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Determination of Gb3 and Lyso-Gb3 in Fabry Disease-Affected Patients by LC-MRM/MS

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Abstract: Limited or absent activity of the enzyme α-galactosidase A (α-Gal A), due to mutation in the related gene on the X chromosome, leads to the development of a rare hereditary and genetic disease known as Fabry disease (FD). This pathology involves a progressive accumulation in various organs of the substrates of the enzyme e.g., globotriaosylceramide (Gb3) and its deacylated form, globotriaosylsphingosine (Lyso-Gb3), suggesting these molecules as biomarkers of Fabry disease. The present paper describes the development of an analytical strategy for the identification and quantification of Gb3 and Lyso-Gb3, in serum and blood samples by using liquid chromatography (LC) coupled to mass spectrometry in multiple reaction monitoring (MRM/MS) ion mode. The best experimental conditions were obtained by extracting the glycolipids with chloroform/methanol/H_2O (2/1/0.3) and by separating them on a C4 column with a linear gradient (A: H_2O with 2 mM ammonium formate. B: methanol with 1 mM ammonium formate, both acidified with 0.2% formic acid). The best transitions (a combination of precursor and fragment ions—m/z) were 786.8 m/z > 268.3 m/z for Lyso-GB3, 1137.3 m/z > 264.3 m/z for Gb3, 1039.3 m/z > 264.4 m/z for N-heptadecanoyl-ceramide trihexoside, and 843.5 m/z > 264.3 m/z for N-glycinated lyso-ceramide trihexoside, the latter being used as an internal standard. The developed method provided a reliable, fast, and effective procedure for direct measurements of GB3 and Lyso-GB3 in serum and blood for diagnosis of Fabry disease, suggesting this method as a complementary assay to the current enzymatic test. Therefore, this approach could open new insights into the clinical diagnostics of lysosomal storage disorders.

Keywords: Fabry disease; glycosphingolipids; ceramides; LC-MS/MS analysis; targeted approach; analytical tool

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1. Introduction

Lysosomal storage disorders (LSDs) are a group of more than 70 genetic diseases showing marked genotypic and phenotypic variability [1,2]. The Anderson–Fabry disease, belonging to this group, is a rare inherited disorder of glycosphingolipid metabolism caused by alterations (mutations) in the α-GaL A (GLA) gene, located on the X chromosome. This gene encodes for a lysosomal enzyme able to hydrolyze glycosphingolipids (GSL), specifically, globotriaosylceramide (GL-3 or Gb3), its deacylated form Lyso-GL-3/Gb3, and related glycolipids, by removing the terminal galactose sugar from the end of these glycolipids. The enzyme deficiency causes a continuous accumulation of Gb3 and related glycolipids, resulting in cell abnormalities and organ dysfunction that particularly affect

small blood vessels, the heart, and the kidney. When the sequence of the GLA gene is altered by mutations, variants of the α-Gal A enzyme are produced that may function less or not at all, different mutations can compromise the activity of the α-Gal A enzyme, resulting in differences in the clinical manifestations of the disease. Mutations that reduced the enzymatic activity of α-Gal A, even at a residual level, were responsible for late-onset variants of FD that generally displayed milder clinical manifestations. Recently, numerous reviews have focused on the consequences of enzymatic impairment, emphasizing the importance of understanding the cellular processes underlying the metabolism of these disorders [3]. Based on these studies, other authors suggested the detection of glycosphingolipids as biomarkers of the LSDs which may reflect disease severity and progression, explaining the heterogeneity found between patients, even those displaying the same GLA mutation [4,5]. These studies showed a complex biomarker profile in LSD's patients, e.g., that of Lyso-Gb3 and its acylated form ceramide trihexoside Gb3, which varied in agreement to gender, types of mutations, and treatment of patients.

Due to the significant relevance of the detection of glycosphingolipids for Fabry disease diagnosis, the current clinical analyses are based on the enzymatic [6], fluorescence assay [7], or genetic studies [8] to identify the presence of Lyso-Gb3 and its acylated form Gb3 lacking the molecular characterization of the nature of the detected sphingolipid. During the last 10 years, liquid chromatography (LC) combined with mass spectrometry (MS) has become a valid alternative to enzymatic tests by passing over the analytical due to the low amount of target molecules in blood and their low ionization efficiency. The protocols of SPE purification using a mixed-mode strong cation exchange (MCX) cartridge [9–11] or liquid/liquid extraction by using methanol [12] or chloroform, methanol, and formic acid to extract ceramides from plasma, were adopted to concentrate the analytes prior to LC-MS/MS analyses [13]. Several authors built up analytical methods based on LC-MS/MS, even utilizing the multiple reaction monitoring (MRM/MS) ion mode, but to exclusively quantify lysoGb3 and its analog in healthy subjects and Fabry patients [10,11,14].

The present paper describes the development of a method for the simultaneous identification and quantification of Gb3 and Lyso-Gb3 by MRM/MS analysis by implementing previously published methods as a valid alternative to laboratory analysis.

The low cost of reagents combined with the simplicity of sample preparation makes the LC-MS/MS MRM method a valid tool for performing research studies or clinical laboratory investigations. The methodology we developed allowed the simultaneous determination of Lyso-Gb3 and GB3 with the opportunity to implement the method with a wide panel of metabolites to be quantified in a single LC run due to the scanning speed of quadrupole mass analyzers.

The main advantage of MRM/MS assay is the inestimable possibility to quantify multiple target metabolites in a single LC-MS analysis by using a few microliters of samples and by reaching sensitivities comparable to conventional clinical methods, as well as its broad generalizability to any metabolite that can be quantified in any biological matrix.

2. Materials and Methods

2.1. Chemicals and Reagents

Pure powders of Gb3, lyso-Gb3, and internal standards (N-heptadecanoyl-ceramide trihexoside, N-glycinated lyso-ceramide trihexoside) of >98% purity were purchased from Matreya LLC. (State College, PA, USA). All solutions and solvents were of the highest available purity and suitable for LC-MS analysis, and were purchased from J. T. Baker (Phillipsburg, NJ, USA). All stock solutions and samples were stored at −20 °C.

2.2. Preparation of Standard Solutions

Gb3, lyso-Gb3, and N-heptadecanoyl-ceramide trihexoside, N-glycinated lyso-ceramide trihexoside of >98% purity standards were dissolved in 1.0 mL of 80:20 chloroform/methanol solution to reach a final concentration of 1000 mg/L for each analyte. Serial dilutions of

stock solutions were prepared in the range of concentration 400–10 µg/L. All standards were stored at −20 °C until LC-MS/MS analysis.

2.3. Sample Preparation

To define the best extraction solvents for the target analytes in real matrices, aliquots of µL 500 serum and blood samples were spiked with the internal standards and separately extracted by using four different extraction solvents: chloroform, methanol, chloroform/methanol (1/1), and chloroform/methanol/H_2O (2/1/0.3), to precipitate proteins. The extractions were repeated three times with each solvent to increase the recovery of the target analytes. The tubes were mechanically and vigorously shaken, sonicated for 10 min, and stored at −20 °C for 30 min. The tubes were centrifuged at 10,000 rpm at 4 °C for 10 min and the organic extract layer was assembled, dried under vacuum, and stored until LC-MS/MS analyses. Prior to the analyses, the extracted samples were resuspended in 500 µL methanol containing 1 mM ammonium formate and 0.2% formic acid and placed on a rotary platform for 10 min to better solubilize the analytes. Each extract was injected several times into the LC-MS/MS apparatus to ensure data reproducibility.

2.4. LC–MS/MS Instrumentation and Conditions

Each extract (3 µL) was analyzed by using an Agilent 6420 Triple Quadrupole LC-MS/MS system equipped with a HPLC 1100 series binary pump (Agilent, Waldbronn, Germany).

A mixture of globotriaosylceramide, lyso-ceramide trihexoside, N-heptadecanoyl-ceramide trihexoside, and N-Glycinated lyso-ceramide trihexoside (100 µg/L each) was separated on a HPLC with C4 column (10 cm × 4.6mm, 5 µm) of Thermo scientific BioBasic (Thermo Fisher Scientific, Waltham, MA USA). The column temperature was set at 40 °C. The mobile phase was generated by mixing eluent A (water, 2 mM ammonium formate, 0.2% formic acid) and eluent B (methanol, 1 mM ammonium formate, 0.2% formic acid), and the flow rate was 0.5 mL/min. The gradient for the LC-MS/MS method started with 80% buffer B and in 11 min it reached 95% of buffer B.

Tandem mass spectrometry was performed by using a turbo ion spray source operating in positive ion mode and triple quadrupole analyzers in multiple reaction monitoring (MRM) ion mode.

All MS parameters, such as precursor and product ion (m/z), dwell, fragmentor, collision energy, cell acceleration voltage, and polarity, were reported for each analyte (Table 1).

Table 1. Mass spectral parameters, including precursor and product ion (m/z), dwell time, collision energy, cell acceleration voltage, and polarity for each analyte.

GSL	Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	Fragmentor (V)	CE (eV)	Cell Acceleration Voltage (V)	Polarity
Gb3	1137.5	282.3	200	161	52	4	positive
		264.3	200	161	55		
Lyso-Gb3	786.8	282.3	200	81	45	4	positive
		264.3	200	81	50		
N-heptadecanoyl-ceramide trihexoside	1039.3	282.3	200	161	80	4	positive
		264.3	200	161	50		
N-glycinated lyso-ceramide trihexoside	843.5	339.3	200	161	70	4	positive
		321.3	200	161	58		
		264.3	200	161	50		

The most intense transitions, defined as *quantifiers*, e.g., 786.8 m/z >268.3 m/z for Lyso-GB3, 1137.3 m/z > 264.3 m/z for GB3, 1039.3 m/z > 264.4 m/z for N-heptadecanoyl-ceramide trihexoside, and 843.5 m/z > 264.3 m/z for N-glycinated lyso-ceramide trihexoside, were used for quantification while the others were used for identification.

2.5. Data Processing

Extracted ion chromatogram (EIC) peaks of target metabolites were integrated using Agilent MassHunter Quantitative Analysis software (B.05.00). Then, each peak area was uploaded on Perseus software (Perseus v. 1.6.15.0) [15] used for the statistical analysis; Perseus analyzed the MRM/MS data, log2 transformed, to obtain a heatmap.

3. Method Validation

Limit of Detection and Quantitation

Limit of detection and quantitation. The limits of detection (LODs) were defined as the lower concentration limit, below which the sample could not be revealed [16,17]. LODs were determined by making 10 replicate measurements of blank samples spiked with low concentrations of each analyte and calculated according to the formula LOD = 3 × σ; where σ represented the standard deviation [17]. The limit of quantitation (LOQ) is the lowest concentration at which the analyte can be reliably detected and quantitated [16]. LOQ was determined as LOQ = 10 × σ, where σ represents the calculated standard deviation.

Matrix effect. Possible matrix effects were evaluated by comparing standard and matrix-matched calibration curves. Standard solutions and spiked blood samples were prepared according to the previous paragraph and analyzed using the LC-MRM/MS method. Each point of the calibration curve was analyzed in triplicate. The matrix effects were evaluated by comparing five-point standard in solution with those defining the matrix-matched calibration curves.

Specificity. The specificity of the assay was demonstrated by checking the presence of interfering peaks at the retention time of the target analytes.

Stability. The stability of analytes was assessed by storing the samples under short-term conditions (−20 °C for 7 days) and by comparing the peak areas of analytes recorded in triplicate in freshly prepared matrices with those in stored matrices. A variation greater than 10% in the peak area of analytes in the stored matrices was considered indicative of non-stability.

Selectivity. Selectivity was assessed by analyzing 20 blank samples. Selectivity was deemed acceptable if no peaks were observed in the blank chromatogram at the analytes' retention times (allowing a retention time variation of ±0.1 min) or if any peak present within the blank did not exceed 30% of the height of the analyte peak at the lowest calibration level.

Linearity. Linearity describes the direct relationship between the concentration of analytes and the corresponding instrumental signals. It was evaluated by analyzing samples with five different concentrations of the analyte, each in triplicate. A regression line or calibration curve was constructed by plotting the instrumental signals against the analyte concentrations. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares were then calculated to assess linearity [18,19].

Precision (Repeatability and Intermediate Precision). Precision was assessed at the lower limit of the working range using matrix samples spiked with known standard solutions. Each spiked sample was analyzed in triplicate on three different days. Repeatability and intermediate precision were calculated by using relative standard deviation (RSD). Intermediate precision was further evaluated through triplicate analyses conducted over one month.

Recovery. Recovery was calculated by measuring standards spiked into test samples at a known concentration. The average recoveries and their relative standard deviations were determined using response factors obtained from calibration solutions.

$$\text{Recovery}(\%) = \frac{C1}{C2} \times 100$$

where $C1$ is the analyte concentration measured after the addition; $C2$ is the added concentration.

Recovery values were not used for the correction of analysis results but to estimate the yield of the sample preparation procedures.

Measurement uncertainty. The measurement uncertainty was estimated using the error propagation law. The sources of uncertainty that were considered included the following: the preparation of standard solutions for instrument calibration, the preparation of spiking solutions for test samples, instrument calibration, the precision of the analyses, and bias uncertainty.

4. Results

In subjects affected by FD, the lack or limited activity of alpha-galactosidase A in lysosomes leads to a progressive accumulation of its substrates within the organs, such as globotriaosylceramide and other derivatives, promoting different pathological mechanisms and clinical manifestations of the disease [20–22]. The incidence of FD is very high, especially in newborn males (1 to 40,000), it being an X-linked pathology, resulting in a more severe manifestation of the symptoms. In female subjects, reduced or absent symptoms lead to an undiagnosed pathology [23]. The main aim of this paper is to set up an analytical method to quantify Fabry disease biomarkers, Gb3 and lyso-Gb3, in the blood samples of affected patients by using mass spectrometry techniques coupled with liquid chromatography in multiple reaction monitoring ion mode.

The first part of the present paper was related to the optimization and validation of the method for target analyte extraction from blood samples, as well as for mass spectrometry analysis. Then, the validated method was used to assess the amount of Gb3 and LysoGb3 in a cohort of blood samples of Fabry disease patients organized for sex and control samples of healthy subjects. The study was approved by the Ethical Committee (Protocol Number: 181/19) of the University of Naples Federico II. In addition, to take part in the work, informed consent was obtained from all participants.

Preliminary analyses were carried out for optimizing the chromatographic separation and MS conditions by using N-heptadecanoyl-ceramide trihexoside, and N-glycinated lyso-ceramide trihexoside as internal standard metabolites to quantify GB3 and lyso-GB3. To this aim, several extraction procedures were compared, and a validation process was performed to calculate the analytical parameters, such as the matrix effect, LOD and LOQ, the precision and accuracy, and the recovery in the matrix before proceeding with the determination of Gb3 and lyso-Gb3 in blood or serum samples.

4.1. Sphingolipids Separation

Preliminary tests, coupling liquid chromatography to the MRM/MS analysis, were performed to find the best chromatographic condition on a C4 column. The optimal separation condition for target molecules, e.g., sphingolipids, was obtained by using a gradient of methanol and water containing a small amount of ammonium formate and formic acid along an 11-gradient chromatographic run. The total ion current (TIC) recorded for a 400 µg/L standard mixture displayed with a good peak shape for all analytes (Figure 1, Panel A). Several injections of the standard mixture were carried out to ensure the repeatability of retention times.

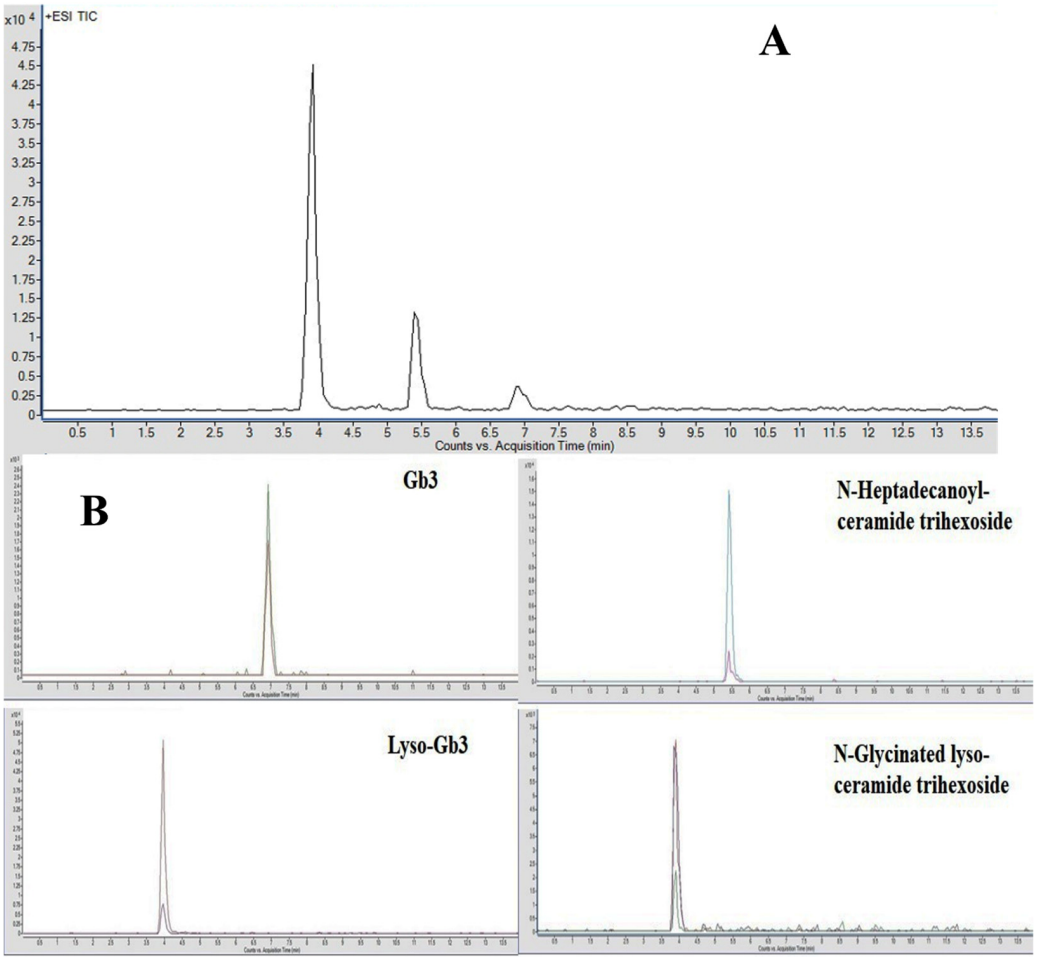


Figure 1. Total ion current chromatogram for the analysis of standard mixture solution at a concentration of 400 μg/L (**A**). MRM transition for the analysis of standard mixture solution at a concentration of 400 μg/L (**B**).

The MRM ion current for specific transitions associated to each analyte was extracted to selectively identify target metabolites (Figure 1, panel B). Indeed, the MRM chromatograms obtained for GB3, lyso-Gb3, N-heptadecanoyl-ceramide trihexoside and N-glycinated lyso-ceramide trihexoside showed a good selectivity, with all transitions detected at the same retention time, confirming the fragmentation reactions associated with the specific chemical structure of each analyte (Figure 1, panel B). Then, a good chromatographic separation was observed for GB3 and N-heptadecanoyl-ceramide trihexoside, detected at a retention time of 6.8 and 5.4 min, respectively. Conversely, the MRM chromatograms obtained for Lyso-GB3 and N-glycinated lyso-ceramide trihexoside (its internal standard) showed just a small difference in retention times (3.9 vs. 3.8 min, respectively).

4.2. Sample Extraction Procedure

The sample extraction procedure was selected in an attempt to reduce the sample manipulation to a minimal level and preserve losses of the analytes within the blood and serum samples. Four different extraction solvents or mixtures of solvents were used to assess the best extraction procedure of target analytes in blood samples: chloroform, methanol, chloroform/methanol (1/1), and chloroform/methanol/H_2O (2/1/0.3). The peak areas of target analytes were extracted and compared to elucidate the best extraction strategy. Figure 2 shows a comparison of peak areas of N-glycinated lyso-ceramide trihexoside in relation to the four extraction solvents.

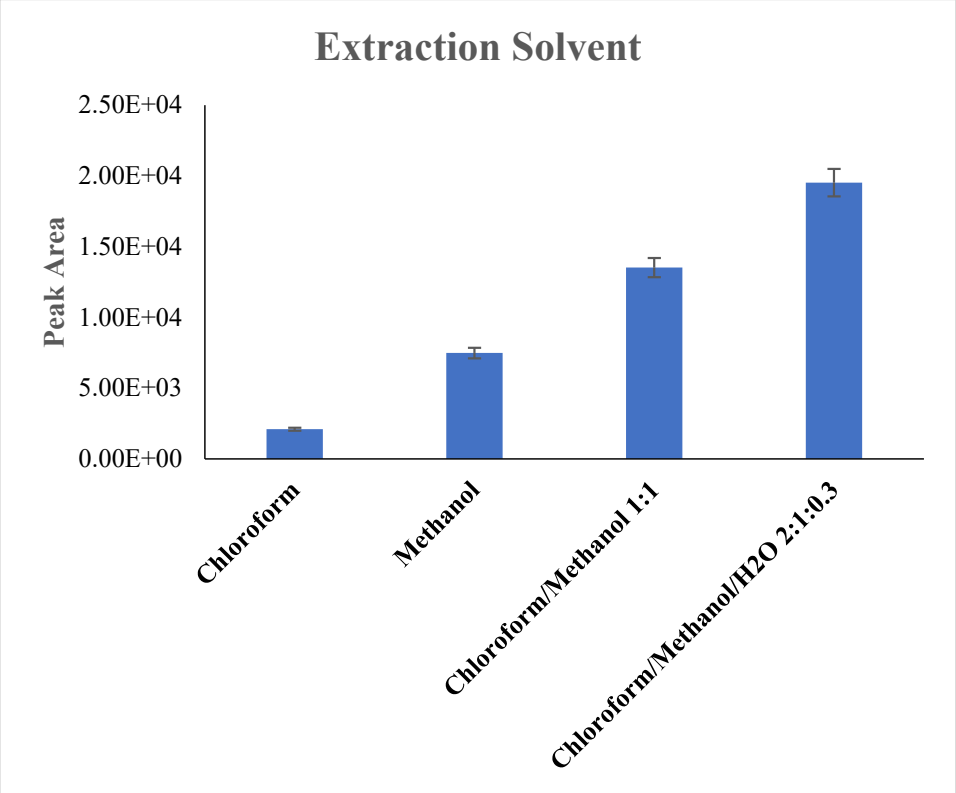


Figure 2. Extraction tests of N-Glycinated lyso-ceramide trihexoside by using several solvents.

The mixture of chloroform/methanol/H_2O (2/1/0.3) was selected as the best solvent for protein precipitation and extraction of sphingolipids by displaying a recovery of 92%, by comparing the EIC peaks for each analyte. The experiments were performed in duplicate and averaged, and the assays were performed by extending the extraction time beyond 30 min (i.e., up to 2 h), demonstrating any increase in the analyte recovery. No transition associated with the internal standards was detected, demonstrating the selectivity of the method resulting from the absence of these metabolites in the matrix and therefore the good choice of internal standards. Furthermore, no interference or carryover peaks were observed in the blank samples (non-spiked matrix) of the MRM analysis.

4.3. Method Validation

The linearity and matrix effects were studied using standard solutions and matrix-matched calibrations prepared by spiking the internal standards, N-heptadecanoyl-ceramide trihexoside and N-glycinated lyso-ceramide trihexoside, at known concentrations, for GB3 and lyso-Gb3, respectively, into the matrix (Table 2). Three replicates were realized to assay the method's precision. Both standard and matrix-matched calibration curves were built up by plotting the peak areas against concentration, and linear functions were applied to the calibration curves. Data were integrated by Mass Hunter quantitative software (version 12.0) showing a linear trend in the calibration range for all molecules. The matrix effect was calculated as the percentage of the matrix-matched calibration slope divided by the standard calibration slope, yielding a matrix effect of 11–15% for the two analytes (Table 2).

Table 2. Reproducibility of the LC-MRM/MS analysis of standard mixtures in solution and spiked into the blood sample. The peak area values of triplicates were averaged. Standard deviation and coefficient of variation expressed as relative standard deviation % (RSD%) are reported.

N-Glycinated Lyso-Ceramide Trihexoside							N-Heptadecannoyl-Ceramide Trihexoside						
µg/L	**Response 1**	**Response 2**	**Response 3**	**Average**	**St. Dev.**	**RSD%**	**µg/L**	**Response 1**	**Response 2**	**Response 3**	**Average**	**St. Dev.**	**RSD%**
25	nd	nd	nd	nd	nd	nd	25	3537	4797	5850	4728	1158.04	24.49
50	5941	9548	8534	8008	1860.21	23.23	50	12,371	7645	11,421	10,479	2499.86	23.86
100	13,745	14,025	12,466	13,412	831.14	6.20	100	15,963	18,135	23,136	19,078	3678.30	19.28
200	25,579	26,436	29,437	27,151	2025.86	7.46	200	55,166	50,451	39,947	48,521	7790.84	16.06
300	52,177	47,129	43,669	47,658	4278.63	8.98	300	80,139	80,826	69,605	76,857	6289.51	8.18
400	66,86	61,56	62,775	63,732	2776.49	4.36	400	125,272	122,059	125,798	124,376	2024.03	1.63
Matrix-N-Glycinated Lyso-Ceramide Trihexoside							**Matrix-N-Heptadecannoyl Ceramide Trihexoside**						
µg/L	**Response 1**	**Response 2**	**Response 3**	**Average**	**St. Dev.**	**RSD%**	**µg/L**	**Response 1**	**Response 2**	**Response 3**	**Average**	**St. Dev.**	**RSD%**
25	nd	nd	nd	nd	nd	nd	25	5596	4567	5990	5384	734.73	13.65
50	6732	8563	9598	8298	1451.31	17.49	50	13,111	9695	12,962	11,923	1930.65	16.19
100	14,745	12,326	12,203	13,091	1433.44	10.95	100	18,993	18,654	20,936	19,528	1231.37	6.31
200	26,699	29,836	30,437	28,991	2007.26	6.92	200	65,261	59,951	59,917	61,71	3075.59	4.98
300	50,763	49,168	55,659	51,863	3382.50	6.52	300	88,963	92,826	89,605	90,465	2070.02	2.29
400	75,55	61,56	69,975	69,028	7042.88	10.20	400	144,972	123,352	135,738	134,687	10,848.23	8.05

The analytical parameters e.g., LOD and LOQ were calculated as reported in Section 2. Data were collected over a period of 10 days and the LODs and LOQs are reported in Table 3. Quantification was obtained using the calibration curves by multiplying the ratio of concentrations obtained by the known concentration of the internal standard.

Table 3. Analytical parameters: LOD, LOQ, linear range, matrix effect (%), and angular coefficient (m) and y-intercept (q) for the calibration curves. Linearity was calculated as R^2 for the standard in solvents and the standard in matrix.

	Compound	m	q	R^2	LOD [μg/L]	LOQ [μg/L]	Linear Range [μg/L]	Matrix Effect %
Solvents	N-Glycinated lyso-ceramide trihexoside	163.05	−2248.7	0.992	3.14	19.05	50–400	-
	N-Heptadecannoyl ceramide trihexoside	309.18	−8055.7	0.991	8.30	25.20	25–400	-
Matrix	N-Glycinated lyso-ceramide trihexoside	179.58	3457.4	0.990	2.57	15.57	50–400	10
	N-Heptadecannoyl ceramide trihexoside	344.13	7724.5	0.990	9.00	27.20	25–400	11

The precision and accuracy at different concentration levels were calculated for each analyte and the results were in the range of %RSD between 1.0 and 24.0%, with the recovery efficiency (%RE) being in the range 9.6–10.3. Data were collected over a period of 10 days and each measurement represents the average of three experiments. Recovery was calculated by comparing the extraction yield before and after the addition of the different standard analytes into the control blood samples. Quantitation was achieved by using the internal standard method. The recovery results for each analyte were higher than 90%.

4.4. Fabry Sample Analysis

The optimized LC-MRM/MS method was then applied to serum and blood samples of patients affected by Fabry disease. The samples were divided into two groups. The first one, consisting of two females and four males, was used to evaluate the best biological matrix for the quantitative analysis of Gb3 and lyso-Gb3, both in serum and blood. The second group, consisting of 13 blood samples (7 males and 6 females) from donors affected by the syndrome, and 12 blood samples (10 males and 2 females) from healthy donors, was analyzed accordingly. The quantitative analysis of GB3 and Lyso-GB3 was conducted using the internal standard method, by spiking the serum and blood samples with an internal standard solution containing 200 μg/L of each standard. These results allowed us to discover the recovery, which was greater than 90% for all samples tested. Each sample was analyzed in triplicate over a period of 10 days with a relative standard deviation of roughly 10%, as reported in Supporting Information (Table S1).

In the first group of samples, a higher content of GB3 was observed in blood samples than in serum samples. A difference of approximately one order of magnitude was found in the comparison between the two TICs. Figure 3 reports the overlapping of the TIC obtained for blood and serum of the sample cohort. Instead, no relevant difference was observed in the comparison of TICs of Lyso-GB3 across the blood and serum matrices. Moreover, no sex discrimination was found by comparing the Gb3 and Lyso-Gb3 levels in the blood and serum samples (Table S1). Because of the higher selectivity of the extraction method used for Gb3, blood was selected as the matrix of choice for determination of sphingolipids in the second group. Each sample was extracted and analyzed by LC-MRM/MS in triplicate, and the results were summarized in Table S2.

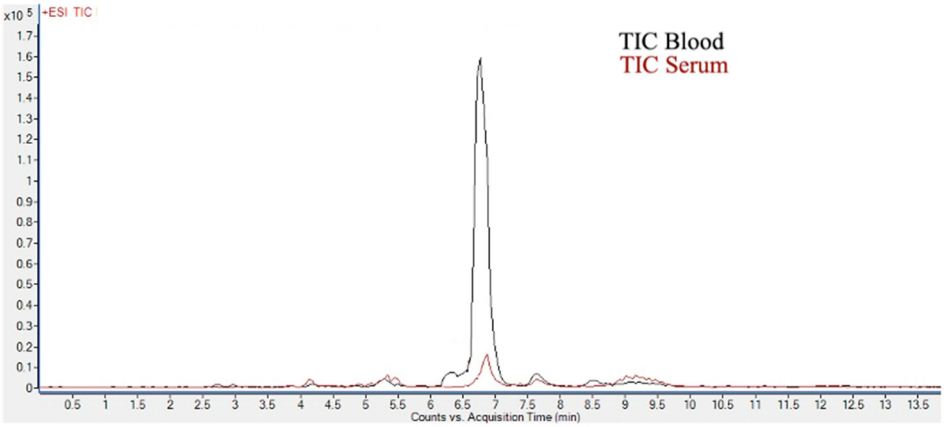


Figure 3. TIC serum and blood of a representative control sample.

A heatmap was obtained to evaluate the different levels of Gb3 and lysoGb3 in the blood samples of male and female Fabry-affected patients and healthy control, and the graphical representation is reported in Figure 4.

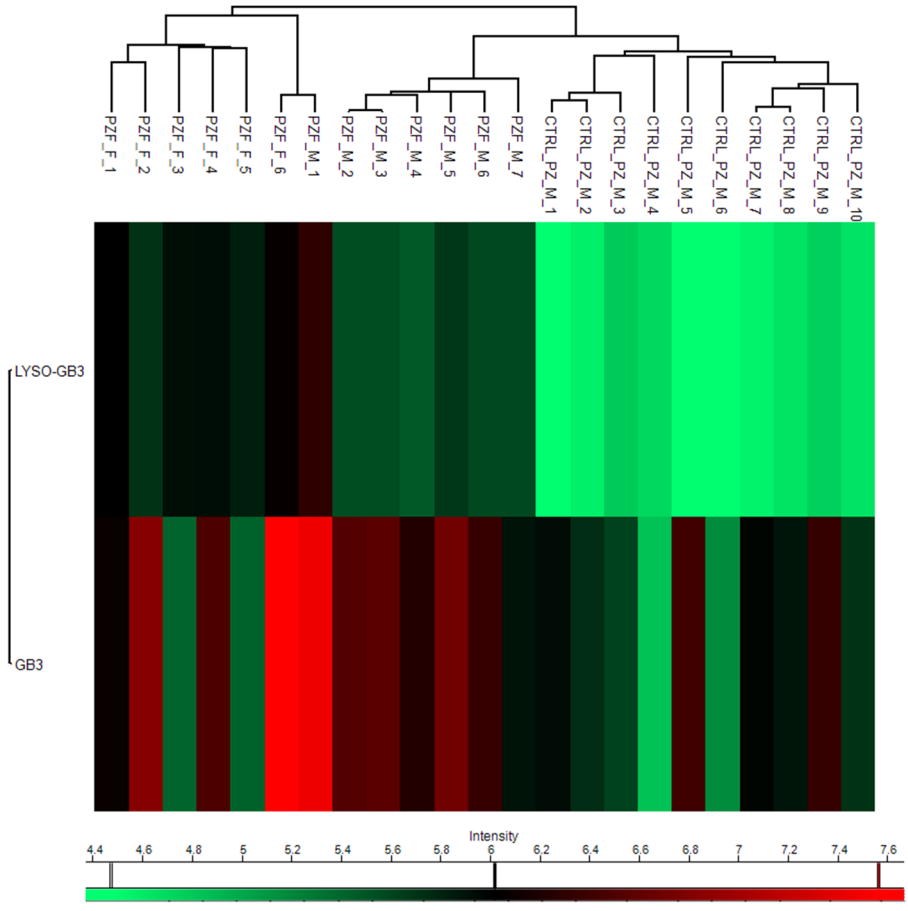


Figure 4. Heatmap hierarchical clustering of GB3 and lyso-Gb3 in blood from Fabry and control subjects.

The results demonstrated good clusterization for Lyso-GB3, which allowed for discrimination of the control samples from the pathological ones, even with a blood concentration higher in females versus males. The higher concentration of lyso-GB3 in FB-affected subjects reflected the impairment of enzymatic activity (α-Gal A) causing an accumulation of lyso-GB3 in blood, as reported by others [14]. Although considerable biological variability was observed for GB3, the average value was higher for all FB subjects than the control

one, similar to lyso-GB3. This finding is in disagreement with other authors [24,25] who differentiate metabolite levels based on the various phenotypic groups, such as the classic or later-onset Fabry, whereas a larger and wider heterogeneity was investigated in the current study. However, the data comparison allowed to obtain an average value for Lyso-GB3 at 44.0 ± 8.0 µg/L (biological replicates), useful for distinguishing a subject affected by FD from a healthy one, independently of the phenotype. Interestingly, a wider biological variability was observed for Gb3, with an average value of 95.7 ± 47.3 mg/L (biological replicate) when excluding two outlier values (Figure 4).

The present work discussed an easy and quick extraction strategy in combination with the targeted approach by using a validated LC-MRM/MS method for the simultaneous quantification of Lyso-Gb3 and GB3. The specificity of triple quadrupole in monitoring fragmentation reactions specific to the chemical structures of molecules, higher sensitivity of targeted approach, and the easy implementation of the method with a larger set of molecules, makes this technology appealing to the analytical field.

These preliminary results encourage further studies on larger cohorts of samples to confirm the experimental evidence of the current study by contemplating a larger biological variability, even correlated to the Fabry phenotypes.

5. Conclusions

Fabry disease is a complex condition that has significant implications for affected individuals. As an X-linked lysosomal storage disorder caused by a deficiency of the enzyme alpha-galactosidase A, it leads to the accumulation of specific glycosphingolipids, which can contribute to various clinical manifestations and organ system involvement. Early diagnosis and management are crucial, as many patients do not exhibit symptoms until later in life, which can complicate treatment and outcomes.

An unexpectedly high prevalence (1 in ~7800 infants) of the disease was observed by neonatal screening carried out within the population of northeastern Italy [26].

Nowadays, due to the relevance of disease, analytical methods capable of making an early diagnosis of the disease to improve patients' lives are encouraged to support specific therapies limiting the symptoms and damage to vital organs. The diagnosis of the disease is made through genetic studies [8], enzymatic, and fluorescence [7] assay to identify the presence of Lyso-Gb3 and its acylated form Gb3.

In the current study, an LC-MRM/MS method was developed to simultaneously identify and quantify the Lyso-Gb3 and Gb3 biomarkers of Fabry disease, by setting the best extraction strategy combined with an efficient chromatographic separation and the detection of the best transitions defined as a *quantifier* (786.8 m/z > 268.3 for Lyso-GB3, 1137.3 m/z > 264.3 m/z for GB3, 1039.3 m/z > 264.4 m/z for N-heptadecanoyl-ceramide trihexoside and 843.5 m/z > 264.3 m/z for N-glycinated lyso-ceramide trihexoside). The optimized method was subsequently applied to biological samples, such as serum and blood. The larger recovery of the target molecules in blood suggested quantifying Lyso-Gb3 and Gb3 in the blood of FD patients and control samples by using a reliable, fast, and effective LC-MRM/MS method.

The low costs of reagents together with the relative simplicity of sample preparation make the LC-MS/MS MRM method well-suited for the measurement of routine clinical samples. The developed methodology allows the simultaneous determination of Lyso-GB3 and GB3, but it can be easily extended to different classes of metabolites, and is easily expandable to a wide variety of clinical screening.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations11080239/s1, Table S1: GB3 and Lyso-GB3 concentration in blood and serum expressed as µg/L; Table S2: Results Fabry patient's female and male vs healthy patients control female and male.

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