

Evaluation of a Liquid Chromatography–Tandem Mass Spectrometry Method for the Analysis of Glucosylceramide and Galactosylceramide Isoforms in Cerebrospinal Fluid of Parkinson's Disease Patients

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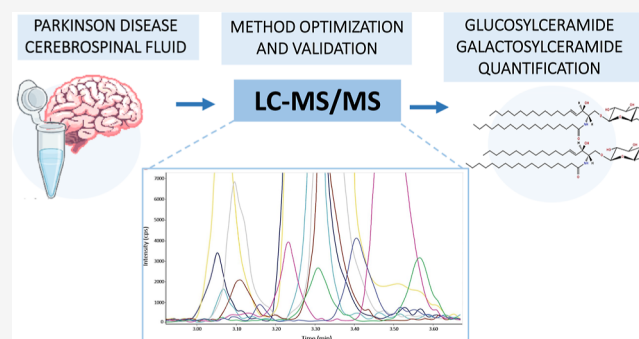


Article Recommendations



Supporting Information

ABSTRACT: Mutations in *GBA1*, encoding glucocerebrosidase beta 1 (GCase), are the most common genetic risk factor for Parkinson's disease (PD). GCase dysfunction leads to an accumulation of glucosylceramide (GluCer) substrates in different organs and fluids. Despite the challenges in quantifying GluCer isoforms in biological samples, their potential clinical interest as PD biomarkers justifies the development of robust assays. An extensively evaluated high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for quantifying 14 GluCer and galactosylceramide (GalCer) isoforms in human cerebrospinal fluid (CSF) samples is presented. Sample pretreatment, HPLC, and MS/MS parameters were optimized. Evaluation was performed according to the recommendations of the Clinical and Laboratory Standards Institute and European Medicines Agency guidelines. Four 7-point calibration curves were generated, with a linearity interval from 2.5 to 200 nM ($R^2 \geq 0.995$). The limit of quantification was set at 5 nM. Between-run precision and accuracy were up to 12.5 and 9%, respectively. After method validation, we measured the levels of GluCer and GalCer isoforms in CSF human samples, including 6 healthy controls (HC), 22 idiopathic *GBA1* wild-type PD (iPD) patients, and 5 *GBA1*-associated PD (PD-GBA) patients. GluCer/GalCer median ratios were found to be higher in the CSF of PD-GBA patients, particularly in severe *GBA1* mutations, than those in iPD and HC. The observed trends in GluCer/GalCer ratios among groups provide novel information for the comprehensive analysis of sphingolipids as potential biomarkers of PD.



Parkinson's disease (PD) is a prevalent neurodegenerative disorder characterized by both motor symptoms, such as tremor, rigidity, and bradykinesia, and nonmotor symptoms, including affective, cognitive, sleep, and autonomic features. The neuropathologic hallmark of PD is the loss of dopaminergic neurons in the brain's substantia nigra and the formation of insoluble deposits of alpha-synuclein (α -syn).¹

The etiology of the majority of cases (85%) is idiopathic, with age being the main risk factor.² Genetics also contribute to susceptibility, particularly genes involved in neuronal homeostasis, such as those of the lysosomal–endosomal pathways.^{3,4} In particular, mutations in the *GBA1* gene are the most common genetic risk factor for PD,⁵ with 2–30% of PD patients carrying *GBA1* mutations.⁶ *GBA1* encodes for the enzyme glucosylceramidase beta 1 (GCase, EC:3.2.1.45), which hydrolyzes the sphingolipid glucosylceramide (GluCer) into glucose and ceramide in the lysosome. Homozygous and compound heterozygous mutations in the *GBA1* gene cause

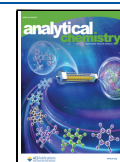
Gaucher disease (GD), which is characterized by reduced GCase activity and subsequent accumulation of GluCer and glucosylsphingosine (GluSph) in various organs.⁷ Individuals carrying pathogenic variants in *GBA1* have an increased risk of developing PD compared to the normal population.^{8–10} PD-GBA patients typically experience earlier onset, a more rapid progression, more severe cognitive impairment, and an increased risk of death compared to those lacking such mutations.^{10–12} Sphingolipid accumulation caused by *GBA1* variants has been linked to dysfunction of the autophagy–

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lysosomal pathway (ALP), which is directly involved in maintaining adequate levels of α -syn.^{13–15}

Measuring GluCer isoforms and derivatives in biological samples is of particular interest because of their potential use as biomarkers in PD to detect, predict, or evaluate disease-modifying treatments.^{15–19} However, it can be challenging due to their structural complexity. Specific and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods are required to measure the diversity of species with different functional groups, chain length, number of double bonds, or carbohydrate structure.²⁰ GluCer is composed of different isoforms that vary depending on the structure of the fatty acid chain. In addition, it is necessary to distinguish between GluCer and its isomer galactosylceramide (GalCer), which can be differentiated by the position of the hydroxyl group on carbon 4 of the pyranose ring²¹ (Figure S1). Due to the inability of triple quadrupole mass analyzers in MRM acquisition mode to discriminate between GluCer and GalCer, chromatographic preseparation of both isomers is required for the accurate quantification of GluCer.²² This is particularly important in the brain and the cerebrospinal fluid (CSF), where the concentration of GalCer is much higher than that of GluCer, as GalCer is a major component of the myelin sheath in the central and peripheral nervous systems.²³

GalCer and GluCer, as well as their derivatives, can be separated using normal phase chromatographic methods or some of their variants, such as hydrophilic interaction liquid chromatography (HILIC). Several LC–MS/MS methods have been developed to quantify these substrates in samples from cellular and animal models for fundamental and translational studies.^{24–26} In recent years, a few LC–MS/MS methods have been developed to quantify GluCer and its derivatives in human brain tissue,^{27,28} blood,²⁹ and CSF^{15,17,18,30} samples. The considerable variability of data from such studies poses a challenge for group comparisons, influenced not only by differences in study design (e.g., sample size, cohort composition, inclusion/exclusion criteria, or origin of the sample type) but also by the lack of methodological harmonization (preanalytical conditions, calibration materials, pretreatment procedures, method conditions, and instrument components). Adherence to international guidelines for the development and verification of bioanalytical methods is essential to obtain more robust and reliable data and reduce the interlaboratory variability.

Therefore, the aim of this study is to evaluate a novel HILIC–ESI–MS/MS method developed for the quantification of GluCer and GalCer isoforms in human CSF, focusing on GluCer (d18:1/C16:0, C18:0, C24:1). Evaluation was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) document C62-A³¹ and the European Medicines Agency (EMA)³² guidelines on bioanalytical method validation.

■ EXPERIMENTAL SECTION

Human Samples and Ethics. CSF samples from the VHIP Collection (Vall d'Hebron Initiative for Parkinson) were collected at the Vall d'Hebron Research Institute including the following: 6 healthy controls (HC), 22 idiopathic *GBA1* wild-type PD patients (iPD), and 5 PD patients with *GBA1* variants (PD-GBA). The classification of variant severity was based on established genotype risks reported previously.³³ Demographic and clinical details are described in Table S1. The study was approved by the Ethics Committee PR(AG)170–2015 and

PR(AG)434–2019, and all participants gave written informed consent.

Chemicals and Reagents. *N*-Hexadecanoyl-glucosylceramide (GluCer C16:0), *N*-octadecanoyl-glucosylceramide (GluCer C18:0), *N*-15Z-tetracosenoyl-glucosylceramide (GluCer C24:1), and *N*-15Z-tetracosenoyl-galactosylceramide (GalCer C24:1) standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Internal standards (IS) *N*-docosanoyl-D4-glucosylsphingosine (GluCer C22:0-d4) and *N*-docosanoyl-D4-galactosylceramide (GalCer C22:0-d4) were obtained from Cayman Chemical (Michigan, USA). LC–MS-grade methanol (MeOH) and chloroform (CHCl₃) were from VWR Chemicals (Radnor, PA, USA). Formic acid (FA, purity: >99%) was purchased from ITW Reagents (Barcelona, ES). Ammonium formate (Amm. Form., purity: >99%) and LC–MS-grade isopropanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). LC–MS-grade acetonitrile (MeCN) was supplied by ThermoFisher Scientific (Waltham, MA, USA). Ultrapure water was generated using a Mili-Q system (Millipore). The artificial CSF (aCSF) matrix was purchased from Tocris Bioscience (Bristol, UK).

Calibration Standards and Quality Control Sample Preparation. The preparation of primary stock, intermediate, and working solutions (WS) is described in Supporting Information. For lipid quantification, a seven-point calibration curve was prepared by serial dilution from WS in aCSF as a surrogate matrix, ranging from 2.5 to 200 nM (2.5, 5, 10, 25, 50, 100, and 200 nM). A blank sample (aCSF matrix sample without analyte and without IS) and a zero sample (a blank sample spiked with IS) were also prepared. Quality control samples (QCs) were prepared in aCSF from independent WS at four different levels to cover the measurement interval as lower limit QC (LLQC), low QC (LQC), medium QC (MQC), and high QC (HQC) to final concentrations of 5, 20, 50, and 100 nM, respectively. When opting to use a surrogate matrix, it is important to test its suitability during method development and evaluation.

Sample Extraction. The liquid–liquid extraction (LLE) procedure was performed based on the methodology previously described.³⁴ In brief, 80 μ L of calibrators, controls, or CSF samples was added to 1.5 mL plastic tubes. Fifteen μ L of working internal solution (WIS) and 120 μ L of deionized water were added and mixed after each addition. Then, 600 μ L of MeOH/CHCl₃ (1/2, v/v) was added and vortexed vigorously for 2 min. The samples were centrifuged at 13 000 rpm for 15 min at 4 °C, and the resulting organic phase (lower) was transferred into glass tubes. In a second step, 600 μ L of MeOH/CHCl₃ (1:2, v/v) was added to the remaining aqueous phase and again vortexed and centrifuged under the same conditions as previously. The two resulting organic phases were pooled, dried under N₂ gas, reconstituted in 250 μ L of organic mobile phase, and transferred to chromatographic vials.

LC–MS/MS Conditions. GluCer and GalCer isoforms C16:0, C18:0, C20:0, C22:0, C23:0, C24:1, and C24:0 were analyzed by LC–MS/MS using a Nexera X2 HPLC system coupled to an LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). Details of the chromatographic parameters and MS conditions are described in Tables S2 and S3. Table S4 summarizes the quantification of the GluCer and GalCer species. All data were analyzed using LabSolutions CS software v5.9 (Shimadzu Corporation, Japan).

Linearity and Calibration Curve. Calibration curves were generated by plotting the peak area ratio of the seven calibration standards to IS against nominal concentrations using weighted ($1/c$) linear regression.³¹ The accuracy of the back-calculated concentrations was assessed by their percentage deviation from nominal concentrations, with an acceptable deviation set at $\pm 15\%$ of the predicted values in at least 75% of the points³² (Table S5). The equivalence of the aCSF and native CSF (nCSF) matrixes was also verified (Table S6).

Limit of Blank, Limit of Detection, and Limit of Quantification. The limit of blank (LoB) for each isoform was estimated from the 95th percentile of zero sample measurements ($n = 8$) on 3 independent days. From this value, the limit of detection (LoD) was calculated as $\text{LoB} + 3$ times the standard deviation of the zero sample results. The limit of quantification (LoQ) was determined based on the precision and accuracy criteria ($<20\%$)³² by processing 5 replicates of the LLQC in two independent runs and ensuring a signal-to-noise ratio (S/N) $> 10:1$.

Precision and Accuracy of the Method. To evaluate precision and accuracy, five replicates of LQC, MQC, and HQC were freshly prepared and measured on three different days. Precision was expressed as the coefficient of variation (CV, %) of replicates, and accuracy was calculated as the percentage deviation from the nominal concentration within each run and between the three runs. Acceptance criteria were set at $\leq 15\%$.

Selectivity Evaluation. Different strategies were employed to assess the selectivity. For analyte identification, retention times (RTs) were compared between the standard calibrators and CSF samples within the same run. To verify the absence of interference of the matrix from the signal of any recorded transitions, a blank sample was injected into each run. Additionally, since all compounds shared the same mass product ion and peaks overlapped in some cases, a “dummy” transition sharing the same product ion (m/z 600 $>$ 264.3) was included and monitored for cross-talk MS analysis at the end of the scan cycle panel.³⁵ An acceptable response was defined as either absent or $<20\%$ of the analyte area and $<5\%$ of the IS area in the LLQC sample. Finally, independent solutions of each standard and IS were injected, and the contribution of each standard to the response of the remaining transitions was evaluated.³⁶

Sample Stability Analysis. To evaluate stability, duplicate aliquots of HQC stored under different conditions were analyzed: after 24 h at 25 °C (S1), 48 h at 4 °C (S2), 14 days at -20 °C (S3), 2 months at -80 °C (S4), and one freeze–thaw cycle (S5). The values were adjusted to the results of freshly prepared HQC in the same run to avoid potential day-to-day variations, and the deviation from the nominal concentration was then estimated. Stability of the samples in the autosampler was also verified by reanalyzing the QC samples at the end of each run.

Carryover Assessment. Carryover study was conducted by alternating injections of high-concentration standard (200 nM) and blank samples three successive times. A response of $<20\%$ of the LoQ in the blank samples was considered acceptable.

Matrix Effect, Recovery, and Overall Process Efficiency. The assessment of matrix effect (ME), recovery (RE), and process efficiency (PE) was based on pre- and postextraction spiking methods, according to previous recommendations (30). The relative ME (CV %) was assessed

by comparing the ME obtained between the different lots of CSF matrices for each standard and IS. When calculated by the area ratio, the relative ME factor (MEF) (CV %) showed the contribution of the IS in compensating for this variability. Preparation and assessment details are described in Supporting Information.

Analysis of GluCer and GalCer Isoforms in Human CSF Samples. Analysis of the CSF samples from the study cohort was performed in a single run. Three QC levels, stored under the same conditions, were quantified. The same pretreatment and LC–MS/MS analysis were applied to all samples, curves, and QCs, as previously described.

Statistical Analysis. Mann–Whitney and Kruskal–Wallis tests were employed to compare medians for stability and patient sample analyses. Outliers were identified using the Grubbs test and only removed if a systematic bias was detected for all standards, allowing a maximum of one replicate per run. Statistical analyses were performed using GraphPad Prism (v8) and Microsoft Excel (v16). $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Chromatographic Separation. A total of 14 analytes in CSF, comprising the 7 GluCer isoforms and their 7 GalCer structural isomers, were detected and quantified within the run time. Representative ion chromatograms of the eluted peak areas of GluCer and GalCer from a 200 nM standard (Figure 1) and a patient CSF sample (Figure S2) are shown,

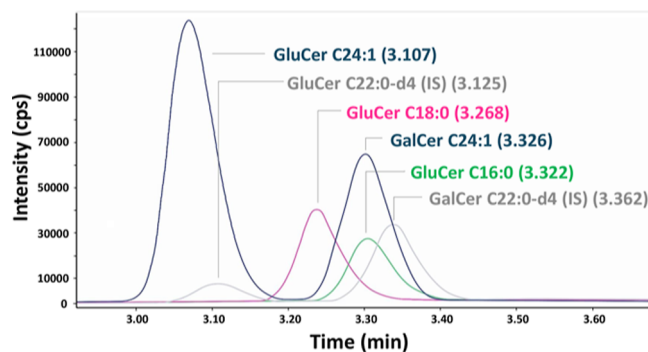


Figure 1. Chromatogram of the 200 nM standard, showing the RTs of GluCer and GalCer isoforms.

demonstrating their effective separation. HILIC methods allow isomer separation but typically require longer chromatographic run times due to extended column equilibration.²⁵ Our method achieves isoform elution in 4 min, but notably, re-equilibration for up to 8 min is essential for consistent results. It should be noted that other methods based on HILIC with zwitterionic stationary phase chromatography (ZIC-HILIC) may offer an alternative resolving power for the identification of minor lipid species.²⁴ The coupling of two or more chromatographic columns³⁷ or the addition of ion mobility spectrometry techniques³⁸ could also improve the identification of lipid profiles.

MS Optimization. The selected product ion represented the major fragment ion, generated through the neutral loss of the acyl chain, glucose (or galactose), and one H_2O molecule, and corresponded to the fragment of the sphingosine moiety (m/z 264), as described in lipid-targeted MS databases³⁹ and previously.^{26,27}

Linearity and Calibration Curve. The 7-point calibration curves exhibited a linear $1/x$ -weighted response with determination coefficients (R^2) ≥ 0.995 (Figure S3). The calculated concentration from the nominal value was within 15%, except for the lower limit of the linearity interval (Table S5).

The calibration curve and QCs were prepared using a surrogate aCSF matrix, which was necessary due to challenges in obtaining a sufficient volume of CSF because of the required depletion strategies. In the matrix equivalence study, the variability ($<15\%$) in the area ratio of the 100 nM standard between both aCSF and nCSF suggested that aCSF was suitable for quantifying CSF patient samples (Table S6). This variability should be considered an approximation, and future research should explore adding more standard concentrations and investigating new nCSF matrices since the presence of endogenous compounds makes it difficult to generate accurate curves. It is recommended that nCSF matrices with minimal endogenous concentrations of the studied compounds be selected for the equivalence study. This will minimize potential biases caused by the endogenous compounds influencing the responses of those of the added compounds. We also prepared calibration curves by serial dilution to reduce the CSF volume required, ensuring prior equivalence with the preparation by using independent standards. Since the concentrations measured in our study cohort samples fell within the calibration curve's measuring interval, we did not consider performing an assessment of dilution integrity.

Limit of Blank, Limit of Detection, and Limit of Quantification. LoB ranged from 0.29 to 1.05 nM, and the estimated LoD ranged from 0.53 to 1.93 nM (Table S7). The LoQ was set at 5 nM, with an accuracy and precision between-run of $\leq 16\%$ for all isoforms (Table S8) and a $S/N > 10:1$. The LoB and LoD were estimated on different runs to account for day-to-day variability, increasing the reliability of the sensitivity evaluation. In addition, the LoQ was also verified for imprecision and accuracy between-runs. Although a lower LoQ (ranging from 1.0 to 3.9 nM) could have been defined using an equation derived from the LoB, this approach may have been less methodologically robust. Even minor differences in precision or accuracy can significantly impact the results, especially for the quantification of GluCer in CSF, whose concentrations were close to the lower limit of the measurement interval for some isoforms and samples.

Precision and Accuracy of the Method. Within-run precisions and accuracies were found to be acceptable for all analytes. The precision and accuracy of the between-runs ranged between 10.9 to 12.5% and 0.6 to 9.0% at 20 nM, 9.2 to 11.3% and 0.6 to 6.4% at 50 nM, and 8.5 to 11.6% and 3 to 8.5% at 100 nM, respectively. The obtained data are summarized in Table S8. Comparing the characteristics of our method with those of others has been challenging as most studies do not provide detailed methodological and validation data. The work of Boutin et al.²⁷ is one of the most informative in this regard. In their study, five GluCer isoforms were analyzed by relative abundance using the commercial standard GalCer(C15:0) as the calibration curve, and the accuracy was validated on the GalCer(C15:0) compound. In our study, we constructed calibration curves with commercial standards of GluCer C16:0, GluCer C18:0, GluCer C24:1, and GalCer C24:1, and this approach allowed us to assess both accuracy and precision.

Selectivity Evaluation. During validation, it is important to consider different approaches for assessing matrix and standard contributions to the remaining monitored transitions. This may include identifying compounds by the standard's RT, demonstrating the absence of significant responses from interfering components at each RT, and implementing strategies to detect potential interferences in the MS analyzer. The responses of the analyte transitions in blank matrix samples were indistinguishable from the signal noise. For identity confirmation, the RT of each pure compound matched that of each compound in the patient CSF sample within $\pm 2.5\%$ (Figure S2). It would be of interest to confirm the identity of the other isoforms that lack a commercial standard. C23:0 was included in the profile based on previous studies,^{15,18,26,30} but it was not included in others.^{16,27} It is essential to rule out the possibility that it represents another structure, such as methylated C22:0 (C22:0)Me, which has previously been structurally elucidated by MS/MS for globotriaosylceramide and galabiosylceramide.^{40–42}

Product ion cross-talk, resulting from MRM overlap due to common product ion fragments and similar chromatographic elution, can be a challenging analytical issue. The addition of a “dummy” transition with the same product ion but a different precursor not present in the solution can be useful in pointing out this type of interference.³⁵ Our results showed no product ion cross-talk (Figure S4), as the “dummy” transition confirmed the absence of interfering peaks at any RT for the analytes.

When monitoring the contribution of each individual standard and IS to the response of the remaining transitions, all monitored signals were considered negligible ($\leq 1\%$), except for the C24:0 transition signal, which was observed to be $<8\%$ when injecting the C24:1 standard at the highest concentration (Table S9). We considered that this interference may be attributed to isobaric overlap resulting from the abundance of natural isotopes, possibly due to C24:1 molecules containing two ^{13}C atoms.³⁸

Sample Stability Analysis. QCs were stable for at least 4 weeks at -20°C , 2 months at -80°C , one freeze–thaw cycle, and when reinjection of reconstituted samples was left overnight in the autosampler. Statistically significant losses of $\sim 68\%$ were observed for all analytes when samples were left at $4\text{--}6^\circ\text{C}$ for 48 h. For GalCer C24:1, a slight but significant increase was found after 24 h at room temperature (Figure S5 and Table S10). The proposed storage conditions were deemed adequate for ensuring sample stability in the current study. However, an extended stability study could be necessary for future investigations.

Carryover Assessment. The peak areas for each isoform at each respective RT in the blank carryover samples injected after 200 nM standards showed no deviation from signal noise (Figure S6).

Matrix Effect, Recovery, and Overall Process Efficiency. The ME study was conducted to ensure method robustness since ME may have an impact on the linearity, precision, and accuracy of the analytical method (Table 1). The ME for standards at both concentrations ranged from 77.2 to 99.0%. The C18:0 isoform had the highest ion suppression (77.2 and 83.2% for 50 and 100 nM, respectively). The ME for IS was 61.5 and 57.9% for 50 and 100 nM, respectively.

The absolute ME was found acceptable for C16:0 and C24:1 isoforms (ranging from 88.2 to 99.0%) and was slightly lower for C18:0 (77.2% at 50 nM and 83.2% at 100 nM). However,

Table 1. ME, RE, PE, MEF in Three Lots of CSF

Conc		GluCer C16:0	GluCer C18:0	GluCer C24:1	GluCer C22:0-d4
50 nM	ME (%)	88.2	77.2	97.5	61.6
	RE (%)	32.5	28.8	35.6	16.9
	PE (%)	25.2	21.3	29.4	10.4
	relative ME (CV, %)	14.7	12.7	12.2	7.43
	relative MEF (CV, %)	12.6	8.4	7.5	n/a
100 nM	ME (%)	93.1	83.2	99.0	57.9
	RE (%)	31.6	27.1	30.7	16.9
	PE (%)	27.3	22.0	27.1	9.79
	relative ME (CV, %)	18.2	18.0	16.1	13.2
	relative MEF (CV, %)	8.6	8.2	8.1	n/a

the ME was more pronounced for the IS (61.6% at 50 nM and 57.9% at 100 nM), indicating increased ion suppression compared to the standards. This resulted in an overall signal enhancement effect of 20–40% when normalized to the IS (MEF values > 1). The higher ion suppression observed in the IS may be due to coelution of target compounds with those of the sample matrix or to the coelution of the different target compounds with each other as some of the chromatographic peak areas of the studied compounds overlap.⁴³ Despite this effect, it was assumed that ME would not impact the LoQ because there would be no over- or underestimation of analytes since the IS was equally added to both samples and calibrators and, in our case, standards did not present as much ion suppression. However, it may affect robustness at low concentrations if the analytical system is not in optimal conditions to maximize the analyte responses. To avoid this potential problem, chromatographic modifications could improve the peak separation. While this may reduce coelution, it is also important to consider that compound ionization efficiency is influenced by solvent composition. In gradient chromatography, the elution of a molecule in a mobile phase with a composition different from that of another molecule used for the calibration curve could potentially introduce a bias in quantification.

Selecting an appropriate IS method posed another challenge. International guidelines recommend the use of stable isotope-labeled IS, with options such as ¹³C, ¹⁵N, or ¹⁷O being preferred.^{31,32,44} Due to the limited commercial availability of isotopically labeled sphingolipids, deuterated isotopes were chosen as the IS. Other commercially available IS were also tested during method development, such as GluCer C18:0-d5 and GluCer C17:0, but there was no improvement in reproducibility.

The relative ME between the three different lots of nCSF had a mean CV of 15.4% (ranging from 12.2 to 18.3%) for standards, indicating that there is variation in the ionization efficiency between different lots. However, when normalized to IS, mean CV decreased significantly to 8.9% (ranging from 7.5 to 12.6) (Table 1), suggesting that the pattern of variation in the standards and IS are similar, and therefore IS compensates for this effect, improving the overall precision of the method.

Sample RE and PE of the standards varied from 27 to 36% and 21 to 30%, respectively. For IS, RE was 16.9% (at both concentration levels), and PE was 10.4% (50 nM) and 9.8% (100 nM). Therefore, RE and, subsequently, PE values for IS were significantly reduced compared to those of the standards. The low RE results may be due to losses during the extraction procedure, which in turn contributed to a decrease in PE. Lipid extraction is a critical step in CSF analysis due to its low lipid content.⁴⁵ The CHCl₃/MeOH mixture is widely considered to be a robust and efficient lipid extraction method,⁴⁶ but other methods have employed solid-phase extraction cartridges.^{17,26,27,29} Although a reduction in sensitivity was described when using LLE due to significant ion suppression effects, the ionization efficiency was not evaluated.²⁷ Our extraction recovery rates were comparable to those reported in this study.

During the batch injection for the ME study, the absolute peak areas for all compounds gradually decreased (mean of 15 to 30%, depending on the analyte) due to instrument or interface instability. This decrease resulted in lower ME, RE, and PE values, as previously reported.³⁶

GluCer and GalCer Isoform Levels and Ratios in Human CSF Samples. The concentrations of GluCer and GalCer isoforms in CSF samples from different groups are shown in Table S11. As expected, GalCer was found to be

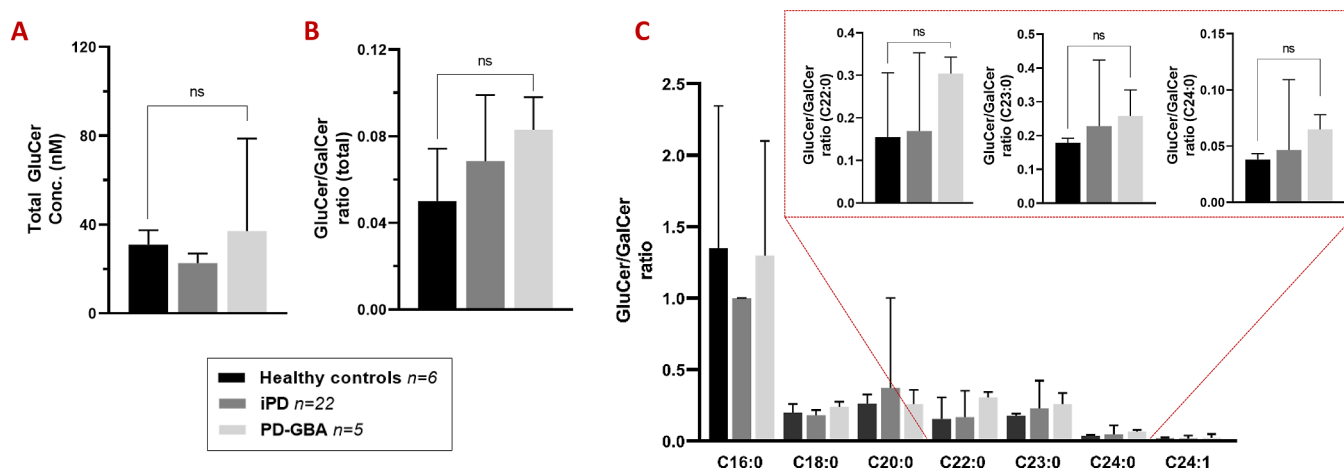


Figure 2. Boxplots (median and interquartile range) for GluCer concentrations and GluCer/GalCer ratios. (A) Sum of the total GluCer isoform concentration and (B) normalization by the total GalCer ratio among the groups of patients. (C) GluCer normalized to the GalCer ratio for each isoform, with a focus on C22:0, C23:0, and C24:0.

more abundant than GluCer, regardless of the patient type. No statistically significant differences were observed between groups when the levels of all isoforms were summed (total GluCer) (Figure 2a). Notably, an upward trend was observed among the HC, iPD, and PD-GBA patient groups when total GluCer concentrations were normalized to their GalCer counterparts (median GluCer/GalCer ratios of 0.050 ± 0.019 , 0.069 ± 0.051 , and 0.083 ± 0.031 , respectively) (Figure 2b and Table S12). Since it is not known whether each isoform contributes differently to the physiopathology of PD, it is deemed important to quantify them separately, in case any of them may provide additional information. In this regard, specific individual isoform ratios, such as C22:0, C23:0, or C24:0, were also found to be of particular interest as they tended to exhibit higher median ratios in PD-GBA compared to those in HC (Figure 2c) and in patients with severe *GBA1* mutations compared to those with mild and risk variants (Table S13).

These trends between patient groups and *GBA1*-type variants indicate the importance of considering the quantification of GalCer isomers and the relative ratio calculation. Interestingly, this increasing trend of GluCer/GalCer ratios with PD has been previously reported in brain tissue.²⁷ As both studies were also performed with a small sample size, further evaluation with a larger cohort would be required to statistically confirm the slight differences observed between the groups. In addition, the CSF GluCer/SM ratio was found to be a potential prognostic marker in patients with idiopathic and PD-GBA.¹⁷

It has been observed that sphingolipid accumulation varies depending on the severity of the *GBA1* mutation.^{15,47,48} Studies in PD human samples have shown variable results regarding sphingolipid levels in brain tissue^{19,27,28} and CSF.^{15–18,30} However, those that studied larger cohorts and stratified individuals by mutation type found higher concentrations in the PD-GBA group and an increasing trend with mutation severity.^{15,17} A reduction in GalCer, and potentially an upregulated ratio, was also found in patients with idiopathic normal pressure hydrocephalus, a parkinsonian disorder.³⁰ These studies suggest a link among lipid metabolism dysregulation, ALP dysfunction, and neurological damage, even in the absence of *GBA* variants, which is also consistent with our findings in the iPD group. Further validation studies are needed to better assess the clinical utility of each isoform and their ratios.

CONCLUSIONS

International guidelines on analytical method validation agree on many recommendations, but minor variations led us to adhere to the evaluation criteria that best suited our laboratory resources, such as limited availability of instruments, time constraints, sample handling, and difficulties in obtaining sufficient CSF volumes for the recommended verification procedures, which justified the adaptation of experimental protocols. Despite these challenges, we present the first extensively evaluated LC–MS/MS method for the quantitative analysis of GluCer and GalCer isoforms in human CSF.

Closely related structures posed an analytical challenge, but the abundance of galactose in CSF^{22,23} justifies its inclusion in the analysis. Although GalCer isoforms have not been as extensively evaluated as GluCer isoforms, they have been used for normalization purposes, and their inclusion allows for a

comprehensive assessment of lipid profiles as potential biomarkers in PD.²⁷

We believe that it is critical to consider the implementation of harmonized methods that could be applied to larger cohorts in order to detect robust changes that facilitate the interpretation of the results.^{15,17} A comprehensive method evaluation according to international bioanalytical guidelines is essential to achieving this aim. This could improve the assessment of lipid profiles and, together with other biochemical variables and genetic and clinical information, improve patient characterization and deepen our understanding of the pathophysiology of the disease.

In summary, a HILIC-ESI-MS/MS method has been developed and evaluated for the quantitative analysis of seven GluCer isoforms and their GalCer isomers in human CSF samples from control individuals and PD patients. The comprehensive optimization and evaluation of the method make it a valuable tool for understanding the lipid profiles associated with PD.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.4c02654>.

Preparation of stock, intermediate, and working solutions; preparation of the ME assay; graphical representations of GluCer and GalCer structures; representative isoform chromatograms from the highest concentration calibrator and a human CSF sample; chromatogram of the “dummy” transition; graphical representation of sample stability evaluation; chromatogram of the carryover assay; demographic and clinical data of patients; HPLC- and MS/MS-optimized parameters; isoforms quantification summary; results of method validation (linearity, precision, accuracy, surrogate matrix equivalence, sensitivity, cross-selectivity, and stability); levels of GluCer and GalCer in human CSF samples of patient groups; ratios of GluCer/GalCer in human CSF samples of patient groups; and ratios of GluCer/GalCer in CSF samples of the PD-GBA group subclassified according to *GBA1* mutation severity (PDF)

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REFERENCES

- (1) Bloem, B. R.; Okun, M. S.; Klein, C. *Lancet* **2021**, 397 (10291), 2284–2303.
- (2) Van Den Eeden, S. K.; Tanner, C. M.; Bernstein, A. L.; Fross, R. D.; Leimpeter, A.; Bloch, D. A.; Nelson, L. M. *Am. J. Epidemiol.* **2003**, 157 (11), 1015–1022.
- (3) Navarro-Romero, A.; Montpeyó, M.; Martínez-Vicente, M. *Cells* **2020**, 9 (11), 2399.
- (4) Klein, A. D.; Mazzulli, J. R. *Brain* **2018**, 141 (8), 2255–2262.
- (5) Sidransky, E.; Lopez, G. *Lancet Neurol.* **2012**, 11 (11), 986–998.
- (6) Riboldi, G. M.; Di Fonzo, A. B. *Cells* **2019**, 8 (4), 364.
- (7) Grabowski, G. A. *Lancet* **2008**, 372 (9645), 1263–1271.
- (8) Bultron, G.; Kacena, K.; Pearson, D.; Boxer, M.; Yang, R.; Sathe, S.; Pastores, G.; Mistry, P. K. *J. Inherited Metab. Dis.* **2010**, 33 (2), 167–173.
- (9) Gan-Or, Z.; Amshalom, I.; Kilarski, L. L.; Bar-Shira, A.; Gana-Weisz, M.; Mirelman, A.; Marder, K.; Bressman, S.; Giladi, N.; Orr-Urtreger, A. *Neurology* **2015**, 84 (9), 880–887.
- (10) Cilia, R.; Tunesi, S.; Marotta, G.; Cereda, E.; Siri, C.; Tesi, S.; Zecchinelli, A. L.; Canesi, M.; Mariani, C. B.; Meucci, N.; Sacilotto, G.; Zini, M.; Barichella, M.; Magnani, C.; Duga, S.; Asselta, R.; Soldà, G.; Seresini, A.; Seia, M.; Pezzoli, G.; Goldwurm, S. *Ann. Neurol.* **2016**, 80 (5), 662–673.
- (11) Alcalay, R. N.; Dinur, T.; Quinn, T.; Sakanaka, K.; Levy, O.; Waters, C.; Fahn, S.; Dorovski, T.; Chung, W. K.; Pauciulo, M.; Nichols, W.; Rana, H. Q.; Balwani, M.; Bier, L.; Elstein, D.; Zimran, A. *JAMA Neurol.* **2014**, 71 (6), 752–757.
- (12) Clark, L. N.; Ross, B. M.; Wang, Y.; Mejia-Santana, H.; Harris, J.; Louis, E. D.; Cote, L. J.; Andrews, H.; Fahn, S.; Waters, C.; Ford, B.; Frucht, S.; Ottman, R.; Marder, K. *Neurology* **2007**, 69 (12), 1270–1277.
- (13) Mazzulli, J. R.; Xu, Y. H.; Sun, Y.; Knight, A. L.; McLean, P. J.; Caldwell, G. A.; Sidransky, E.; Grabowski, G. A.; Krainc, D. *Cell* **2011**, 146 (1), 37–52.
- (14) Navarro-Romero, A.; Fernandez-Gonzalez, I.; Riera, J.; Montpeyó, M.; Albert-Bayo, M.; Lopez-Royo, T.; Castillo-Sanchez, P.; Carnicer-Caceres, C.; Arranz-Amo, J. A.; Castillo-Ribelles, L.; Pradas, E.; Casas, J.; Vila, M.; Martínez-Vicente, M. *npj Parkinson's Dis.* **2022**, 8 (1), 126.
- (15) Lerche, S.; Schulte, C.; Wurster, I.; Machetanz, G.; Roeben, B.; Zimmermann, M.; Deuschle, C.; Hauser, A. K.; Böhringer, J.

- Krägeloh-Mann, I.; Waniek, K.; Lachmann, I.; Petterson, X. M. T.; Chiang, R.; Park, H.; Wang, B.; Liepelt-Scarfone, I.; Maetzler, W.; Galasko, D.; Scherzer, C. R.; Gasser, T.; Mielke, M. M.; Hutten, S. J.; Mollenhauer, B.; Sardi, S. P.; Berg, D.; Brockmann, K. *Mov. Disord.* **2021**, *36* (5), 1216–1228.
- (16) den Heijer, J. M.; Cullen, V. C.; Pereira, D. R.; Yavuz, Y.; de Kam, M. L.; Grievink, H. W.; Moerland, M.; Leymarie, N.; Khatri, K.; Sollomoni, I.; Spitalny, L.; Dungeon, L.; Hilt, D. C.; Justman, C.; Lansbury, P.; Groeneveld, G. J. *Mov. Disord.* **2023**, *38* (5), 783–795.
- (17) Huh, Y. E.; Park, H.; Chiang, M. S. R.; Tuncali, I.; Liu, G.; Locascio, J. J.; Shirvan, J.; Hutten, S. J.; Rotunno, M. S.; Viel, C.; Shihabuddin, L. S.; Wang, B.; Sardi, S. P.; Scherzer, C. R. *npj Parkinson's Dis.* **2021**, *7* (1), 102.
- (18) Niimi, Y.; Mizutani, Y.; Akiyama, H.; Watanabe, H.; Shiroki, R.; Hirabayashi, Y.; Hoshinaga, K.; Mutoh, T. *J. Parkinson's Dis.* **2021**, *11* (1), 221–232.
- (19) Huebner, M.; Moloney, E. B.; Van Der Spoel, A. C.; Priestman, D. A.; Isacson, O.; Hallett, P. J.; Platt, F. M. *Mol. Neurodegener.* **2019**, *14* (1), 40.
- (20) Shaner, R. L.; Allegood, J. C.; Park, H.; Wang, E.; Kelly, S.; Haynes, C. A.; Sullards, M. C.; Merrill, A. H. *J. Lipid Res.* **2009**, *50* (8), 1692–1707.
- (21) Reza, S.; Ugorski, M.; Suchański, J. *Glycobiology* **2021**, *31* (11), 1416–1434.
- (22) Milenkovic, I.; Blumenreich, S.; Futerman, A. H. *Curr. Opin. Neurobiol.* **2022**, *72*, 148–154.
- (23) Merrill, A. H. *Chem. Rev.* **2011**, *111* (10), 6387–6422.
- (24) Sidhu, R.; Mikulka, C. R.; Fujiwara, H.; Sands, M. S.; Schaffer, J. E.; Ory, D. S.; Jiang, X. *Biomed. Chromatogr.* **2018**, *32* (7), No. e4235.
- (25) Nakajima, K.; Akiyama, H.; Tanaka, K.; Kohyama-Koganeya, A.; Greimel, P.; Hirabayashi, Y. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2016**, *1031*, 146–153.
- (26) Hamler, R.; Brignol, N.; Clark, S. W.; Morrison, S.; Dungan, L. B.; Chang, H. H.; Khanna, R.; Frascella, M.; Valenzano, K. J.; Benjamin, E. R.; Boyd, R. E. *Anal. Chem.* **2017**, *89* (16), 8288–8295.
- (27) Boutin, M.; Sun, Y.; Shacka, J. J.; Auray-Blais, C. *Anal. Chem.* **2016**, *88* (3), 1856–1863.
- (28) Gegg, M. E.; Sweet, L.; Wang, B. H.; Shihabuddin, L. S.; Sardi, S. P.; Schapira, A. H. V. *Mov. Disord.* **2015**, *30* (8), 1085–1089.
- (29) Zheng, K.; Ji, A.; Chung, L. L.; Culm-Merdek, K.; Liu, H.; Richards, S.; Sung, C. *Mol. Genet. Metab. Rep.* **2016**, *8*, 77–79.
- (30) Mutoh, T.; Niimi, Y.; Ito, S.; Akiyama, H.; Shiroki, R.; Hirabayashi, Y.; Hoshinaga, K. *Biochem. Biophys. Res. Commun.* **2023**, *639*, 84–90.
- (31) Clarke, W.; Molinaro, R. J.; Bachmann, L. M.; Botelho, J. C.; Cao, Z.; French, D.; Garg, S.; Gawoski, J. M.; Grant, R. P.; Hoofnagle, A. N.; Iyer, B.; Kulasingam, V.; Mason, D. S.; Rappold, B.; Tacker, D. H.; Truscott, S. M.; Yu, C.; Zhu, Y. *C62 ED2 Liquid Chromatography-Mass Spectrometry Methods*; CLSI, 2022.
- (32) EMA Guideline M10 on Bioanalytical Method Validation and Study Sample Analysis Step5; EMA, 2022.
- (33) Parlar, S. C.; Grenn, F. P.; Kim, J. J.; Baluwendrat, C.; Gan-Or, Z. *Mov. Disord.* **2023**, *38* (3), 489–495.
- (34) Basit, A.; Piomelli, D.; Armirotti, A. *Anal. Bioanal. Chem.* **2015**, *407* (17), 5189–5198.
- (35) Morin, L. P.; Mess, J. N.; Furtado, M.; Garofolo, F. *Bioanalysis* **2011**, *3* (3), 275–283.
- (36) Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M. *Anal. Chem.* **2003**, *75* (13), 3019–3030.
- (37) Berkecz, R.; Tömösi, F.; Körmöczy, T.; Szegedi, V.; Horváth, J.; Janáky, T. *J. Pharm. Biomed. Anal.* **2018**, *149*, 308–317.
- (38) Köfeler, H. C.; Ahrends, R.; Baker, E. S.; Ekroos, K.; Han, X.; Hoffmann, N.; Holčapek, M.; Wenk, M. R.; Liebisch, G. *J. Lipid Res.* **2021**, *62*, 100138.
- (39) Peng, B.; Kopczynski, D.; Pratt, B. S.; Ejsing, C. S.; Burla, B.; Hermansson, M.; Benke, P. I.; Tan, S. H.; Chan, M. Y.; Torta, F.; Schwudke, D.; Meckelmann, S. W.; Coman, C.; Schmitz, O. J.; MacLean, B.; Manke, M. C.; Borst, O.; Wenk, M. R.; Hoffmann, N.; Ahrends, R. *Nat. Commun.* **2020**, *11* (1), 2057.
- (40) Auray-Blais, C.; Boutin, M. *Curr. Med. Chem.* **2012**, *19* (19), 3241–3252.
- (41) Abaoui, M.; Boutin, M.; Lavoie, P.; Auray-Blais, C. *Clin. Chim. Acta* **2016**, *452*, 191–198.
- (42) Boutin, M.; Auray-Blais, C. *J. Am. Soc. Mass Spectrom.* **2015**, *26* (3), 499–510.
- (43) Liang, H. R.; Foltz, R. L.; Meng, M.; Bennett, P. *Rapid Commun. Mass Spectrom.* **2003**, *17* (24), 2815–2821.
- (44) FDA Bioanalytical Method Validation Guidance for Industry Biopharmaceutics; FDA, 2018.
- (45) Millán, L.; Fernández-Irigoyen, J.; Santamaría, E.; Mayo, R. *Methods Mol. Biol.* **2019**, *2044*, 353–361.
- (46) Aldana, J.; Romero-otero, A.; Cala, M. P. *Metabolites* **2020**, *10* (6), 231–232.
- (47) Sun, Y.; Zhang, W.; Xu, Y. H.; Quinn, B.; Dasgupta, N.; Liou, B.; Setchell, K. D. R.; Grabowski, G. A. *PLoS One* **2013**, *8* (3), No. e57560.
- (48) Huh, Y. E.; Chiang, M. S. R.; Locascio, J. J.; Liao, Z.; Liu, G.; Choudhury, K.; Kuras, Y. I.; Tuncali, I.; Videnovic, A.; Hunt, A. L.; Schwarzschild, M. A.; Hung, A. Y.; Herrington, T. M.; Hayes, M. T.; Hyman, B. T.; Wills, A. M.; Gomperts, S. N.; Growdon, J. H.; Sardi, S. P.; Scherzer, C. R. *Neurology* **2020**, *95* (6), E685–E696.