

# Development and Validation of a Multiplex LC-MS/MS Assay for Lysosphingolipid Quantification in the Diagnosis of Gaucher and Fabry Diseases

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## Abstract

**Objectives:** Lysosphingolipids (LysoSLs), the N-deacylated forms of sphingolipids, are increasingly recognized as toxic metabolites that accumulate in various lysosomal storage disorders (LSDs), collectively known as sphingolipidoses. These compounds, due to their amphiphilic nature and bioactive properties, can contribute to cellular dysfunction and disease pathogenesis. Recent studies have demonstrated that specific LysoSLs, such as hexosylsphingosine (HexSph), globotriaosylsphingosine (LysoGb3), and lysosphingomyelin (LysoSM), are elevated in plasma, serum, or dried blood spots from patients with Gaucher, Fabry, Krabbe, or Niemann-Pick diseases. These findings have paved the way for the use of LysoSLs as sensitive and specific biomarkers in the diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses. This study aimed to develop and validate a method for the simultaneous quantification of three LysoSLs—HexSph, LysoGb3, and LysoSM—using liquid chromatography–tandem mass spectrometry (LC-MS/MS), for use in the diagnosis and follow-up of sphingolipidoses.

**Materials and Methods:** A multiplex analytical method was optimized for quantifying HexSph, LysoGb3, and LysoSM by ultra-fast LC coupled to tandem MS (UFLC-MS/MS) in positive-ion mode with electrospray ionization (LC-20 AD UFLC XR; Shimadzu 8040, Japan), employing multiple reaction monitoring. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantification, and recovery rates. Following method validation, plasma samples were collected from 14 patients diagnosed with Gaucher disease or Fabry disease at Akdeniz University Hospital and from 20 healthy adult volunteers. Sample preparation involved methanol/acetone/water extraction prior to analysis of LysoSL concentrations.

**Results:** The total analysis time for all three analytes was determined to be 10 minutes. Calibration curves demonstrated strong linearity, with coefficients of determination ( $r^2$ ) of 0.995 for LysoSM, 0.982 for HexSph, and 0.961 for LysoGb3. Plasma concentrations of LysoSLs were measured in both the patient and control groups. Significantly elevated levels of LysoSM and HexSph were observed in Gaucher patients compared to healthy controls. While LysoGb3 levels were higher in Fabry patients than in controls, the difference did not reach statistical significance.

**Conclusion:** This validated multiplex LC-MS/MS assay enables rapid and reliable simultaneous quantification of key LysoSLs in plasma. The assay holds promise as a diagnostic and monitoring tool for sphingolipidoses and may be further expanded to include additional biomarkers relevant to other LSDs. Quantification of LysoSLs can support clinical decision-making, including therapeutic selection and monitoring of patients undergoing enzyme replacement therapy.

**Keywords:** Lysosphingolipid, Hexosylsphingosine, Globotriaosylsphingosine, Lysosphingomyelin, LC-MS/MS



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## INTRODUCTION

Sphingolipidoses are a subgroup of lysosomal storage disorders (LSDs) caused by inherited defects in lysosomal degradation pathways of sphingolipids.<sup>1,2</sup> This group of inborn metabolic diseases includes primary sphingolipidoses, such as Niemann-Pick types A, B, and A/B (NPA/B), Fabry disease (FD), Krabbe disease (KD), Gaucher disease (GD), and Tay-Sachs disease, all of which are caused by mutations in genes encoding specific lysosomal enzymes. Secondary sphingolipidoses, such as NPC, as well as disorders related to saposin deficiencies, are included.

Due to overlapping clinical manifestations among various sphingolipidoses, accurate clinical diagnosis is often challenging. Therefore, identification and analysis of molecular biomarkers that can facilitate differential diagnoses have become increasingly important. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a widely used, highly sensitive, and highly specific analytical technique for biomarker quantification. One of its key advantages is the ability to simultaneously measure multiple analytes within a single run.

In recent years, the metabolism and biological functions of sphingolipids have been the focus of extensive research, and LC-MS/MS has become an essential tool in sphingolipid profiling. Although increased levels of glycosphingolipids have been reported in several sphingolipidoses, their relatively low sensitivity and specificity limit their utility as diagnostic biomarkers.<sup>3</sup> In contrast, lysosphingolipids (LysoSLs)—the N-deacylated derivatives of glycosphingolipids—have been found to accumulate markedly in the plasma and urine of patients with sphingolipidoses. These findings support their potential as sensitive and specific biomarkers for both diagnosis and therapeutic monitoring.<sup>4</sup>

Various forms of LysoSLs (LysoSLs) have been found to be selectively elevated in specific sphingolipidoses. For instance, significantly increased levels of globotriaosylsphingosine (LysoGb3) have been detected in both plasma and urine of male and female patients with FD.<sup>5-7</sup> In Krabbe disease, galactosylsphingosine (GalSph) is markedly elevated,<sup>8,9</sup> while glucosylsphingosine (GlcSph) shows a significant increase in GD.<sup>3</sup> Additionally, lysosphingomyelin (LysoSM), the N-deacylated derivative of sphingomyelin, has been reported to accumulate in patients with NPA, NPB, and NPC.<sup>10,11</sup>

The aim of this study was to establish and validate a robust analytical method for the simultaneous quantification of hexosylsphingosine (HexSph), LysoGb3, and LysoSM using LC-MS/MS. This method was optimized for use in clinical laboratories to support the diagnostic workflow for suspected sphingolipidoses and to monitor biochemical responses in patients receiving

specific therapies, such as enzyme replacement therapy or substrate reduction therapy. The development of a multiplex assay for LysoSLs not only improves diagnostic efficiency but also facilitates the early detection and longitudinal assessment of disease activity, potentially leading to better patient outcomes. Our study introduces a locally optimized, rapid multiplex LC-MS/MS workflow for simultaneous quantification of LysoGb3, LysoSM, and HexSph with a 10-minute run time, which is shorter than that of comparable published methods.<sup>12</sup>

## MATERIALS AND METHODS

### Reagents and Chemicals

The following chemicals and reagents were used for LysoSL analyses: GlcSph (bovine buttermilk), N-omega-CD3-octadecanoyl-ceramide trihexoside (internal standard 1537; Matreya Chemicals), GalSph, LysoSM, LysoGb3, acetonitrile, methanol, acetone, chloroform, formic acid, and ultrapure water.

### Sample Collection

Ethical approval was obtained from the Ethics Committee of Akdeniz University Faculty of Medicine (decision number: 373, dated: 30.05.2018). After obtaining informed consent, venous blood samples were collected into K2-EDTA tubes from 14 patients diagnosed with Fabry or GD and from 20 healthy adult volunteers at Akdeniz University Hospital. Plasma samples were separated and stored for analysis. Diagnoses were confirmed in all patients through demonstration of significant lysosomal enzyme deficiencies and/or mutation analysis.

### Preparation of LysoSL Calibration Standards

Stock standard solutions were prepared by dissolving purified standards in a chloroform:methanol (2:1, v/v) mixture at the following concentrations: GlcSph, 0.5 mg/mL; LysoSM, 1 mg/mL; and LysoGb3, 0.1 mg/mL. GlcSph was used as the standard for HexSph. Working solutions at a concentration of 10 µmol/L for each analyte were prepared in the same solvent mixture and stored at -20 °C. These working solutions were diluted with methanol/acetone/water (45:45:10, v/v) to a final concentration of 10 nmol/L to prepare the precipitation solution containing the internal standard. A deuterium-labeled internal standard, N-omega-CD3-octadecanoyl-ceramide trihexoside (C18-CD3-Gb3), was used for quantification.

Calibration curves were constructed using six concentrations prepared by serial dilution of the stock solutions. The calibration ranges were 0-200 nmol/L for LysoGb3 and LysoSM, and 0-1000 nmol/L for HexSph. The accuracy and precision of the assay were evaluated using QC samples prepared at two concentration levels.

## Sample Preparation

To 100 µL of plasma, calibrator, or QC sample, 500 µL of working precipitation solution was added. After vortexing for 30 seconds, the mixture was sonicated for 1 minute and subsequently centrifuged at 16,200 × g for 10 minutes. The clear supernatant was transferred to a new tube and evaporated under nitrogen. The dried residue was reconstituted in 100 µL of acetonitrile/water (1:1, v/v) containing 0.1% formic acid and centrifuged again at 16,200 × g for 10 minutes prior to LC-MS/MS analysis.

## LC-MS/MS Method Optimization

Chromatographic separation of LysoGb3, HexSph, and LysoSM was achieved using a reverse-phase Raptor acid-resistant C18 column (2.1 × 100 mm, 2.7 µm particle size; Restek, USA). Because GlcSph and GalSph co-elute on this column, the two analytes were quantified together as HexSph (GlcSph + GalSph). Unlike most published studies focusing on a single analyte–disease pair, we provide a multiplexed approach applicable to multiple sphingolipidoses.

Mass spectrometric detection was performed on a UFC-MS/MS system (LC-20 AD UFC-XR, Shimadzu 8040, Japan) equipped with a positive-mode electrospray ionization source. For each analyte, the precursor ions and optimized transitions were selected and monitored in multiple reaction monitoring mode. Detailed LC-MS/MS parameters are provided in Table 1.

## Method Validation

The accuracy of the method was evaluated using quality control (QC) samples. Accuracy was calculated as the relative

**Table 1. Detailed LC-MS/MS parameters.**

Parameter	Description
Mass spectrometer	Shimadzu 8040
Ionization mode	ESI, positive mode (+)
Column	Raptor ARC-18, 2.1 × 100 mm, 2.7 µm particle size
Column temperature	45 °C
Mobile phase A	H <sub>2</sub> O + 0.1% FA
Mobile phase B	ACN + 0.1% FA
Gradient program (Pump B)	Time (min) %B 0–4 80 4–6 100 6–10 40
Flow rate	0.5 mL/min
Injection volume	2 µL
Total run time	10 minutes

H<sub>2</sub>O 0.1% FA; water containing 0.1 percent FA, ACN 0.1% FA; acetonitrile containing 0.1 percent FA. ARC-18, acid-resistant C18; ESI, electrospray ionization; FA, formic acid.

error (%), which was obtained by subtracting the nominal value from the mean measured value, dividing the difference by the nominal value, and multiplying by 100. Intra-day and inter-day precision were assessed by analyzing each standard in triplicate over consecutive days. Linearity was evaluated using six-point calibration curves for each biomarker. The limit of detection (LOD) and limit of quantification (LOQ) were determined for all analytes. Carryover effects were assessed by injecting blank samples after the highest calibration standard.

## Statistical Analysis

Statistical analyses were performed using SPSS version 20.0. Differences between groups were assessed using a two-tailed Student's t-test. A p-value ≤ 0.05 was considered statistically significant.

## RESULTS

### Chromatographic Analysis of LysosSLs

Chromatographic separation of LysosSLs was achieved with retention times of 1.57 minutes for LysoGb3, 1.72 minutes for LysoSM, and 1.98 minutes for HexSph. However, to ensure complete column elution and prevent carryover, the total run time was set to 10 minutes (Figure 1).

### Linearity, Detection, and Quantification Limits

Repeated calibration curves were generated to assess linearity for LysoGb3, LysoSM, and HexSph, and the r<sup>2</sup> values were 0.995 for LysoSM, 0.982 for HexSph, and 0.961 for LysoGb3. A blank sample was injected after the highest calibration standard, and no carry-over was observed. The LOD and LOQ for LysosSLs were calculated from the mean and standard deviation of blank samples, which were run to obtain those statistics. The LOD and LOQ for LysosSLs were 0.012 and 0.036 nmol/L for LysoSM, 0.01 and 0.03 nmol/L for HexSph, and 7.18 and 21.54 nmol/L for LysoGb3, respectively (Table 2).

### Precision and Accuracy

Precision and accuracy for each analyte were determined intraday (n = 5) and interday (n = 5). The % CV was below 20% for LysoSM and HexSph at all concentrations tested, whereas for LysoGb3 it was below 20% at 25 nmol/L and above.

### LysosSL Levels in Healthy Controls and Lysosomal Disease (LD) Patients

Demographic characteristics of the study groups are summarized in Table 3. All Gaucher patients had Type 1 disease. Our study cohort consisted of seven male patients with the classic Fabry phenotype and three female patients with a stroke-associated Fabry phenotype. All patients were genetically confirmed. None of the patients were on enzyme replacement therapy at the time

of sampling. Plasma concentrations of LysoGb3, LysoSM, and HexSph in patients and controls are shown in Table 4.

Patients with GD exhibited significantly higher mean plasma levels of LysoSM and HexSph than those in healthy individuals. Although LysoGb3 levels were numerically higher in Fabry patients than in controls, the difference did not reach statistical significance ( $p = 0.08$ ). The lack of statistical significance is likely due to the limited sample size ( $n=10$ ) and the considerable

**Table 2. Mass shifts and retention times of Lysos in LC-MS/MS.**

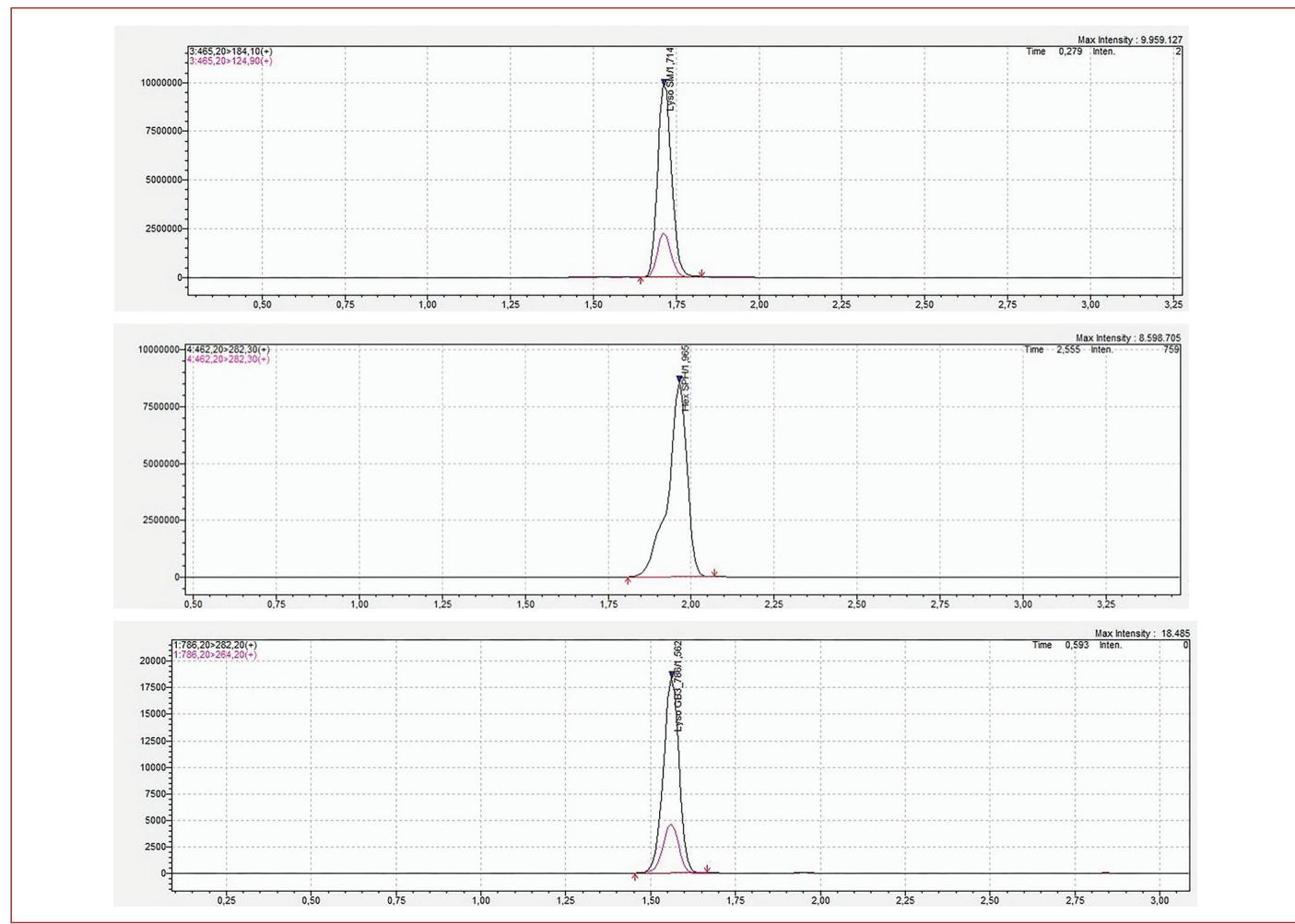
Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)
LysoGb3	786.20	282.20	1.57
LysoSM	465.20	184.10	1.72
HexSph	462.20	282.30	1.98
LysoGb3-IS	843.20	264.30	1.40

**Table 3. Demographic characteristics of patients and healthy controls.**

Group	Controls	FD	GD
Sample size	20	10	4
Sex (M/F)	10/10	7/3	4/0
Age (mean, range)	37 (21-77)	37 (22-51)	40 (38-42)

**Table 4. Plasma Lysos levels in Gaucher and Fabry patients vs. healthy controls.**

Lysos	Group	Mean $\pm$ SEM (nmol/L)	p-value
LysoSM	Gaucher (n=4)	0.87 $\pm$ 0.28	0.001
	Control (n=20)	0.25 $\pm$ 0.02	
HexSph	Gaucher (n=4)	0.83 $\pm$ 0.39	0.001
	Control (n=20)	0.02 $\pm$ 0.002	
LysoGb3	Fabry (n=10)	35.63 $\pm$ 15.89	0.08
	Control (n=20)	10.03 $\pm$ 0.28	



**Figure 1.** Chromatographic separation of Lysos with ARC-18 column in the 100 nmol/L calibrator (A. LysoSM 1.72, B. HexSph 1.98, C. LysoGb3 has an elution time (retention time) of 1.57 min).

variability between patients. In addition, differences in matrix and calibration, which affect absolute concentrations, may have masked numerical differences, even though they do exist.

## DISCUSSION

LysoSLs have emerged as reliable and informative biomarkers for the diagnosis, prognosis, and therapeutic monitoring of sphingolipidoses, a group of inherited LSDs characterized by the accumulation of sphingolipid substrates due to enzyme deficiencies.<sup>3,5</sup> Numerous LC-MS/MS methods have been developed for the quantification of LysoSLs in various biological matrices, including plasma, urine, and dried blood spots.<sup>6,9,11–15</sup> However, a significant limitation of many previously published assays is their focus on a single analyte associated with a specific disease. While such approaches are effective for targeted diagnostics, they may fail to capture the broader metabolic alterations observed in LDs with overlapping or non-specific symptoms.

Recent studies have highlighted the value of multiplex panels that allow for the simultaneous measurement of multiple LysoSLs, suggesting that such panels can improve diagnostic yield, reduce turnaround time, and enhance cost-efficiency, particularly in high-throughput clinical settings.<sup>14</sup> The ability to evaluate multiple biomarkers from a single sample can also be particularly advantageous in early screening programs or when patient sample volume is limited. Moreover, multiplexing supports a more comprehensive understanding of disease pathology and may help differentiate between related disorders or phenotypic variants.

In this study, we developed and validated a rapid and accurate UFLC-MS/MS method for the simultaneous quantification of three key LysoSLs—HexSph (a combination of GlcSph and GalSph), LysoGb3, and LysoSM—in human plasma. The method is based on simple protein precipitation, avoiding the need for complex and time-consuming liquid–liquid extraction protocols. This streamlined sample preparation significantly reduces overall analysis time and enables high-throughput testing without compromising sensitivity or accuracy. The developed method is rapid, reproducible, and suitable for clinical laboratories. It simplifies sample preparation, reduces run time, and provides robust quantification.

Chromatographic separation was performed on a reversed-phase C18 column, which enabled efficient retention and resolution of LysoSLs. Due to the structural similarity between GlcSph and GalSph, these analytes were not resolved and were therefore reported collectively as HexSph. The use of a deuterated LysoGb3 internal standard ensured analytical robustness and reproducibility. The method demonstrated excellent linearity,

precision, and accuracy, with detection and quantification limits comparable to or better than those reported in prior literature.

The analysis enabled the quantification of plasma LysoSL levels in healthy controls and in subjects with confirmed Gaucher and FD. Among Gaucher patients, LysoSM and HexSph were significantly elevated compared with controls, suggesting their relevance as disease-specific biomarkers. These findings are consistent with previous studies reporting elevated levels of LysoSM and GlcSph in GD, which is caused by  $\beta$ -glucocerebrosidase deficiency. In Fabry patients, plasma LysoGb3 levels were increased; however, this difference did not reach statistical significance, potentially owing to the limited sample size and the cross-sectional design of the study. Larger cohort studies may be needed to fully validate the diagnostic sensitivity and specificity of LysoGb3 in this population. During the study period (2018–2019), commercial control materials were not yet available in our laboratory.

Importantly, our method's short run time (10 minutes per sample) and compatibility with standard LC-MS/MS instrumentation make it well-suited for routine clinical application. The proposed panel could be expanded in future studies to include additional LysoSLs or disease-specific lipid species, thereby increasing its diagnostic utility. Furthermore, monitoring LysoSL levels over time may provide insights into disease progression and response to treatment, particularly in patients receiving enzyme replacement therapy or substrate reduction therapy.<sup>16</sup>

## CONCLUSION

This study presents a robust, high-throughput UFLC-MS/MS method for the simultaneous quantification of clinically relevant LysoSLs in plasma. The approach holds promise for improving the efficiency and diagnostic accuracy of sphingolipidosis screening and may serve as a valuable tool in both clinical and research settings. Further validation in larger and more diverse patient populations is warranted to fully establish its diagnostic and prognostic utility.

## Ethics

**Ethics Committee Approval:** Ethical approval was obtained from the Ethics Committee of Akdeniz University Faculty of Medicine (decision number: 373, dated: 30.05.2018).

**Informed Consent:** Informed consent was obtained from the parents of all patients included in this paper.

## Footnotes

## Authorship Contributions

**Surgical and Medical Practices:** H.A., E.S., **Concept:** H.A., **Data Collection or Processing:** H.A., B.K.E., N.Ö., **Analysis or**

Interpretation: H.A., E.S., Literature Search: H.A., A.E.B., B.K.E., Writing: H.A., A.E.B., B.K.E.

**Conflict of Interest:** The authors have no conflicts of interest to declare.

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