Multiplex Quantification of Plasma Biomarkers for Patients with Gaucher Disease Type 1

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Gaucher disease (GD) is a lysosomal storage disorder caused by a deficiency of the enzyme beta-glucocerebrosidase. This leads to the accumulation of glycolipids in macrophages and ultimately results in tissue damage. Recent metabolomic studies highlighted several potential biomarkers in plasma specimens. In hopes of better understanding the distribution, importance, and clinical significance of these potential markers, a UPLC-MS/MS method was developed and validated to quantify lyso-Gb_1 and six related analogs (with the following modifications on the sphingosine moiety: $-C_2H_4$ (-28 Da), $-C_2H_4+O$ (-12 Da), $-H_2$ (-2 Da), $-H_2+O$ (+14 Da), $+O$ (+16 Da), and $+H_2O$ (+18 Da)), sphingosylphosphorylcholine, and *N*-palmitoyl-O-phosphocholineserine in plasma specimens of treated and untreated patients. This 12-min UPLC-MS/MS method involves a purification step via solid-phase extraction followed by evaporation under nitrogen flow and resuspension in an organic mix compatible with HILIC chromatography. This method is currently used for research purposes and might be used for monitoring, prognostics, and follow-up. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

Keywords: biomarker • Gaucher disease • Lyso-Gb_1 • Lyso-Gb_1 analogs • mass spectrometry • *N*-palmitoyl-O-phosphocholineserine • sphingosylphosphorylcholine

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INTRODUCTION

Gaucher disease (GD) is a lysosomal storage disorder caused by biallelic pathogenic variants in the *GBA1* gene (Özdemir, 2022). This results in enzymatic deficiencies of beta-glucocerebrosidase (GCase, EC 3.2.1.45), which is involved in the degradation pathway of glucosylceramide and leads to the accumulation of several glycolipids within the lysosomes of tissues including the spleen, liver, bone marrow, and occasionally the lungs, ultimately resulting in multisystemic complications (Grabowski, Antommaria, Kolodny, and Mistry, 2021).

GD is characterized by marked phenotypic and genotypic variability (D'Amore et al., 2021; Grabowski et al., 2021; Özdemir, 2022). Over 300 different mutations have been described for the *GBA* gene (D'Amore et al., 2021) and result in phenotypes ranging from asymptomatic to in-utero death (Arévalo et al., 2022; Weinreb et al., 2021. Although GD phenotypes span a wide continuum, three types can be distinguished (Schiffmann et al., 2020; Silva García et al., 2021). Type 1 GD (OMIM 230800) accounts for approximately 90% to 95% of all GD cases, with an estimated disease prevalence of 1 in 52,000 individuals in the general population (Biegstraaten et al., 2018; Chis, Chis, and Dumitrascu, 2021; Giraldo, 2021). This number can, in certain communities, be significantly increased. Indeed, GD affects approximately 1 newborn out of 800 in Ashkenazi Jewish communities (Mistry et al., 2020; Stirnemann et al., 2017). Type 1 GD is known for being a more attenuated form of the disease (Mistry et al., 2020). Typically, neurological involvement is not observed in GD type 1, although some have described attenuated neurological features. Other clinical manifestations may include hepatosplenomegaly, cytopenia, and bone or pulmonary involvement (Essabar et al., 2015; Miyamoto et al., 2021). GD type 3 (OMIM 231000), known as the chronic neuronopathic form of the disease, generally involves some degree of neurological impairment in addition to the somatic symptoms potentially observed in GD type 1 (Donald et al., 2020). Finally, GD type 2 (OMIM 230900) is the most severe form of the disease. Neurological involvement may appear in the first months of life and lead to death within the first years of life (Gary, Ryan, Steward, and Sidransky, 2018; Marshall et al., 2016; Sam, Ryan, Daykin, and Sidransky, 2021).

Enzyme replacement therapy (ERT) and substrate reduction therapy are available (Gary et al., 2018; Marshall et al., 2016). Although some challenges remain, such as the inability of ERT to cross the blood-brain barrier, studies have demonstrated the clinical efficacy of these treatments (Andrade-Campos et al., 2017). Moreover, experts have argued about the potential benefits of early treatment initiation (Andrade-Campos et al., 2017; Charrow, 2015; Mistry et al., 2009). It is believed that starting treatment early would prevent the accumulation of cytotoxic metabolites that lead to permanent and irreversible tissue damage (Charrow, 2015; Mistry et al., 2009). To achieve this, sensitive molecular and biochemical tests are required. Several biomarkers are used in clinical labs: chitotriosidase, angiotensin-converting enzyme (ACE), tartrate-resistant acid phosphatase (TRAP5b), and C-C motif ligand 18 (CCL18) can be quantified in blood specimens for routine follow-up (Giraldo, López de Frutos, and Cebolla, 2018; Stirnemann et al., 2017). Unfortunately, these markers are not specific to GD and are not well adapted for diagnostic purposes (Giraldo et al., 2018; Stirnemann et al., 2017). In recent years, the analysis of lyso-Gb$_1$ emerged as a gold standard for monitoring disease progression (Dinur et al., 2021; Revel-Vilk, Fuller, and Zimran, 2020). Studies revealed that lyso-Gb$_1$ is a highly sensitive biomarker (Dinur et al., 2021; Revel-Vilk et al., 2020) and that lyso-Gb$_1$ plasma levels correlate with disease severity (Revel-Vilk et al., 2020). Although lyso-Gb$_1$ represents a significant upgrade from previous markers, it is possible, considering the major phenotypic variability associated with the disease, that other markers in patient plasma may correlate with specific clinical manifestations. To investigate this hypothesis, our research group used a metabolomic approach to identify metabolites potentially associated with the condition in untreated patient plasma samples (Menkovic et al., 2020). This specific metabolomic study using ultra-performance liquid chromatography time-of-flight mass spectrometry highlighted several potential biomarkers, namely lyso-Gb$_1$ and four related analogs (with the following modifications on the sphingosine moiety: -C$_2$H$_4$ (-28 Da), -H$_2$ (-2 Da), -H$_2$+O (+14 Da), and +H$_2$O (+18 Da)), sphingosylphosphorylcholine (SPC), and *N*-palmitoyl-O-phosphocholineserine (NPOPCS) (Menkovic et al., 2020). After structural identification of these metabolites, we developed and validated a quantification method for these compounds and two other lyso-Gb$_1$ analogs (see Background Information) using ultra-performance liquid chromatography/tandem mass

spectrometry (UPLC-MS/MS) to investigate marker distributions and their clinical significance (Menkovic et al., 2022a). The validation step revealed that the method had high sensitivity with low limits of quantification and detection as well as high levels of accuracy and precision (Menkovic et al., 2022a). Herein, we provide an in depth protocol for the method developed to study the distribution of metabolites identified in our metabolomic study.

Materials

Surrogate matrix (see recipe)
H_3PO_4 (2% v/v in water) (see recipe)
Calibration curve/QC working solutions (see recipe)
Internal standard working solution (see recipe)
Methanol:Dimethyl sulfoxide (DMSO) 95:5 v/v solution (see recipe)
Formic acid 2% v/v solution in water (see recipe)
Formic acid 0.2% v/v in methanol (see recipe)
Ammonium hydroxide 2% v/v in methanol solution (see recipe)
Nitrogen evaporator (homemade)
Mobile phase A solution (see recipe)
Mobile phase B solution (see recipe)

Sodium citrate (light blue) blood collection tubes
Centrifuge (Beckman GPR Centrifuge Model 7C008)
1.5-ml polypropylene tubes (Sarstedt, cat. no. 72.690.300)
13 × 100–mm disposable culture tubes (Fisher Scientific, cat. no. 14-961-27)
16 × 150–mm disposable culture tubes (Fisher Scientific, cat. no. 14-961-31)
2-ml vials (Waters Corp. Milford, MA, USA, cat. no 186000307C)
Oasis MCX cartridges, 60 µm, 30 mg, LP (Waters, cat. no. 186000782)
Inserts, 250-µl, 6 × 29 mm (Chromatographic Specialities, cat. no. C221020M)
Preslit polytetrafluoroethylene (PTFE) silicone septum (Chromatographic Specialities, cat. no.C779200XBBM)
Halo HILIC 2.7 µm 4.6 × 150 mm (Advanced Materials Technology, cat. no. 92814701)
Xevo TQ-S mass spectrometer coupled with an Acquity I-Class UPLC system (Waters)

Plasma collection

1. Collect blood samples in sodium citrate vacutainer blood collection tubes (light blue).

2. Centrifuge at room temperature for 10 min at 1000 × *g* to extract plasma from the whole blood samples.

3. Transfer the plasma specimens into polypropylene tubes.

4. Keep at −20°C until shipping and analysis.

Experimental Protocol

5. Thaw the following solutions:

   - Quality control plasma specimens (healthy control samples)
   - Calibration curve/QC working solutions
   - Plasma samples to analyze
   - Internal standard working solution

6. Before use, vortex each solution for 10 s, followed by 1 min of sonication.

7. Repeat 3 times.

   This is quite important to solubilize SPC standards.

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Sample Preparation

QC samples and calibration curve points

8. In fourteen 1.5-ml Eppendorf polypropylene tubes, add 200 µl of the surrogate matrix.

9. Add 500 µl H_3PO_4 (2% v/v in water).

10. Add 20 µl calibration curve or QC working solution.

11. Add 500 µl internal standard working solution.

12. Vortex 10 s.

Patients and Controls

13. In a 1.5-ml Eppendorf polypropylene tube, add 200 µl of plasma.

14. Add 500 µl H_3PO_4 (2% v/v in water).

15. Add 20 µl methanol:dimethyl sulfoxide (DMSO, 95:5 v/v).

16. Add 500 µl internal standard working solution.

17. Vortex 10 s.

Plasma specimen SPE extraction

18. Label one 13 × 100–mm disposable culture tube, one 16 × 150–mm disposable culture tube, and one 2-ml borosilicate vial for each sample ID.

19. Deposit an Oasis MCX cartridge into each 16 × 150–mm disposable culture tube.

20. Cartridge conditioning 1: Add 1200 µl methanol to the cartridge using an automatic pipette and let it pass through via gravity.

21. Cartridge conditioning 2: Add 1200 µl H_3PO_4 (2% v/v in water) to the cartridge using an automatic pipette and let it pass through via gravity.

22. Transfer previously prepared samples onto their assigned conditioned Oasis MCX cartridge using an automatic pipette and let it pass through the cartridge via gravity.

23. Wash step 1: Add 1200 µl formic acid (2% v/v in water) using a repeater pipette and let it pass through the cartridge via gravity.

24. Wash step 2: Add 1200 µl formic acid (0.2% v/v in methanol) using a repeater pipette and let it pass through the cartridge via gravity.

25. Transfer Oasis MCX cartridges to the assigned 13 × 100–mm disposable culture tubes.

26. Using a repeater pipette, elute samples with 1200 µl ammonium hydroxide (2% v/v in methanol) and let it pass through the cartridge via gravity.

27. Evaporate to dryness under a nitrogen flow at room temperature.

28. Resuspend with 100 µl mobile phase A with a pipette.

29. Vortex each tube for 15 s.

30. Sonicate for 30 s.

31. Transfer suspension to a 250-µl glass insert deposited in the assigned 2-ml glass vial with a pipette, then cap the vials.

32. Centrifuge the vials for 20 s at room temperature and 1300 × *g*.

Table 1 Ultra-Performance Liquid Chromatographic Separation Method Parameters

Parameter	Description
Autosampler Temperature	10°C
Column Temperature	60°C
Weak Wash Solvent	ACN
Strong Wash Solvent	ACN
Injection Mode	Partial Loop Injection
Injection Volume	10 µl
Mobile Phase A	94.5:2.5:2.5:0.5 ACN: MeOH:H_2O:FA + 5 mM Amm. Form.
Mobile Phase B	10:89.5:0.5 ACN:H_2O:FA + 5 mM Amm. Form.
Flow rate	0.85 ml/min
Gradient (% Mobile Phase B)	0.0-1.0 min: 15%
	1.0-6.0 min: 15%
	6.0-7.0 min: 15%-60% (Linear Gradient)
	7.0-10.0 min: 60%
	10.0-12.0 min: 15%

33. Prepare the UPLC-MS/MS for sample analysis.

System preparation

34. Install the Halo HILIC chromatographic column on the UPLC system.

35. Install the following solutions on the UPLC system:

   - Mobile phase A
   - Mobile phase B
   - Weak needle wash
   - Strong wash

36. Set up the chromatographic separation parameters on the UPLC system, as shown in Table 1.

37. Set up the tandem mass spectrometry method parameters on the tandem mass spectrometry system, as shown in Table 2.

   When implementing a new method, voltages, dwell times, temperatures, and gas flows should always be optimized.

38. Set up the multiple reaction monitoring transitions in positive electrospray ionization mode, as shown in Table 2.

39. Inject 10 µl of the samples in the prepared UPLC-MS/MS system.

   Prepared samples are injected a single time and are stable in the UPLC injector at 10°C for at least 24 hr.

Data processing

40. Integrate the chromatographic peaks for each analyte and internal standard using TargetLynx software (Waters).

41. Calculate the ratio of the area of each analyte to the area of its corresponding internal standard. Lyso-Gb$_1$-($^{13}C_6$) is used as the internal standard for lyso-Gb$_1$ and its analogs, while SPC-(D$_9$) is used as the internal standard for SPC and NPOPCS. A chromatogram is shown in Figure 1.

Table 2 Tandem Mass Spectrometry Method Parameters

Mass Spectrometry Parameters

Capillary voltage	3.2 kV
Desolvation temperature	550°C
Desolvation gas flow	750 L/hr
Cone gas flow	150 L/hr
Source temperature	150°C

Analytes

Compound	Transitions (m/z)	Cone voltage (V)	Collision energy (V)	Dwell time (s)
Lyso-Gb$_1$ -28 Da	434.31 > 254.25	38	18	0.028
Lyso-Gb$_1$ -12 Da	450.31 > 252.23	38	18	0.028
Lyso-Gb$_1$ -2 Da	460.32 > 280.26	38	18	0.028
Lyso-Gb$_1$	462.34 > 282.29	38	18	0.028
Lyso-Gb$_1$-(^{13}C$_6$)	468.32 > 282.28	38	18	0.028
Lyso-Gb$_1$ +14 Da	476.36 > 296.29	38	18	0.028
Lyso-Gb$_1$ +16 Da	478.34 > 280.31	38	18	0.028
Lyso-Gb$_1$ +18 Da	480.36 > 318.31	38	18	0.028
SPC	465.35 > 184.05	18	21	0.028
SPC-(D$_9$)	474.40 > 193.13	18	21	0.028
NPOPCS	509.34 > 104.11	18	21	0.028
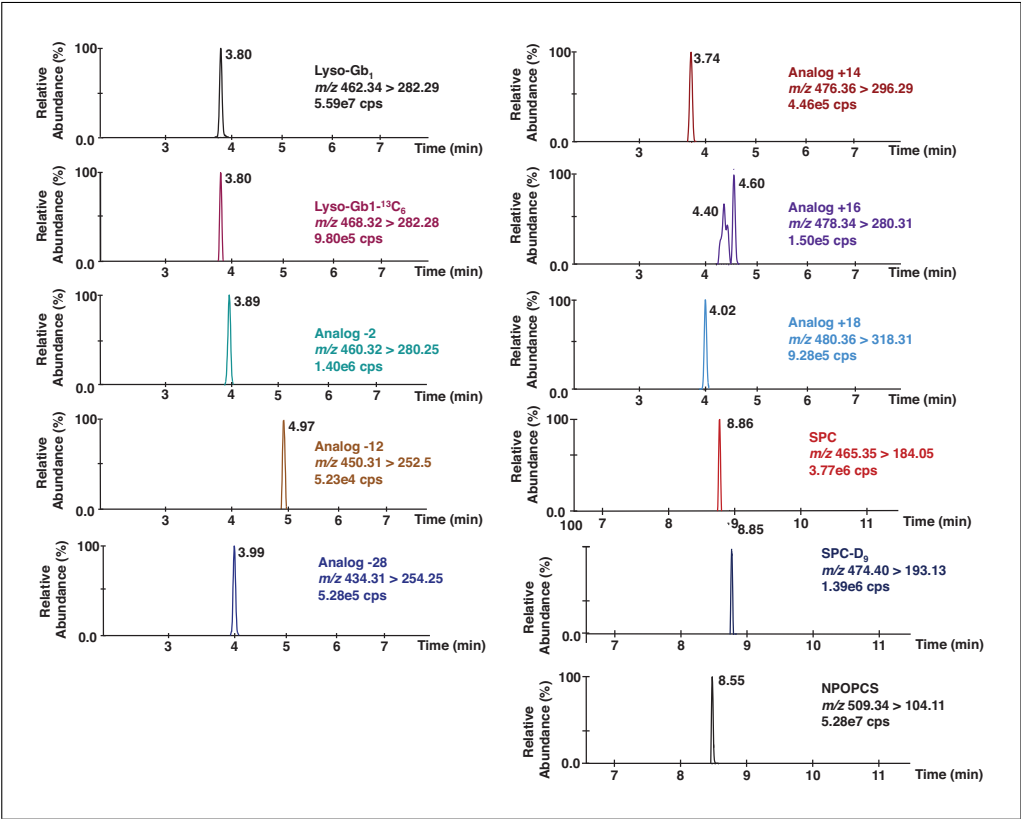
Figure 1 Chromatographic separation of lyso-Gb$_1$, lyso-Gb$_1$ analogs -28, -12, -2, +14, +16 and +18 Da, SPC, and NPOPCS.

42. Calculate the analyte concentrations using the calibration curves (area ratios obtained in the calibrators plotted against the nominal concentration). A lyso-Gb$_1$ calibration curve is used to quantify lyso-Gb$_1$ and its analogs, while an SPC calibration curve is used to quantify SPC and NPOPCS.

   A quadratic calibration curve with weighting 1/x is used for quantitation, and a fresh calibration curve is prepared with each batch of samples.

43. For both lyso-Gb$_1$ and SPC calibration curves, ensure that a minimum of 8 standards out of 10 have an accuracy (%bias) within ±15%, except for P1 (limit of quantitation), which should have an accuracy (%bias) within ±20%.

44. Ensure that the calibration curves have a coefficient of determination (r^2) >0.995.

REAGENTS AND SOLUTIONS

Use Optima LC/MS grade water (Fisher, cat. no. W6-4) for all solutions unless otherwise indicated.

Calibration curve and quality control working solutions

Using Table 3 below, add specific volumes of each solution in a 4-ml amber glass vial with PTFE black phenolic cap to obtain the calibration curve and QC working solutions. All preparations should be made fresh, at room temperature, and used the same day.

Formic acid, 2% v/v in water

1. In a 500-ml clear glass bottle, add 490 ml Optima LC/MS-grade water.
2. Add 10 ml formic acid (FA; >99%; Acros Organics, cat. no. EW-88147-15).

Keep at room temperature for up to 6 months.

Formic acid, 0.2% v/v in methanol

1. In a 250-ml clear glass bottle, add 100 ml Optima LC/MS-grade methanol and 99.6 ml Optima LC/MS-grade water.
2. Add 0.4 ml formic acid (FA; >99%; Acros Organics, cat. no. EW-88147-15).

Keep at room temperature for up to 6 months.

H$_3$PO$_4$ 2% v/v solution in water

1. In a 500-ml clear glass bottle, add 336 ml Optima LC/MS-grade water (Fisher Scientific, cat. no. W6-4).
2. Add 8 ml ACS-certified O-phosphoric acid (H$_3$PO$_4$; 85%; Fisher Scientific, cat. no. A242-500).

Keep at room temperature for up to 18 months.

Internal standard working solutions (SPC-(D9): 10.6 nM and lyso-Gb$_1$-($^{13}C_6$): 10.7 nM)

1. In a 250-ml amber glass bottle, mix 40 µl SPC-(D$_9$) stock solution (528 µM solution) with 40 µl lyso-Gb$_1$-($^{13}C_6$) internal standard (535 µM solution).
2. Complete volume with Optima LC/MS-grade methanol to 200 ml.
3. Proceed with a 1:10 dilution to obtain the internal standard working solution (20 ml internal standard solution added to 180 ml Optima LC/MS-grade methanol).

Keep at 4°C for up to 6 months.

Lyso-Gb$_1$ stock solution (0.2 mM)

1. In a 20-ml clear glass vial with polytetrafluoroethylene (PTFE) black phenolic cap, weigh 0.923 mg glucosylsphingosine (lyso-Gb$_1$; Matreya, cat. no. 1306) and dissolve in 10 ml Optima LC/MS-grade methanol.

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Table 3 Preparation of Calibration Curve and Quality Control Working Solutions

Calibration Point	Lyso-Gb₁ working solutions			SPC working solutions		DMSO (µl)	MeOH (µl)	Total Vol. (µl)	Lyso-Gb₁ Conc. (nM)	SPC Conc. (nM)
	200 nM (µl)	4000 nM (µl)	20,000 nM (µl)	200 nM (µl)	20,000 nM (µl)					
P0	-	-	-	-	-	50	950	1000	0	0
P1	5	-	-	10	-	49	936	1000	1	2
P2	50	-	-	100	-	40	810	1000	10	20
P3	250	-	-	500	-	0	250	1000	50	100
P4	-	50	-	-	20	48	882	1000	200	400
P5	-	200	-	-	50	45	705	1000	800	1000
P6	-	600	-	-	80	42	278	1000	2400	1600
P7	-	-	200	-	125	38	637	1000	4000	2500
P8	-	-	300	-	160	34	506	1000	6000	3200
P9	-	-	400	-	320	18	262	1000	8000	6400
P10[a]	-	-	500	-	600	50	950[a]	1000	10,000	12,000
LQC	150	-	-	300	-	20	530	1000	30	60
MQC	-	625	-	-	130	37	208	1000	2500	2600
HQC	-	-	250	-	375	12	363	1000	5000	7500

[a] Add 500 µl of lyso-Gb₁ 20,000 nM working solution and 600 µl of 20,000 nM working solution. Evaporate to dryness under a nitrogen stream and resuspend in 1 ml of a 95:5 MeOH:DMSO solution. Vortex for 10 s and sonicate for 30 s.

2. Sonicate for 30 s and verify that dissolution is complete.

Keep at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

Lyso-Gb$_1$-(^{13}C$_6$) internal standard stock solution (535 μM)

1. Dissolve 1 mg of ^{13}C$_6$-glucosylsphingosine (lyso-Gb$_1$-(^{13}C$_6$); Matreya, cat. no. 2209) in its original container with small amounts of Optima LC/MS-grade methanol and transfer to a 4-ml amber glass vial with PTFE black phenolic cap (Duran Wheaton Kimble, cat. no W224682).
2. Bring volume to 4000 μl with Optima LC/MS-grade methanol.
3. Sonicate for 30 s, vortex for 10 s, and verify that dissolution is complete.

Keep frozen at −20 or −80°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

Lyso-Gb$_1$ working solution 1 (20,000 nM)

In a 20-ml clear vial with PTFE black phenolic cap, dilute 1 ml lyso-Gb$_1$ stock solution (0.2 mM) with 9 ml Optima LC/MS-grade methanol.
Keep frozen at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

Lyso-Gb$_1$ working solution 2 (4000 nM)

In a 20-ml clear vial with PTFE black phenolic cap, dilute 2 ml lyso-Gb$_1$ working solution 1 (20,000 nM) with 8 ml Optima LC/MS-grade methanol.
Keep at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

Lyso-Gb$_1$ working solution 3 (200 nM)

In a 20-ml clear vial with PTFE black phenolic cap, dilute 0.1 ml lyso-Gb$_1$ working solution 1 (20,000 nM) with 9.9 ml Optima LC/MS-grade methanol.
Keep frozen at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

Methanol:Dimethyl sulfoxide (DMSO) 95:5 v/v solution

1. In a 20-ml clear glass vial with PTFE black phenolic cap (Duran Wheaton Kimble, cat. no W224589), add 19 ml Optima LC/MS grade methanol (Fisher Scientific, cat. no. A456-4).
2. Add 1 ml dimethyl sulfoxide (DMSO; >99%; Sigma Aldrich, cat. no. M81802).

Keep at 4°C for up to 1 week.

NH$_4$OH 2% v/v in methanol

1. In a 250-ml clear glass bottle, add 56 ml Optima LC/MS grade methanol.
2. Add 4 ml ACS-grade ammonium hydroxide (NH$_4$OH; 28%-30%; Fisher Scientific, cat. no. AC423300250).

Prepare fresh on the day of extraction.

SPC stock solution (0.2 mM)

1. In a 20-ml clear vial with PTFE black phenolic cap, weigh 0.929 mg sphingosylphosphorylcholine (SPC; Matreya, cat. no. 1318) standard and dissolve in 10 ml of 90:10 MeOH:DMSO.
2. Sonicate for 30 s and vortex for 10 s and verify that dissolution is complete.

Keep frozen at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

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SPC working solution 1 (20,000 nM)

1. In a 20-ml clear vial with PTFE black phenolic cap, dilute 1 ml SPC stock solution (0.2 mM) with 9 ml of 90:10 MeOH:DMSO.
2. Vortex (10 s) and sonicate (30 s) the SPC stock solution before use.

Keep frozen at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

SPC working solution 2 (200 nM)

1. In a 20-ml clear vial with PTFE Black Phenolic Cap, dilute 0.1 ml SPC working solution 1 (20,000 nM) in 9.9 ml of 90:10 MeOH:DMSO.
2. It is extremely important to vortex (10 s) and sonicate (30 s) the SPC working solution before use.

Keep frozen at −20°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

SPC-(D_9) internal standard stock solution (528 µM)

1. Dissolve 1 mg deuterated sphingosylphosphorylcholine (SPC-(D_9); Avanti Polar Lipids Alabaster, cat. no. 860685) in its original container with small amounts of Optima LC/MS-grade methanol and transfer to a 4-ml amber glass vial with PTFE black phenolic cap.
2. Add Optima LC/MS methanol to 4000 µl.
3. Vortex for 10 s and sonicate for 30 s.
4. Verify that dissolution is complete.

Keep frozen at −80°C until use. Stable while frozen. Once thawed, use immediately and do not refreeze.

Strong Wash–ACN 100%

In a 1000-ml clear glass bottle, add 1000 ml OmniSolv LC-MS-grade acetonitrile (ACN; EMD Chemicals, cat. no. AX0156-1).
Store at room temperature in a well-ventilated place for up to 2 months.

Surrogate matrix

Dissolve 75 mg fatty-acid-free bovine serum albumin (BSA; ≥98%; Sigma Aldrich, cat. no. A7030) in 10 ml PBS (1×, pH 7.4) (Wisent Bioproduct, cat. no. 311-010-CL).
Keep at 4°C for up to 2 weeks.

Weak Needle Wash–ACN 100%

In a 1000-ml clear glass bottle, add 1000 ml OmniSolv LC/MS-grade acetonitrile (ACN; EMD Chemicals, cat. no. AX0156-1).
Store at room temperature in a well-ventilated place for up to 2 months.

Background Information

Most biomarkers measured in GD are non-specific to the disease, thus limiting their utility (Nagral, 2014). Given the large clinical spectrum and important phenotypic variability associated with GD, other plasma biomarkers should be identified. Lyso-Gb$_1$ has recently emerged as a biomarker of choice for GD monitoring and follow-up (Cozma et al., 2020; Dinur et al., 2021). Some experts argue that this biomarker could be used for diagnosis (Dinur et al., 2022). This is a major departure from the previous dogma, which held that GD biomarkers are only useful for patient monitoring and follow-up. We performed a semi-targeted metabolomic study of plasma specimens obtained from patients with untreated GD and healthy controls (Menkovic et al., 2022a) based on our previous experience with Fabry disease (Auray-Blais et al., 2012; Dupont, Gagnon, Boutin, and Auray-Blais, 2013). Based on the results of this study

and the research done by Mirzaian and colleagues (Mirzaian et al., 2015), we developed and validated a UPLC-MS/MS method to quantify lyso-Gb$_1$ and lyso-Gb$_1$ analogs at –28 Da, –12 Da, –2 Da, +14 Da, +16 Da, and +18 Da in addition to *N*-palmitoyl-O-phosphocholineserine (NPOPCS) and sphingosylphosphorylcholine (SPC) to study the distribution of these markers in treated and untreated patients (Menkovic et al., 2022b). This paper is a detailed protocol for the quantification method.

Critical Parameters

One of the main challenges is the incorporation of SPC due to its limited solubility in the solvents compatible with this method. To address this issue, we added several vortexing (10 s) and sonication (30 s) cycles prior to any step that requires using an SPC solution.

Also, a relative quantification approach was used because standards and internal standards are unavailable for most of the compounds. Analytes for which standards and internal standards are not available are quantified based on the response factor of either lyso-Gb$_1$ (for lyso-Gb$_1$ analogs) or SPC (for NPOPCS). It is important to monitor and document any important changes in signals or retention times because such changes could suggest that the chromatography is affected by interferents and may lead to erroneous results due to matrix effects. Nevertheless, the method described herein has been validated to provide high precision, accuracy, and stability for lyso-Gb$_1$ and SPC.

Understanding the Results

A proxy matrix was used to simulate plasma for the calibration curve point samples because lyso-Gb$_1$ and SPC are found in healthy plasma. It is important to verify whether the chromatography efficiently separates lyso-Gb$_1$ from its structural isomer galactosylsphingosine (psychosine), a biomarker for Krabbe disease. The resolution of these two compounds is important and should be optimized, given that the chromatography might vary due to inter-laboratory differences. In fact, psychosine was found in the surrogate matrix used to prepare the calibration curve points and in healthy reference controls. Split peaks are visible in the chromatogram for the lyso-Gb$_1$ analog at +16 Da. Both peaks are isomers of the lyso-Gb$_1$ analog +16 Da.

Lyso-Gb$_1$ and its analogs are expected to be elevated in patients with Gaucher disease type 1. Lyso-Gb$_1$ is barely detectable (1.22 to 1.44 nM) in healthy controls, and lyso-Gb$_1$ analogs are not detectable. Nevertheless, this method should be used for research purposes, and positive results should be confirmed by established diagnostic assays such as a marked β-glucosidase deficiency in peripheral blood leukocytes and *GBA* gene sequencing. The levels of lyso-Gb$_1$ and its analogs were higher in patients with severe Gaucher disease type 1 versus those with the attenuated form. Moreover, patients undergoing treatment had a lower concentration of these molecules than naive patients. SPC and NPOPCS were observed in healthy controls and patients with GD type 1, and levels did not correlate with disease severity or treatment status. SPC is also increased in Niemann-Pick types A and B, while NPOPCS is increased in Niemann-Pick type C.

Time Considerations

A technician will need approximately 5 hr to prepare 48 samples, including calibrators and QCs. The UPLC-MS/MS analysis requires 12 min per sample. Data processing generally requires 60 to 90 min for a batch of 48 samples.

Ethics Statement

Patient samples were obtained with signed informed consent under protocols approved by the Research Ethics Board (REB) of the Faculty of Medicine and Health Sciences of the Centre Hospitalier Universitaire de Sherbrooke (CHUS) under project ID MP-31-2017-1414 in accordance with the ethical standards laid down in the World Medical Association Declaration of Helsinki.

Author Contributions

Iskren Menkovic: Formal analysis, methodology, validation, visualization, writing original draft. **Michel Boutin:** Supervision, writing review and editing. **Pamela Lavoie:** Visualization, writing review and editing. **Christiane Auray-Blais:** Conceptualization, data curation, funding acquisition,

project administration, resources, supervision, visualization, writing review and editing.

Conflict of Interest

I.M. received a student grant from Shire/Takeda Pharmaceutical. M.B. received a salary from Shire/Takeda Pharmaceutical. C.A.-B. received research grants and honoraria from Shire/Takeda Pharmaceutical.

Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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