, we did not split the cutoff values between the different groups. With nonoverlapping 95% confidence interval, however, a group specific cutoff value was calculated (see Table 5).

When we recalculate the amount of retests with the cutoff values that are adjusted to sex and gestation age, we find the same amount of retests for GLA (6 retests or 0.03%), but a significant decrease in the amount of retests for GAA (from 54 to 35) and IDUA (from 54 to 40).

Discussion

We validated and optimized the assay to measure GAA, GLA, and IDUA enzymatic activity in DBS using LC-MS/MS and evaluate this method very positively as robust and suitable for large-scale screening studies. Sample preparation time, with 3-hour incubation, was minimal. The total run time per sample (3.2 minutes) is short so that LSD enzyme activity could be measured on the same tandem mass spectrometer that was available in the laboratory for other routine screening. No additional technical personnel were necessary, reducing the cost to reagents and consumables (0.78 euro per sample).

Adam et al²⁴ found that frozen DBS stored at low humidity kept high enzyme activities for a year. We however noted an important enzyme activity loss for GAA, GLA, and IDUA when stored over 3 weeks at the various temperatures (Figure 2). The majority of all samples from the newborn screening presented in the laboratory within maximum 2 weeks after blood collection and were kept at room temperature. As enzyme activity of GAA, GLA, and IDUA is sufficiently stable for 3 weeks at room temperature, all samples could be analyzed. In addition, we keep the analysis turnaround time as short as possible and not longer than 1 weeks.

Müller et al²⁵ highlights the importance of establishing specific reference values for lysosomal enzymes in each center. Therefore, we conducted a pilot project to determine the reference ranges in our laboratory. In total, we analyzed around 20 000 newborn screening samples for each GAA, GLA, and IDUA enzyme activity (Table 3). One of the advantages of multiplexing was that samples with low activities for 2 or more enzymes were excluded for retesting as they indicated questionable sample integrity. We noticed a low recall rate of 0.05%, 0.005%, and 0.015% for GAA, GLA, and IDUA, respectively, which is suitable for newborn screening.

We conducted a large-scale study with high statistical power and noted significant higher GAA, GLA, and IDUA enzyme activities in female newborns compared to male newborns. Moreover, a negative correlation between all investigated enzyme activities and the gestational age was seen together with a statistically significant higher enzyme activity in premature newborns (gestational age <37 weeks) compared to in-term newborns. This is in line with a study where 205 control individuals (of all ages) were evaluated and it was shown that the enzyme activity is affected by age. They noticed significantly lower enzyme activity in individuals older than 18 years compared with those in newborns.²⁵ In a Turkish study on 130 neonates, where GAA, β -glucosidase, and GLA activities in DBS samples of newborns were determined fluorometrically, GLA activities of newborns who were delivered before 38 weeks were significantly lower than those who were delivered at 39 to 40 weeks.²⁶ A recent study of the group of Giugliani (Schmitt et al) provided the same findings of higher activities of lysosomal activities (MS/MS) in newborns compared to children and adults.²⁷

The differences in enzyme activity levels with regard to the newborn sex and gestational age point out the importance of defining specific reference intervals for lysosomal enzyme

activities as has been shown by this study. Specific cutoff values (see Table 5) were established and implemented in our laboratory with the ultimate aim to reduce false positives (and false negatives) to a minimum.

We validated and optimized the assay to measure GAA, GLA, and IDUA enzymatic activity in DBS using LC-MS/MS and evaluated that this method is accurate, fast, has low cost, and is easy to implement next to the routine newborn screening and therefore suitable large-scale screening studies. For the first time, the data of a large-scale LSD study were used to assess statistical differences in enzyme activity in the newborn population, and with these data, we highlighted the importance to use reference intervals for lysosomal enzyme activities in function of sex and gestational age.

Authors' Note

Each author states to have substantially contributed to the conception or design of the work presented, approved the final version of the work to be published, and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Declaration of Conflicting Interests

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