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A new multiplex analysis of glucosylsphingosine and globotriaosylsphingosine in dried blood spots by tandem mass spectrometry

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ABSTRACT

Background: Gaucher's and Fabry's disease are two of the most common treatable lysosomal storage diseases, and have a wide spectrum of clinical symptoms. Early detection is important, because timely initiation of treatments can improve the disease status and prevent complications. However disease manifestations develop in childhood, diagnosis is delayed until adulthood partly due to the limitations of the currently used diagnostic pathway. The aim of this research is to develop and validate a multiplex assay and defining reference ranges, which do not exist at this moment, to improve and facilitate the entire diagnostic work up and enable treatment in an earlier stage of disease.

Methods and findings: Biomarkers glucosylsphingosine (GlcSph) and globotriaosylsphingosine (Lyso-Gb3) were detected and quantified using LC-MS/MS on dried blood spots. We developed an improved and new extraction method that allowed to measure GlcSph and Lyso-Gb3 in a multiplex analytical platform. After validation of the method, samples of 1480 individuals with normal enzymatic activity were collected to determine age and gender-related reference ranges.

Our combination method showed a good linearity, precision, accuracy and limit of quantification with lack of carry-over following the specific international CLSI guidelines. The suggested protocol is robust, efficient, sensitive, specific, comprehensive and relatively cheap in order to accelerate the diagnostic process for both lysosomal storage diseases. The samples, with normal enzymatic activity, defined statistical relevant and clinical correct reference ranges for each specific age group by gender.

Conclusion: We report a multiplex LC-MS/MS method and relevant reference ranges that are appropriate for the targeted screening, diagnosis and follow-up of Fabry and Gaucher disease.

1. Introduction

Lysosomal storage diseases (LSDs) are a heterogeneous group of inherited diseases caused by specific mutations affecting genes that encode either the function of the lysosomal enzymes required for the degradation of a wide range of complex macromolecules, or for the specific transporters of these degradation products to export them out of the cell [1–4]. Gaucher disease (GD) and Fabry disease (FD) are two of the most common LSDs. The deficiency of β-glucocerebrosidase and α-galactosidase, respectively, results in the accumulation of the

Abbreviations: LC-MS/MS, Liquid chromatography with tandem mass spectrometry; CLSI, Clinical and Laboratory Standards Institute; LSD, Lysosomal storage diseases; GD, Gaucher disease; FD, Fabry disease; GlcCer, Glycosphingolipid glucosylceramide; GlcSph or Lyso-Gb1, Glucosylsphingosine; Gb3, Globotriaosylceramide; Lyso-Gb3, Globotriaosylsphingosine; ERT, Enzyme replacement therapy; SRT, Substrate reduction therapy; ASMD, Acid Sphingomyelin Deficiency; DNA, Deoxyribonucleic acid; CCL18, C-C Motif Chemokine Ligand 18; DBS, Dried blood spot; QC, Quality Control; DMSO, Dimethyl sulfoxide; MeOH, Methanol; RBC, Red blood cells; K3-EDTA, K3 potassium salt of Ethylene Diamine Tetra Acetic acid; WBC, White blood cells; ESI, Electrospray ionization; MRM, Multiple Reaction Monitoring; IS, Internal standard; LOQ, Limit of quantification; CV%, Coefficients of variance; SD, Standard deviation; S/N, Signal-to-noise ratio.

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corresponding substrates giving rise to cellular and organ dysfunction [5–7].

Gaucher's disease has an incidence in the general population that varies between 0.4 and 5.8/100.000 inhabitants, but has been reported to be much more prevalent in certain populations such as the Ashkenazi Jewish population with an incidence of 1/800–1000 [7]. Beta-glucocerobrosidase is responsible for the degradation of the glycosphingolipid glucosylceramide (GlcCer) and results in the accumulation of GlcCer and the deacylated form glucosylsphingosine (GlcSph or Lyso-Gb1) [1,2,5,7]. GlcCer is only found intracellular whereas GlcSph, can also be detected in blood and plasma due to its increased solubility in water. The deficiency is caused by a mutation in the *GBA1 gene*, located on chromosome one (*1q21*) [2,8]. GlcCer and GlcSph accumulate in the lysosomes of the macrophages, known as Gaucher cells. Macrophages occur in all tissues but in excess in the liver the bone lung, bone marrow and spleen [5,7,9]. Due to the occurrence of Gaucher cells throughout the body, GD is characterized by a very diverse clinical presentation which hampers early diagnosis. One of the hallmarks is the presence of (hepato)splenomegaly whether or not in combination with thrombocytopenia [2,5,9,10].

Fabry's disease occurs with an incidence in general population of 1/8454–1/17000 live male births [3,11,12]. It is caused by an X-linked mutation of the *GLA-gene*, which impairs the normal function of α-galactosidase A [3,13]. Alpha-galactosidase A deficiency causes the accumulation of globotriaosylceramide (Gb3) and its derivative globotriaosylsphingosine (Lyso-Gb3) [6,11,14]. Due to the progressive accumulation of glycosphingolipids in lysosomes diverse symptoms appear [3,6,15,16]. Specific to FD is that males are more severely affected than female heterozygotes, whose manifestation can range from as severely affected to, more frequently, asymptomatic depending on the random X-chromosomal inactivation [15,17,18]. FD is a multisystem disorder involving cardiac/renal disease, skin disorders (angiokeratoma), neurologic diseases (acroparesthesias, autonomic dysfunction and hypohidrosis), ocular abnormalities (cornea verticillata), sensorineural hearing loss and cerebrovascular diseases-stroke [3,6,19,20].

In GD and FD, the majority of patients develop gradually disease manifestations during childhood [7]. A swift diagnosis is often impeded by the rare nature of LSDs and their diverse clinical presentation associated with a rather poor awareness among clinicians [18]. The delayed diagnosis results in a delayed treatment, which is associated with increased disease complications [3,21]. Advanced complications, both mental as physical, jeopardize the overall benefit of treatment [3]. Timely initiation of effective treatments, such as enzyme replacement therapy (ERT), substrate reduction therapy (SRT) and chaperone therapy, are able to improve the disease status and outcome [9,18].

When clinically suspected, the analytical confirmation of GD and FD is not straightforward. Currently, the golden standard is the measurement of enzyme activity, confirmed by genetic analysis [19,21,22]. Due to the highly variable phenotypic presentation and the clinical overlap with multiple other disorders, e.g. with Acid Sphingomyelin Deficiency (ASMD) type B, a multiplex enzymatic assay is recommended [23]. Neither the specific mutation nor the remaining enzyme activity is associated with the severity of the disease in GD in contrast to FD in which classical male patients have the lowest enzyme activities. Females with FD can have normal enzyme activities [6,24]. The analytical scope for diagnosing GD and FD was broadened by the introduction of biomarker research [13,15,21,24,25].

Biochemical investigations of the nature of these LSD's provide more relevant information and facilitate the diagnostic process. As a result of recent research the detection of the deacylated forms of Gb1 and Gb3, Lyso-Gb1 (GD) and Lyso-Gb3 (FD), have become a valuable and useful sensitive and specific tool both in the diagnostic procedure as in the follow-up [8,13,14,16,23,25–27]. These biomarkers are related to the clinical manifestations and, probably, the severity of the disease [5,7,15,21,26,28–30]. By extension, GlcSph is correlated to the therapeutic results, making this biomarker exceptionally useful in the follow-up of patients [7,25,26,31,32]. Compared to other suggested biomarkers for GD and FD, GlcSph and Lyso-Gb3 are relatively specific to their underlying disorder [5,7,13,22,32,33]. Heterozygous female FD patients are notoriously hard to diagnose as they often display a normal residual enzyme activity. Lyso-Gb3 has been shown to be elevated in cases of this population, making it a relevant additional parameter, as there are currently no better alternative options within the diagnostic process for female FD patients [16,17,30]. The monitoring of GlcSph in Gaucher disease, and Lyso-Gb3 in Fabry disease offers advantages in the diagnosis and treatment of these sphingolipidoses [7,14,22,34].

Latest developments concern the analysis of these biomarkers in dried blood spots (DBS) [5,6,9,14,35,36]. A DBS sample offers several advantages, such as the ease of sampling and stability of the compounds of interest during transport and storage [9,14,35]. Moreover, the DBS matrix allows simultaneous detection of GlcSph, Lyso-Gb3 and the corresponding enzyme activities enabling a faster diagnosis [6,7,9]. To date, very little data about the detection of either biomarker in DBS, is available. Additionally there are no reports on the possibility to combine both biomarkers in one analysis. Because the use of these biomarkers and certainly the detection in DBS is so innovating, there are no universal decision limits determined for the use in clinical practice. A few small studies tried to estimate possible cut-off values, but each time on a limited number of samples and therefore not representative for a clinical relevant framework [7,12,37,38].

The aim of our study is to accelerate the diagnostic process and initiation of therapy. This in first stage by developing an efficient, sensitive and specific screening method based on simultaneous measurement of the biomarkers GlcSph and Lyso-Gb3 in dried blood spot and in second stage by providing reference ranges for these biomarkers.

2. Materials and methods

2.1. Chemicals and reagents

Globotriaosylsphingosine (Lyso-Gb3)(Purity ≥98%, molecular weight 786 g/mol) and Glucosylsphingosine (GlcSph)(Purity ≥98%, molecular weight 462 g/mol) were purchased from Matreya LLC, State Collega, PA, USA and dissolved in Chloroform:Methanol (2:1) to make a 1 mg/mL (1 g/L; 0.0013 M for Lyso-Gb3 and 0.0022 M for GlcSph respectively) stock solution. $^{13}C_6$-Lyso-Gb3 (Purity ≥98%, molecular weight 791.87 g/mol, 0.0013 M) was used as internal standard and purchased from GelbChem Seattle WA, USA.

Analytical chemicals and solvents include Formic acid (Purity 99–100%), purchased from VWR Chemicals, France. Acetonitrile UPLC, Water ULC/MS (Purity ≥99%), Methanol ULC/MS-CC/SFC (MeOH, Purity 99.98%) and Isopropranol UPLC (Purity ≥99%) were all purchased from Biosolve chimie SARL, France. Dimethylsulfoxide (DMSO, Purity 99.9%) and Chloroform (Purity 99–99.4%) were purchased from Merck, Sigma Aldrich, Germany. Physiological water (Purity ≥99%) was purchased from Baxter, Switzerland.

2.2. Standards, internal standards and quality controls

The validation protocol defined by CLSI EP05 and C62 was used. Standard and Quality Control (QC) values were determined based on a literature search in Pubmed (see discussion). Standards were made by serially dilution of the stock solution with DMSO:MeOH (1:1) at concentrations of 100, 500, 1000, 2000, 10,000, 50,000 and 100,000 ng/mL (10^{-6} g/L) for GlcSph and 20, 50, 200, 800, 8000, 16,000 and 40,000 ng/mL (10^{-6} g/L) for Lyso-Gb3. Ten μL of the above dilutions for both GlcSph and Lyso-Gb3 were combined and diluted 100 times in a 1:1 ratio washed red blood cells (RBC) and DMSO:Sodium Chloride 0.9% (52%:48%). After this dilution, new concentrations were obtained of 1, 5, 10, 20, 100, 500 and 1000 ng/mL (10^{-6} g/L) for GlcSph and 0.2, 0.5, 2, 8, 80, 160 and 400 ng/mL (10^{-6} g/L) for Lyso-Gb3, used as calibration standards (S1 Table). Quality controls (QC) were analogously prepared

from the stock solutions (1 g/L) at concentrations of 400, 1000, 4000, 20,000 and 80,000 ng/mL (10^{-6} g/L) for GlcSph and 120, 300, 1000, 5000 and 20,000 ng/mL (10^{-6} g/L) for lyso-Gb3, followed by dilution with washed RBC, DMSO and Sodium Chloride 0.9%. The obtained QC concentrations were 4, 10, 40, 200 and 800 ng/mL (10^{-6} g/L) for GlcSph and 1.2, 3, 10, 50, 200 ng/mL (10^{-6} g/L) for Lyso-Gb3, representing values expected to be close to LOQ, medium and high levels (S2 Table). Red blood cells were obtained from one healthy volunteer. Whole blood was collected with K3-EDTA as anticoagulant and washed three times with physiological water by centrifugation (Beckan Coulter, Allegra x-15R) at 4000 rpm for five minutes. Plasma and white blood cells (WBC) were removed after the first time of centrifugation and physiological water was added to the remaining RBCs (Hematocrit 50%). Afterwards the remaining upper layer was removed, leaving the washed red blood cells for preparation of the GlcSph and Lyso-Gb3 mixtures as stated above. 70 μL of each standard or QC level was spotted onto filter paper (PerkinElmer, Turku, Finland) and air-dried for at least 12 h. All standards and QC levels were stored in zip-lock plastic bags at −20 °C with desiccant until further analysis.

Samples for the reference range calculation were collected in the University Hospital in Antwerp, Belgium. Collection took place over a period of 3 years, from 2020 until 2022. A posteriori selection process was performed to collect nonclinical indicated blood samples [39]. EDTA-anticoagulated blood samples from 1480 anonymous individuals in which the corresponding beta-glucocerebrosidase (GD) and alfa-galactosidase (FD) enzymatic activity were normal, were collected. Blood samples were spotted on filter paper and air dried at room temperature for at least 12 Hours. The DBS was sealed in a plastic bag and stored at −20 °C until further analysis. A minimum of 120 samples for each specific age group by gender was adhered, following literature and the CLSI guideline EP28-A3C, to establish age and gender-related reference ranges [40–41]. Age categories were defined as 0–4; 4–12; 12–18; 18–40; 40–60 and above 60 years old.

2.3. Sample preparation

Three punches (1/8 in., punch tool Kangaro, India) of 3.2 mm DBS equal a volume of 9.3 μL [5,27,36]. Standards, QCs and blank filter paper were punched into a 96-well microplate (Waters, US-made in Mexico) and 150 μL of extraction solution (DMSO:MeOH; 1:1) with the internal standard $^{13}C_6$-Lyso-Gb3 was added (2 10^{-5} g/L) [SS]. Extraction was obtained by incubation for 20 min at 37 °C on an orbital shaker at 300 rpm (PerkinElmer, DELFIA PlateShake). Afterwards the samples were centrifuged (Beckman Coulter, Allegra x-15R) for ten min at 4750 rpm and 20 °C to obtain a clear supernatant. 100 μL of the supernatant layer, was transferred to a 96-well microplate (Waters, USA) and centrifuged for ten min for additional purification of the sample before analysis. Two μL of the extracted mixture was injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system. Summary of the new detection method is provided in Table 1.

2.4. Liquid chromatography with tandem mass spectrometry

The analysis was performed on a QTRAP5500 (AB Sciex, USA) detector with Nexera X2 LC-30AD ultra-high performance liquid chromatography pumps (Shimadzu Scientific Instruments, Columbia, Maryland). Specific settings for the QTRAP5500 system are provided in Table 2. Electrospray ionization (ESI) in positive mode was used for peak detection. The settings for the Multiple Reaction Monitoring (MRM) transition of $^{13}C_6$-Lyso-Gb3 were 792.392 > 282.3 *m/z*, of GlcSph were 462.294 > 282.3 m/z and for Lyso-Gb3 786.392 > 282.3 m/z. Separation of the prepared samples was achieved on a C18 column (Acquity UPLC CSH C18 1.7 μm, 2.1mmx50mm, Waters, USA) with 40 °C as column temperature. To protect the column from contamination a pre-column (VanGuard Acquity UPLC CSH C18 1.7 μm pre-column, Waters, USA) was used.

A gradient elution utilizing 0.1% formic acid in water as solvent A and 0.1% formic acid in 80% acetonitrile and 20% MeOH as solvent B was performed, the flow rate was 0.50 mL/min. The gradient was set at 75%A-25%B), changed gradually to 0%A-100%B at 2.5 min, and returned to initial conditions after 0.1 min. The total run time was equal to five minutes. The washing solution was 30%MeOH:30%Water:30% Acetonitrile:10%Isopropranol.

Retention times are 1.70–1.72 min for Lyso-Gb3 and $^{13}C_6$-Lyso-Gb3 and 1.82 min for GlcSph.

Table 1
Summary of the new detection method.

Working solution	Extraction method
50% MeOH + 50% DMSO + 20 μL IS on 10 mL	20 min shaker at 37 °C + 10 min centrifuging at 4750 rpm

DMSO: Dimethyl sulfoxide; IS: internal standard $^{13}C_6$-Lyso-Gb3; MeOH: Methanol.

2.5. Method validation

According to the CLSI EP05 and C62 guidelines a minimum of 20 individual runs with three series of the predetermined samples give rise to 60 analytical results to verify the within-run and between-run precision and accuracy, carry-over and limit of quantification (LOQ). The current validation protocol encompasses in total 33 individual runs resulting in 99 analyses. Statistical analyses were carried out using R statistical soft-ware v2.10.1 (Revolution analytics, Palo Alto, CA, USA).

2.5.1. Linearity

The linearity of the method was determined the seven calibration standards in the 33 different runs. The linear calibration curve was generated by plotting the ratio of the peak area of the detected versus the spiked concentrations. A weighing of 1/x was used. The correlation of the measured results and the target values represents the accuracy of this standard level and needs to be <15% following the CLSI EP06 guideline. The method was accepted as being linear within the 95% confidence interval. The slope and intercept did not deviate from 1 and 0, respectively with a certainty of $p < 0.05$. Calculations were made by using the Passing Bablok regression, Spearmans correlation and the Bland-Altman test.

2.5.2. Precision

Precision (CV%) was carried out by analyzing the QC levels in triplet for all 33 individual runs replicates for intra-day test and from the 99 analyses in total for the inter-day test (100 x (the standard deviation/calculated mean)). The precision was determined by comparing the measured and spiked concentrations. CV% requires to be <15% as defined in the CLSI C62 and EP05 guidelines. To compare the obtained results with the determined cut-off of 15% a Chi-quadrate-test is used. Significant differences were assumed when $p < 0.05$.

2.5.3. Accuracy

Accuracy (Bias%) was carried out by analyzing the QC levels in 33 replicates for inter-day test and in triplet for inter-day test ((calculated mean – nominal value)/nominal value x 100). The accuracy was determined by comparing the measured and spiked concentrations. To compare the obtained results with the determined cut-off of 15%, defined in the CLSI C62 and EP05 guidelines, a Chi-quadrate-test is used. Significance was assumed when $P < 0.05$.

2.5.4. Carry-over

A carry-over analysis was performed by analysis of six QC low (QC1) after QC low levels and comparing these with five QC low levels analyzed after QC high (QC4) levels. The difference between low after

Table 2
Specific settings used on the QTRAP5500 and LC system.

Compound parameters					
MRM	Test	Dwell time (ms)	DP (Volts)	CE (Volts)	CXP (Volts)
1	Lyso-Gb3-IS	53.0	171.0	45.0	20.0
2	GlcSph	53.0	171.0	45.0	20.0
3	Lyso-Gb3	53.0	176.0	31.0	20.0

LC-MS/MS source settings							
Total Flow	Pressure Limits	Needle Stroke	Sampling speed	Cooler Temp	Oven Temp	ESI Needle	Rinsing Volume
0.50 mL/min	14000 psi	50 mm	2.0 µL/s	15 °C	40 °C	50 mm	500 µL

EP	**CUR**	**CAD**	**IS[a]**	**TEM**	**GS1**	**GS2**
10 V	35 psi	medium	5500 V	600 °C	60 psi	50 psi

CE: capillary electrophoresis; CUR: Curtain gas; CXP: Collision Cell Exit Potential; DP: declustering potential; EP: Enterance potential; ESI: Electrospray ionization; GS1: ion source gas 1; GS2: ion source gas 2; IS: internal standard; IS[a]: Ionspray voltage; LC-MS/MS: Liquid Chromatography with tandem mass spectrometry: MRM: Multiple Reaction Monitoring; ms: milliseconds; psi: Pound-force per square inch; TEM: temperature; V: volts.

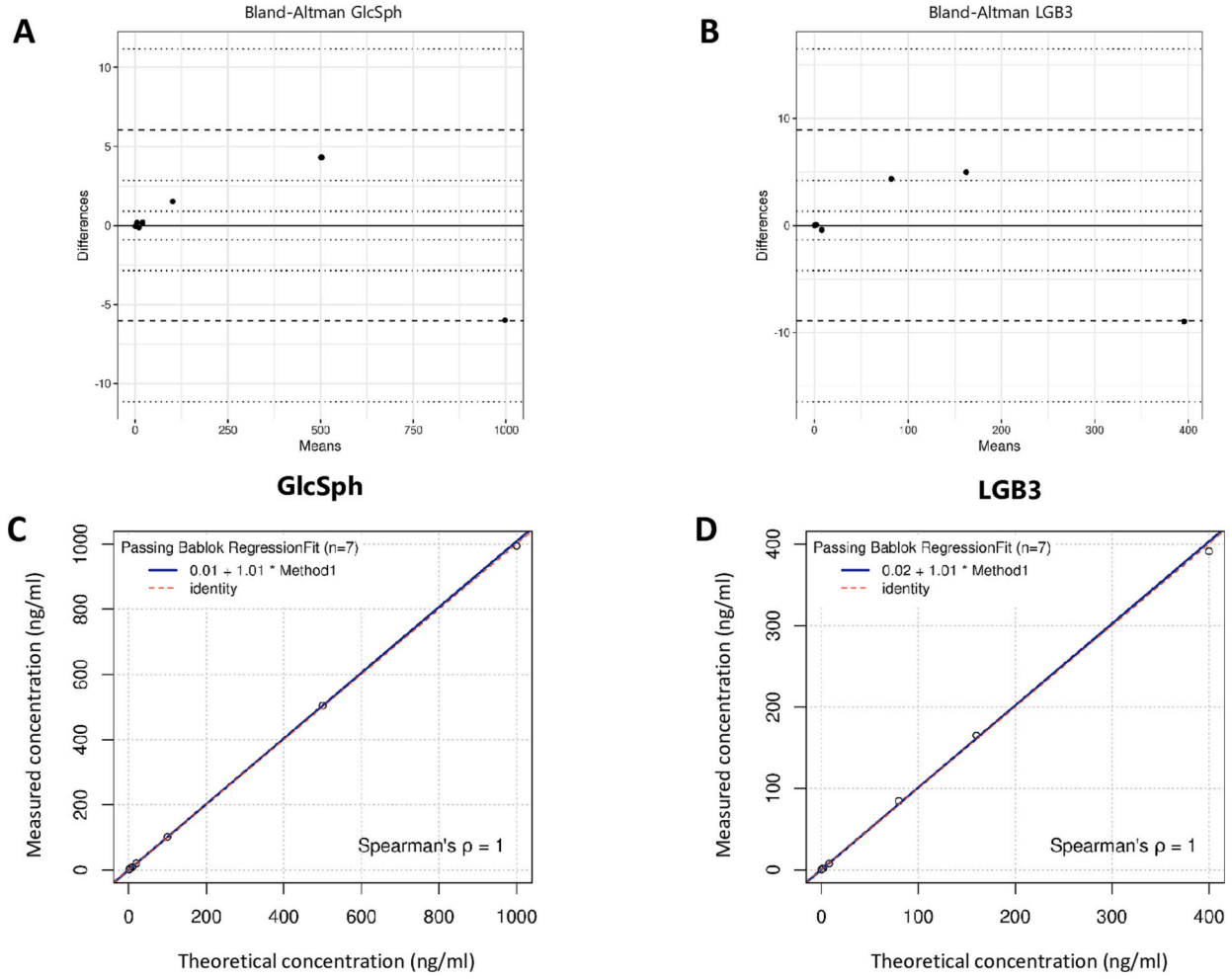

Fig. 1. Linearity of the calibration curve.
Representation of the linearity based on the 7 standard values for each biomarker. Analyzation by comparing the spiked concentrations with the measured concentrations. Concentration units are expressed in ng/mL (or 10^{-6} g/L). (A) Bland-Altman curve for GlcSph. (B) Bland-Altman curve for Lyso-Gb3. (C) Passing Bablok Regression and the Spearman's test for GlcSph. (D) Passing Bablok Regression and the Spearman's test for Lyso-Gb3.

high and low after low was calculated using the difference between the mean detected concentrations. When the value for carry-over is lower than three times the standard deviation (SD) of the lowest QC value, then the methodology is free from carry-over according to the CLSI EP10 guideline.

2.5.5. Lower limit of quantification

The LOQ was assessed by using the signal-to-noise method, defined by the CLSI E17 and C50 guidelines. The LOQ can be calculated by the auto-integrator of the instrument or manually on a chromatogram printout. The ratio between the peak signal over the noise signal, the signal-to-noise ratio (S/N), should be >10 [42]. To account for any variation between runs, the LOQ of ten different runs was calculated for each biomarker, the mean was used as the finale and representing LOQ. The used area to calculate the LOQ is defined as the full peak width from starting point until the ending point at baseline.

2.5.6. Reference ranges

The statistically sufficient group of minimum 120 samples per category was obtained following literature and the CLSI EP28 A3C guideline [40,41]. Based on these CLSI EP28 A3C guidelines calculation of the parametric central 95% to obtain reference ranges was performed, using the statistical program MedCalc Statistical Software version 20.027 (MedCalc Software Ltd., Ostend, Belgium) [41]. Data were Gaussian distributed using logarithmic mathematical transformation for GlcSph and a square transformation for Lyso-Gb3, in combination with a box-cox transformation. One round of outliner detection according to Tuckey test was performed and the results excluded. Gender and age related subgroups were compared based on the Z scores, described in the CLSI EP28 A3CE guideline. [41,43].

3. Results

3.1. Optimization of the LC-MS/MS method

Preliminary experiments were performed to optimize the mass spectrometric parameters, mobile phases, columns and the extraction solution. The optimized ion source parameters were determined via direct infusion of GlcSph, Lyso-Gb3 and $^{13}C_6$-Lyso-Gb3 10 ppm solutions in positive ion mode.

3.2. Analytical validation

The calibration curve for GlcSph and Lyso-Gb3 based on seven different standard concentrations (Fig. 1) was linear with $R^2 = 1$, Spearman $r = 1$, significant with <0.001, Passing Bablok regression and Bland-Altman supporting the almost perfect linearity and no bias. The obtained regression equation for GlcSph was defined as $Y = 1.01X + 0.01$ and for Lyso-Gb3 $Y = 1.01X + 0.02$.

Data for intra-assay and inter-assay accuracy and precision were determined based on the QC-values and reported in Tables 3 and 4. For all QC values the accuracy and precision were < 15% (S1 and S2 Fig).

The difference between the low after low runs versus the low after high runs in absolute numbers were 0.65 ng/mL (10^{-6} g/L) for GlcSph and 0.53 ng/mL (10^{-6} g/L) Lyso-Gb3. The limit for carry-over, defined by 3 times the SD of the lowest QC was respectively 1.37 ng/mL (10^{-6} g/

Table 3
Results for the precision, expressed as the percent of coefficient of variance.

GlcSph	QC level	Precision, CV% Intra-assay	Precision, CV% Inter-assay
	QC 1	10.3	6.1
	QC 2	6.4	4.8
	QC 3	6.3	5.4
	QC 4	7	6
	QC 5	7	4.6
Lyso-Gb3	**QC level**	**Precision, CV% Intra-assay**	**Precision, CV% Inter-assay**
	QC 1	14.5	6.5
	QC 2	12.5	4.3
	QC 3	11.4	6.3
	QC 4	6.3	5.2
	QC 5	6.3	3.8

CV%: percent of coefficient of variance; QC: Quality control; Cut-off defined by the CLSI C62 and CLSI EP05 guidelines is 15%. All calculations are clearly below the defined cut-off.

Table 4
Results for the accuracy, expressed as the percent of coefficient of variance.

GlcSph	QC level	Accuracy, RE% Inter-assay
	QC 1	−5
	QC 2	−1.8
	QC 3	−0.9
	QC 4	−1.9
	QC 5	4.5
Lyso-Gb3	**QC level**	**Accuracy, RE% Inter-assay**
	QC 1	3.3
	QC 2	8.7
	QC 3	8.8
	QC 4	9.1
	QC 5	9.3

CV%: percent of coefficient of variance; QC: Quality control; Cut-off defined by the CLSI C62 and CLSI EP05 guidelines is 15%. All calculations are clearly below the defined cut-off.

Table 5
Results of the Carry-over analysis.

	Cut-off	Low-low mean	High-low mean	Carry-over Absolute	Conclusion
GlcSph	1.37	3.81	4.46	0.65	Below cut-off
Lyso-Gb3	1.033	1.40	1.92	0.53	Below cut-off

The mean value of the low after low analysis is represented in the 'low-low mean' column. The mean value of the low concentrations measured after a high concentration is represented in the 'high-low mean' column. Concentration units are expressed in ng/mL (or 10^{-6} g/L). The difference between the mean detected concentrations is represented in the 'carry-over absolute' column. The defined cut-off is represented in the 'cut-off' column.

Table 6
Identified reference ranges.

Age in years	Number of samples	GlcSph (ng/mL)		Lyso-Gb3 (ng/mL)	
		Female	Male	Female	Male
0–4	N = 233	0,282–6,890		0,044–1,518	0,057–1,952
4–12	N = 245	0,235–5,409		0,262–2,217	0,223–2,326
12–18	N = 242	0,237–6,385		0,138–2,014	
18–40	N = 232	0,272–7,111		0,145–1,971	
40–60	N = 233	0,319–6,392		0,085–2,014	
>60	N = 234	0,317–4,928		0,118–1,774	

The 95% central parametric reference ranges for enzymatic normal samples, are represented above. Only for Lyso-Gb3 there is a difference between gender for the age-categories 0–4 years and 4–12 years. Measurements are expressed in ng/mL (or 10^{-6} g/L).

L) and 1.03 ng/mL (10^{-6} g/L) (Table 5). Both results are significantly lower than their defined limit values; concluding that the carry-over does not significantly impact the obtained data.

The S/N was found to be 151.23 at the concentration level of 1 ng/mL (10^{-6} g/L) and 22.08 at concentration level 0.2 ng/mL (10^{-6} g/L) respectively for GlcSph and Lyso-Gb3. Both above ten and thus defining the significant increase of the measured value versus the detectable noise in the sample (S3A and S3B Fig.).

3.3. Reference ranges

Gaussian distribution was achieved. Exclusion of the outliers resulted in 1370 remaining samples for GlcSph and 1419 for Lyso-Gb3. the central 95% defining the reference ranges are shown in Table 6. After comparison of each age and gender category using z-scores, we could conclude that only the age categories 0–4 and 4–12 years of Lyso-Gb3 were clinically different.

4. Discussion

GD and FD are two of the most common inherited lysosomal storage disorders. Early diagnosis and prompt initiation of specific treatment are critical in achieving positive patient outcomes. The biomarkers GlcSph and Lyso-Gb3 are shown to be a useful tool both in the diagnostic procedure as in the follow-up.

However, currently there is a clear lack of data related to the measurement of GlcSph and Lyso-Gb3 in DBS. Moreover, the scarce publications differ widely in methodology and suggested cut off values to distinguish the healthy from the affected population. A literature overview was made prior to the analytical work in order to establish bench marking for the standard calibration and QC levels used in the current study. PubMed was consulted to collect data from the last five years using the search terms ‘Lyso-Gb1’, an alternative name for GlcSph, and ‘Lyso-Gb3’. In literature the concentrations were subdivided between healthy population, patients with GD or FD and patients under therapy. Based on the limited data found in the literature, the reference range for GlcSph should be between 2.1 and 9.7 ng/mL (10^{-6} g/L) (14.3 ng/mL in neonates) [36], as for Lyso-Gb3 between 1.4 and 3.5 ng/mL (10^{-6} g/L) [19,38]. For GD, concentrations above 6.8 ng/mL (10^{-6} g/L) were considered as suggestive for the disease [4]. In patients reported results were between 190.5-2380.6 ng/mL (10^{-6} g/L) and 16.6-207 ng/mL (10^{-6} g/L) [8,37]. For Lyso-Gb3 results in other studies with patients were 3.13–25.6 ng/mL (10^{-6} g/L) and 2.23–17.9 ng/mL (10^{-6} g/L) [12,38]. Additionally a differentiation between gender can be made, range between 0.5 and 29.1 ng/mL (10^{-6} g/L) for males and between 0.8 and 4.7 ng/mL (10^{-6} g/L) for females [12,38]. The different diagnostic limits values reflect the different populations and different measurement methods in studies [7]. Based on this information standard and QC values were chosen (S1 and S2 Table). Because the observed effect of different measurement methods we did a first exploratory screening on anonymous, healthy samples with our new multiplex-method. We saw that with this method we obtained target values for GlcSph between 0.106 and 5.829 ng/mL (10^{-6} g/L) and for Lyso-Gb3 between 0.073 and 2.240 ng/mL (10^{-6} g/L). These values are nicely within the range of the previously mentioned studies, pointing at the accuracy of the analytical data comparing with earlier published alternatives. Based on these reference limits, the clinical use during diagnostics comes within reach. The above data indicate the need for further research concerning biomarker levels in healthy and disease populations.

The subsequent method development was based on current insights in detection of GlcSph and Lyso-Gb3, and contributes substantially to the existing literature, which focusses mainly on analysis in plasma. [2,6,13,15,19,22,30,44–48]. Adaptations were made to the composition of the working solution, efficiency of the extraction protocol and volume of the injected sample in order to guarantee the most accurate and sensitive multiplex detection on the QTRAP5500 system. A summary is provided in Table 1.

The used volume unit mL of the DBS samples represents the volume of blood spotted on the DBS circles. Namely 70 μL blood was used to fill a full circle, where a 3.2 mm punch is known to be equivalent to 3.1 μL of blood [5,27]. Due to the need to properly prepare the DBS samples, the use of a standardized method to collect blood samples on DBS is recommended.

To further improve accuracy, precision and stability of the described method some suggestions can be made. Firstly in the literature search a lack of harmonization between laboratories was prevalent. Improved standardization could be achieved by the use of a dedicated internal standard for GlcSph namely $^{13}C_6$-GlcSph (Purity ≥98%, 468 g/mol) which is available from Matreya LLC, USA. Secondly, in the current study set up, the concentration of biomarkers naturally present in the healthy population was not taken into account during the validation of the method. To exclude or determine this concentration an analysis of blank values could be performed. The mean of the blank values subtracted from each enzyme activity could be taken into account for the in-source fragmentation from substrate to product and is recommended for upcoming research.

As mentioned above the use of DBS ensures an easy way of collection the necessary samples and simplified their transport and preservations. Follow-up of patients could be improved and facilitated (e.g. home-monitoring).

At this moment the method is BELAC accredited according to ISO 15189, not only for analysis on DBS but also for the use on plasma samples. On weekly basis, 30 samples are analyzed in our lab (University Hospital Antwerp, Belgium).

The recently published guidelines for laboratory diagnosis of Gaucher disease type 1 by the International Working Group of Gaucher Disease (IWGGD) recommend the use of DBS for detection of GlcSph and the enzymatic activity [49]. It emphasizes the importance of detection of GlcSph on DBS to facilitate timely and accurate diagnosis independent of the accessibility to health care [49]. The development of the method for GlcSph and Lyso-Gb3 on DBS offers the opportunity for multiplex analyses of biomarkers and enzyme activity on the same DBS sample. Currently we are conducting an additional study related to the use of DBS for the multiplex detection of enzymatic activity and biomarkers.

Further investigations are planned using this multiplex analysis in order to facilitate the diagnostic process, as well as the opportunities in follow-up of patients with Gaucher and Fabry disease. Additionally analogous investigations are planned to expand this method through the detection of other lysosphingolipids correlated with LSDs.

Until now the use of variable methodology, employed by different laboratories, makes it difficult to compare findings and define a universal cut-off value. By describing our validated method in detail, we aim to obtain a transparent culture with consensus about the used detection method. This accredited method proved to be accurate, robust and fast with a duration of the whole assay of 40 min.

By obtaining (clinical) relevant reference ranges, based on until now largest number of enzymatic normal samples, conclusions about the most accurate limit values, can be made. These are crucial in order to improve and facilitate the entire diagnostic work up, enable treatment in an earlier stage of disease and to closer follow-up efficacy of treatment.

5. Conclusion

We present a simplified and robust method for the detection of both biomarkers GlcSph and Lyso-Gb3 in a multiplex analysis, that has been proven being sensitive, specific, robust and accurate. By using the most advantageous matrix, DBS, we create opportunities for the follow-up of patients from their home as well as facilitating the exchange of samples between labs. Additionally this method brings practical improvement for the lab, due to the simple an rapid pre-analytical process with limited workload for lab technicians. Using this method, for the first time in literature statistical relevant reference ranges were defined on a large

number of enzymatic normal samples, making the detection method relevant for clinical practice.

List of human genes

GBA1: Glucosylceramidase beta 1, HGNC ID 4177.
Alias symbols: GBA, GLUC, Alias names: glucocerebrosidase.
GLA: Galactosidase alpha, HGNC ID 4296.
Alias symbols: GALA

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The above described disclosures played no role in the conception and design of the study, acquisition of data, analysis and interpretation of data, preparation of manuscript or final approval of manuscript.

Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Informed consent

Not applicable.

Ethical approval

The local Institutional Review Board of the University Hospital Antwerp, Belgium approved the project description with Central EC (ID 2021 0550).

Declaration of Competing Interest

The author François Eyskens is the president of Metabolics.be and a member of the MetabERN Medical Executive Committee. Additionally he attended advisory boards and received speaking fees and travel grants from Takeda, Sanofi, Recordati, Protalix, Amicus, Acthelion, Alexion, Chiesi and Ultragenyx.

The other authors have nothing to disclose.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2023.100993.

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