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Tandem Mass Spectrometry Multiplex Analysis of Glucosylceramide and Galactosylceramide Isoforms in Brain Tissues at Different Stages of Parkinson Disease

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Abstract

Previous studies demonstrated that Parkinson disease (PD) is associated with a decreased activity of the glucocerebrosidase (GCase) enzyme in brain tissues. The objective of this study was to determine if GCase deficiency is associated with the accumulation of its glucosylceramide (GluCer) substrate in PD brain tissues. A UPLC-MS/MS method was developed, optimized and validated for the multiplex analysis of GluCer isoforms (C18:0, C20:0, C22:0, C24:1 and C24:0) in brain tissue samples. These molecules were chromatographically separated from their isobaric galactosylceramide (GalCer) counterparts using normal phase chromatography. The analysis was performed by tandem mass spectrometry in the multiple reaction monitoring (MRM) acquisition mode. Limits of detection ranging from 0.4 to 1.1 nmol/g brain tissue were established for the different GluCer isoforms analyzed. For the first time, GluCer isoform levels were analyzed in temporal cortex brain tissue samples from 26 PD patients who were divided into three PD disease stages (IIa, III, and IV) according to the Unified Staging System for Lewy Body Disorders. These specimens were compared with brain tissue samples from 12 controls and 6 patients with Incidental Lewy Body Disease. No significant GluCer concentration differences were observed between the 5 sample groups. The GluCer isoform levels were also normalized with their matching GalCer isoforms. The normalized results showed a trend for GluCer levels which increased with PD severity. However, the differences observed between the groups were not significant, owing likely to the high standard deviations measured.

Key Words

Tandem mass spectrometry, High performance liquid chromatography, Parkinson Disease, Incidental Lewy Body Disease, Glucosylceramide, Galactosylceramide, Human brain tissues

INTRODUCTION

Mutations in the human *GBA* gene, associated with deficiency of glucocerebrosidase (GCase) activity, are a significant genetic risk factor for Parkinson Disease (PD)[1-3], and homozygosity also causes Gaucher disease (OMIM #230800)[4,5]. GCase deficiency was recently reported in PD brain tissues[6,7] and was shown to promote alpha-synuclein accumulation in neurones in culture[8]. GCase is a lysosomal enzyme which cleaves the *beta*-glucosidic linkage of glucosylceramide (GluCer), also known as glucocerebroside. Figure 1a shows the structure of GluCer and the cleavage site of GCase. GluCer is comprised of different isoforms owing to different fatty acid moieties. GluCer isoforms are isobaric structural isomers of galactosylceramide (GalCer) isoforms. Both species can be differentiated only by the axial or equatorial configuration of one hydroxyl group (Figure 1).

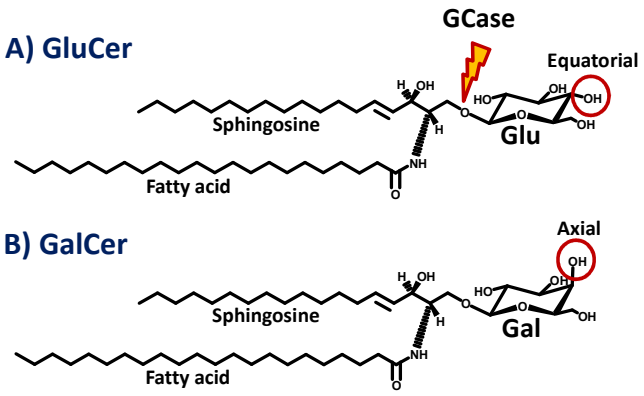

Figure 1. Structures of: (A) Glucosylceramide (GluCer), and (B) Galactosylceramide (GalCer). Both molecules are comprised of different isoforms according to different fatty acid chains. The cleavage site of the glucocerebrosidase (GCase) enzyme is indicated on the GluCer molecules. Red circles indicate the hydroxyl group differentiating GluCer and GalCer by its axial or equatorial configurations. Glu = glucose, Gal = galactose.

The main objective of this study was to evaluate if the GCase deficiency observed in PD patient brain tissues was reflected by the accumulation of its GluCer substrate. In order to attain this objective, the main challenge was to separate chromatographically low abundance GluCer isoforms from their high abundance GalCer counterparts in complex brain tissue matrices. Zama *et al.*[9] quantified simultaneously GluCer and GalCer in zebra fish embryos using the sphingolipid ceramide *N*-deacylase enzyme to remove the fatty acids of the analytes, followed by derivatization of the resulting free NH_2 groups with *O*-phthalaldehyde, separation by normal phase chromatography, and detection by fluorescence. The main disadvantages of this approach were the loss of information caused by removal of the fatty acid moieties before the analysis and the low specificity of

the fluorescence detection technique. Few liquid chromatography methods allowing the separation of GluCer and GalCer isomers and using a normal stationary phase have been reported.[10-13]. For these latter methods, the detection was performed using tandem mass spectrometers. The separation of GluCer and GalCer on a hydrophilic interaction chromatography (HILIC) stationary phase was also achieved using supercritical fluid carbon dioxide as the mobile phase[14]. A complex, time consuming and less accessible not-stop-flow online normal-/reversed-phase two-dimensional liquid chromatography-quadrupole time-of-flight mass spectrometry method was also developed for the analysis of different lipids, including GluCer and GalCer isoforms.[15] We referred to the UPLC-MS/MS protocol from a conference abstract[10] and developed a method for this study owing to its compatibility with our mass spectrometry instruments and to its capability to maintain a highly efficient separation of GluCer/GalCer mixtures, even in the presence of a particularly lipid-rich tissue matrix in brain.

Recently, Gegg et al.[13] analyzed GluCer isoforms in brain tissues (putamen and cerebellum) from controls (n = 5 for putamen, n = 7 for cerebellum), PD patients (n = 7 for putamen, n = 13 for cerebellum), and PD patients with a *GBA* mutation (n = 5 for putamen, n = 14 for cerebellum). This study revealed no evidence of GluCer accumulation in brain tissue even with the *GBA* mutation (PD patients with Gaucher disease). These results were normalized to the protein content of the samples. In the current study, we preferred to normalize GluCer isoform levels with their GalCer counterparts. GalCer has similar chemical properties as GluCer, but is not a substrate of GCase. The current study

was performed on larger cohorts with temporal cortex brain tissue specimens from 12 controls, 6 patients with incidental Lewy body disease (ILBD) and 26 PD patients. PD patients were divided into subgroups according to disease severity (Stages IIa, III and IV) determined by the Unified Staging System for Lewy Body Disorders[16]. A rigorous validation of the method used to analyze GluCer isoforms in brain tissues was also performed.

EXPERIMENTAL SECTION

Ethics Approval

This project was approved by the Research Ethics Board (REB) of the Faculty of Medicine and Health Sciences and the Centre hospitalier universitaire de Sherbrooke (CHUS), as well as the Institutional Review Board (IRB) for Human Use by the University of Alabama at Birmingham.

Cases

De-identified, frozen, post-mortem human brain tissue specimens were obtained by the Shacka lab from the Arizona Parkinson's Disease Consortium (Dr. Thomas Beach, Director) following institutional investigational review board approval for their ethical use. Frozen brain tissue samples (temporal cortex) were subsequently shipped from the Shacka laboratory to our laboratory for tandem mass spectrometry analysis. The sample classification was based on the Unified Staging System for Lewy body disorders[16] and on

the level of neocortical phosphorylated (p)-alpha synuclein.[17,18] Patient cases included 12 neurologically normal controls, 6 patients with incidental Lewy body disease (ILBD), 6 PD patients at stage IIa, 10 PD patients at stage III, and 10 PD patients at stage IV. Patients at PD stage IIa have Lewy body pathology limited to the olfactory bulb and brainstem. For patients at PD stage II, the Lewy pathology was robust in olfactory bulb, brainstem and limbic system, but sparse in the neocortex. Finally, for PD patients at stage IV, the Lewy boby pathology was robust in the neocortex and throughout the brain.

Gaucher Disease Mouse Model Brain Tissue Specimens

Neonatal brain tissue specimens were collected from Gba1 +/- and -/- mice (a Gaucher disease mouse model) to use for protocol optimization in our study.[19,20] Homozygous Gba1 -/- mice developed rapid progressive neurological manifestations with increased levels of GlcCer, as well as reduced GCase activity in different organs, such as the brain, liver and spleen.

Reagents

Deuterated N-hexadecanoyl-D_3-glucopsychosine (GluCer(C16:0)D_3) (>98%), N-pentadecanoyl-psychosine (GalCer(C15:0)) (>98%), glucocerebroside (GluCer) isoform mixture from Gaucher spleen (>98%), and cerebroside (GalCer) isoform mixture (>98%) were purchased from Matreya (Pleasant Gap, PA). HPLC grade methanol (MeOH) and acetonitrile (ACN), and A.C.S. grade acetone were from EMD Chemicals Inc. (Darmstadt,

Germany). Optima LC/MS grade water and A.C.S. Reagent grade ammonium formate (Amm. Form.) were supplied by Fisher Scientific (Fair Lawn, NJ). Formic acid (FA) (>99%) was from Acros Organics (Morris Plain, NJ) and ReagentPlus® grade dimethylsulfoxide (DMSO) (≥99.5%) from Sigma-Aldrich (Saint-Louis, MO).

Sample Preparation

Homogenization was performed using 100 µg of a frozen human or murine brain tissue specimen (100 µg) which was deposited in a 2 mL reinforced plastic tube with screw cap and O-ring (Omni International, Kennesaw, GA). Six zirconium oxide beads (2.8 mm diameter) (Omni) and 400 µL of MeOH were then added. Tissue samples were homogenized using a Bead Ruptor 12 (Omni) bead mill for 45 s at high speed (5 m/s). Another 600 µL of MeOH was added to the tube, followed by a second 45 s homogenization cycle at low speed (3 m/s), to obtain a homogenate corresponding to 0.1 g of tissue/mL MeOH. Methanol was the solvent used for the homogenization process in order to denature proteins and prevent enzymatic reactions.

The solid phase extraction procedure was adapted from Brignol *et al*.[10] Briefly, 100 µL of brain tissue homogenates (0.1 g/mL MeOH) were deposited in 2 mL plastic tubes containing 1000 µL of MeOH, 55 µL of DMSO, and 20 µL of the internal standard (GluCer(C16:0)D_3) at 10 µmol/L in DMSO. Acetone (625 µL) was added to the tubes and the sample mixtures were vortexed for 30 min, and 300 µL of water was then added to each tube. Sample mixtures were vortexed for 30 s and centrifuged at 9 455 g for 1 min

to retrieve the supernatant. The supernatants were diluted with 300 µL of water and loaded on a hydrophilic-lipophilic balance (HLB) solid phase extraction cartridge (30 mg, Oasis, Waters Corp., Milford, US) preconditioned with 1 mL of MeOH. The cartridge was washed with 2 mL of MeOH 60%/H_2O 30%/acetone 10% and the samples were eluted with 2 mL of acetone 90%/MeOH 10%. Finally, the sample was evaporated to dryness under a nitrogen stream and resuspended with 50 µL of DMSO and 200 µL of Phase A (ACN 95%/MeOH 2.5%/H_2O 2.5%/FA 0.5%/5 mM Amm. Form.) for analysis.

For the calibration curve, 7 stock solutions containing 0; 0.25; 1.25; 6.25; 12.50; 25.00; and 62.50 µmol/L GalCer(C15:O) in DMSO were prepared. The calibration curve points were prepared as described for the samples, except that 100 µL of pooled human brain (0.1 g/mL MeOH) was used as the matrix and the 55 µL of DMSO added to the sample was replaced by 20 µL of one of the calibration curve stock solutions in DMSO plus 35 µL of DMSO. The final concentrations of the calibration curve points corresponded to 0, 0.5; 2.5; 12.5; 25.0; 50.0, and 125.0 nmol/g brain.

UPLC-MS/MS Analysis of GluCer Isoforms in Brain Tissue Specimens

An ACQUITY UPLC I-Class system from Waters was used for the separation of GluCer and GalCer isoforms in brain tissue samples. The parameters of the isocratic normal-phase UPLC method are shown in Table 1. We must emphasize that the UPLC parameters were critical in obtaining an efficient separation of GluCer and GalCer isoforms. Even a slight change in the concentration of methanol, water, or formic acid in the mobile phase might

significantly affect the retention times. Moreover, we noticed that up to 16 "conditioning injections" before the sample run were necessary to obtain reproducible results. Maximum separation efficiency was achieved using a mobile phase containing a minimum water concentration (2.5 %) needed to maintain the aqueous-rich layer at the surface of the stationary phase and to sustain analyte partitioning.

The UPLC system was coupled to a Xevo TQ-S (Waters) tandem mass spectrometer operated in the multiple reaction monitoring (MRM) mode. The mass spectrometer parameters are presented in Table 1. Using this mode, the precursor ion of the analyte is isolated in the first quadrupole, fragmented in the collision cell, and one of its specific fragment ions is isolated in the last quadrupole for quantitative analysis. Collision-induced dissociation (CID) with argon atoms was used for the fragmentation. The seven MRM reactions analyzed corresponded to the calibration curve standard GalCer(C15:0) (*m/z* 686.55), the internal standard GluCer(C16:0)D_3 (*m/z* 703.59), and the five GluCer/GalCer couples analyzed: GluCer/GalCer(C18:0) (*m/z* 728.60), GluCer/GalCer(C20:0) (*m/z* 756.64), GluCer/GalCer(C22:0) (*m/z* 784.67), GluCer/GalCer(C24:1) (*m/z* 810.68) and GluCer/GalCer(C24:0) (*m/z* 812.70). The same fragment ion (*m/z* 264.27), corresponding to the di-dehydrated sphingosine moiety, was monitored for all the molecules analyzed. Results were analyzed with the use of the TargetLynx 4.1 software (Waters). To evaluate the relative abundance of the 5 GluCer isoforms analyzed, GluCer(C16:0)D_3 was used as the internal standard and the GalCer(C15:0) response as the calibration curve. The later was quadratic with a 1/x

weighing function and the origin was excluded. The areas of the GalCer isoform peaks were used for normalization purposes.

Fragmentation studies of the isoforms C18:0 and C24:1 of GluCer and GalCer were performed on a quadrupole time-of-flight (QTOF) (Synapt G1, Waters) mass spectrometer to confirm the attribution of the molecules. Supplementary Table S-1 shows the mass spectrometer parameters for the fragmentation studies.

Table 1. UPLC and MS parameters for the analysis of GluCer isoforms in brain tissue samples

UPLC Parameters		MS Parameters	
Column	Halo HILIC 2.7	Capillary voltage	3.0 kV
	Advanced Materials Technology (Wilmington, DE)	Sampling cone voltage	10 V
	Length: 150 mm; internal diameter: 4.6 mm	Source offset voltage	50 V
	Particle diameter: 2.7 μm	Desolvation temperature	350°C
Column temperature	30° C	Desolvation gas flow	1000 L/h
Mobile phase	ACN 95%/MeOH 2.5%/H_2O 2.5%/FA 0.5%/ 5mM Amm. Form.	Cone gas flow	150 L/h
Weak wash solvent	Mobile phase	Nebulized pressure	7.0 Bar
Strong wash solvent	ACN	Collision gas flow	0.15 mL/min
Flow rate	0.5 mL/min	Collision energy	32 V
Injection volume	5 μL	Dwell time	0.1 s

Method validation

For the method validation, brain tissue homogenates from controls (0.1 g/mL MeOH) were pooled, mixed, and separated in 100 μL aliquots. From these aliquots, low concentration quality controls (LQC) and high concentration quality controls (HQC) were prepared by spiking GalCer(C15:0) to reach concentrations of 1.34 and 7.5 nmol/g brain tissue, respectively. LQCs and HQCs were used to evaluate intraday (n = 5) and interday

(n = 5) precisions for GalCer(C15:0) and the 5 GluCer isoforms analyzed in brain samples. LQCs and HQCs were also used to evaluate accuracy for GalCer(C15:0). LOD and LOQ were defined as 3 and 10 times, respectively, the standard deviations of the concentrations measured during the intraday. Extraction recovery for GalCer(C15:0) was measured using LQCs and HQCs where the GalCer(C15:0) standard was spiked before (n = 3) and after (n = 3) the extraction procedure. To evaluate the stability of GalCer(C15:0) and 5 GluCer isoforms, LQCs and HQCs (n = 2) were analyzed after 5 h at 22^{o}C, 48 h at 4^{o}C, 11 days and 16 weeks at -20^{o}C, and 2 freeze/thaw cycles. Adsorption of the analytes on glass and plastic ware after 3 transfers (n = 2), and stability of the reconstituted samples (n = 2) at room temperature in the UPLC sample manager were also evaluated.

RESULTS AND DISCUSSION

UPLC-MS/MS Analysis of GluCer Isoforms in Brain Tissues

The main challenge for the analysis of GluCer isoforms in brain tissue samples was to separate GluCer isoforms from their GalCer isobaric counterparts, which are differentiated only by the equatorial or axial configuration of one hydroxyl group (Figure 1). In brain tissue samples, the abundance of GluCer isoforms was considerably lower than that of their GalCer counterparts, which further complicated the analytical process. Five GluCer isoforms (C18:0, C20:0, C22:0, C24:1, and C24:0) were detected and analyzed in human brain tissue samples. Figure 2 shows ion chromatograms of these

latter GluCer isoforms which were well separated from their GalCer structural isomers.

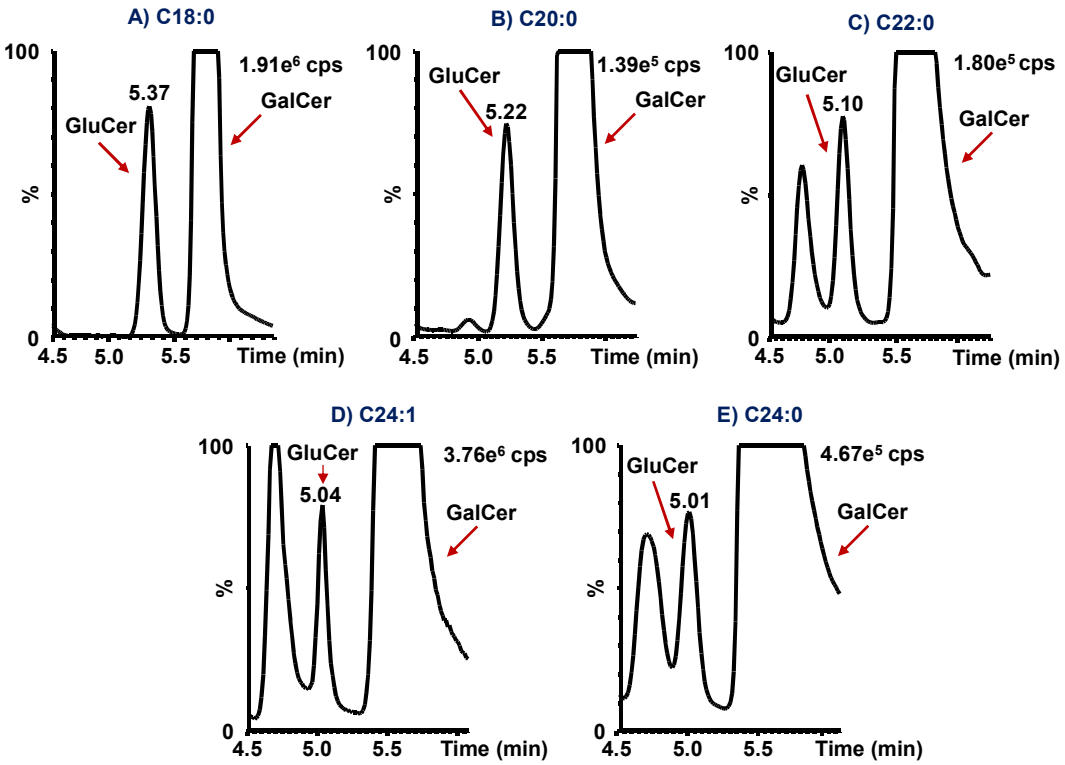

Figure 2. Ion chromatograms of glucosylceramide (GluCer) and galactosylceramide (GalCer) structural isomers, analyzed in human brain tissue samples, with different fatty acid moieties: A) C18:0; B) C20:0; C) C22:0; D) C24:1; and E) C24:0. Cps = counts per second.

Figure 3 shows the results obtained for the analysis of GluCer(C20:0) and GalCer(C20:0) in a Parkinson disease brain tissue sample and in different positive and negative controls. Similar results were obtained for other GluCer and GalCer isoforms analyzed (C18:0, C22:0, C24:1, and C24:0). Brain tissues from the Gaucher mouse model (Figure

3b), and a commercial mixture of GluCer isoforms (Figure 3d) were used as positive controls for the GluCer isoforms. As expected, GluCer isoforms were significantly more abundant in brain tissue from the Gaucher mouse model (Figure 3b), compared to brain tissue from the wild-type mouse (Figure 3c), owing to its GCase deficiency causing the storage of GluCer isoforms in brain tissue.[21] The retention times of the GluCer isoforms detected in Parkinson disease brain tissues were the same as the ones measured in the two GluCer positive controls. Furthermore, a commercial mixture of GalCer isoforms (Figure 3e) was used as a positive control for GalCer isoforms. The retention times of GluCer isoforms detected in PD brain samples were the same as the ones measured from the commercial mixture of GalCer isoforms. Additionally, the identification of the two most abundant GluCer isoforms (C18:0 and C24:1) and their GalCer counterparts in brain tissue samples was also supported by fragmentation spectra obtained with the UPLC-QTof mass spectrometer. The interpreted spectra are presented in Figure 4 for GluCer/GalCer(C18:0) and Supplementary Figure S-1 for GluCer/GalCer(C24:1). Other GluCer isoforms were not sufficiently abundant to generate good quality fragmentation spectra.

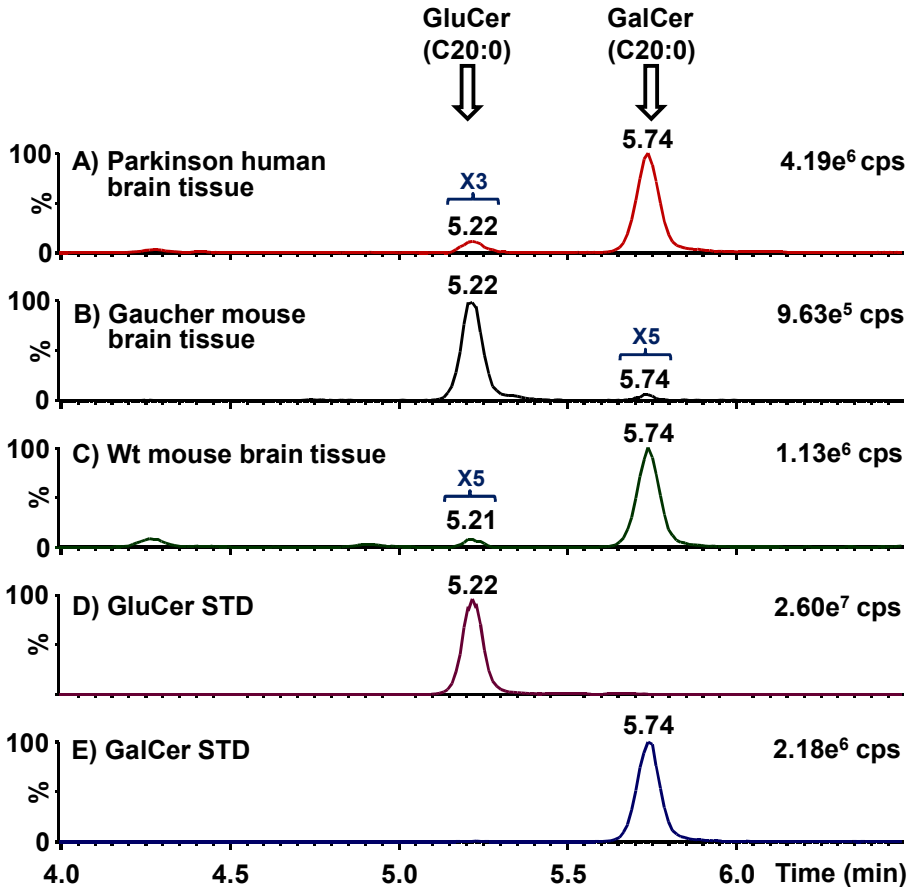

Figure 3: Ion chromatograms of glucosylceramide (GluCer) and galactosylceramide (GalCer) structural isomers with an arachidic fatty acid (C20:0) in: A) Parkinson human brain tissues (0.1 g/mL MeOH; B) Gaucher mouse brain tissues (0.01 g/mL MeOH); C) Wild-type (wt) mouse brain tissues (0.01 g/ mL MeOH); D) Commercial mixture of GluCer isoforms (10 µg/mL); and E) Commercial mixture of GalCer isoforms (10 µg/mL). Cps = counts per second.

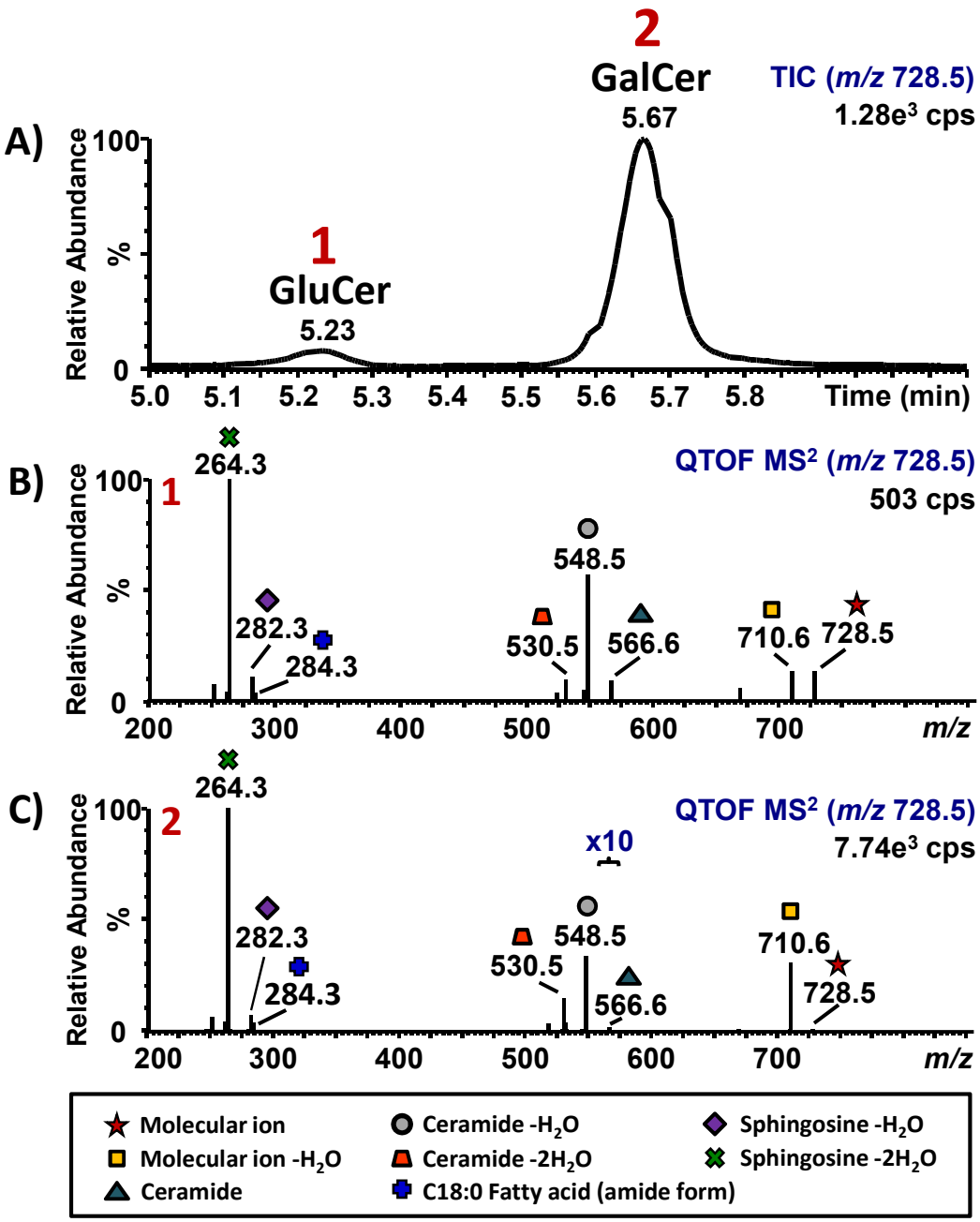

Figure 4. GluCer and GalCer(C18:0) fragmentation studies in a human brain tissue sample. A) GluCer and GalCer (C18:0) ion chromatogram; B) Fragmentation spectrum of GluCer(C18:0); C) Fragmentation spectrum of GalCer(C18:0). Rowling collision energy of 20-40 V for both fragmentation studies.

Method Validation

The semisynthetic GalCer(C15:0) isoform, not present in brain tissue, was used as the standard for the calibration curve and for the QCs. Unlike its GluCer counterpart, GalCer(C15:0) was commercially available. Since GluCer and GalCer isoforms are differentiated only by the axial or equatorial position of one hydroxyl group, very similar chemical properties are expected for these structural isomers and thus very similar behaviour during the sample preparation and the mass spectrometry analysis. Table 2 summarizes the method validation results. The concentrations shown in Table 2 for 5 GluCer isoforms correspond to the mean concentrations measured during the intraday in the pooled brain tissue matrix. The LOD measured for GluCer isoforms ranged between 0.4 and 1.1 nmol/g brain whereas the LOQ measured ranged between 1.2 and 3.7 nmol/g brain. Intra- and interday precisions were <16% for GluCer(C15:0) in LQCs and HQCs, and for the 5 GluCer isoforms detected in the pooled control brain tissue matrix. The intra- and interday accuracies were <24% and <9% for LQCs and HQCs, respectively. For the stability assays, GluCer(C20:0) and GluCer(C22:0) were not taken into account owing to their concentrations at the LOD level in the pooled brain tissue matrix used for the validation. The spiked GalCer(C15:0) and the endogenous GluCer isoforms in the QCs were stable (Bias <15%) for at least 5 h at 22^{o}C, 48 h at 4^{o}C, 11 days at -20^{o}C, and 2 freeze/thaw cycles. After 16 weeks at -20^{o}C, significant losses of GalCer(C15:0) and GluCer isoforms were noticed. No significant adsorption (bias <15%) of GalCer(C15:0) and GluCer isoforms was observed on plastic or glass ware even after three transfers. Bias ranging between -9.3 and 20.2% were observed for GalCer(C15:0)

and GluCer isoforms in the reconstituted samples left 24 h at room temperature in the UPLC autosampler. The mean correlation coefficient (R^2) measured for the 7-point calibration curve was 0.994 (n = 5). The extraction recoveries for GluCer(C15:0) in LQCs and HQCs were 32.3 and 25.2% respectively. A liquid-liquid extraction protocol with chloroform[22-24], previously used for the analysis of other sphingolipids, was also tested for the analysis of GluCer and GalCer isoforms. However, with this latter approach, the sensitivity was quite low owing to major ion suppression effects.

Table 2. Method Validation Results for the Analysis of GluCer Isoforms in Human Brain Tissue Samples. RSD = relative standard deviation, nd = not detected, na = not applicable.

Validation parameter	GalCer(C15:0) (LQC) 1.34 nmol/g brain	GalCer(C15:0) (HQC) 7.5 nmol/g brain	GluCer(C18:0) 7.7 nmol/g brain	GluCer(C20:0) 0.3 nmol/g brain	GluCer(C22:0) 0.5 nmol/g brain	GluCer(C24:1) 7.9 nmol/g brain	GluCer(C24:0) 1.5 nmol/g brain
Intraday (n = 5)							
Precision: RSD%	7,4	8,3	7,8	8,6	15,7	14,9	4,1
Accuracy: Bias (%)	23,7	8,3	na	na	na	na	na
Interday (n = 5)							
Precision: RSD%	9,9	6,0	9,1	nd	nd	6,8	15,3
Accuracy: Bias (%)	14,1	2,2	na	na	na	na	na
Stability (n = 2)							
(5 h, 22°C) Bias (%)	9,7	-8,0	2,6	na	na	-11,2	-7,7
(48 h, 4°C) Bias (%)	-10,1	-7,8	-6,0	na	na	-4,7	-3,9
(11 days, -20°C) Bias (%)	-5,3	-8,7	8,7	na	na	-13,7	11,4
(16 weeks, -20°C) Bias (%)	-25,8	-24,5	-15,5	na	na	-33,8	-24,1
2 freeze thaw cycles Bias (%)	-6,3	0,8	0,2	na	na	-6,9	-10,0
(24 h in injector) Bias (%)	7,4	15,2	20,2	na	na	-9,3	4,7
(Glass adsorption, 3 transfers) Bias (%)	-1,3	0,8	0,8	na	na	4,0	1,7
(Plastic adsorption, 3 transfers) Bias (%)	7,4	13,1	4,0	na	na	7,3	5,1
Extraction recovery (%)	32,3	25,2	na	na	na	na	na
LOD	0,4	na	0,7	0,4	0,5	1,1	0,8
LOQ	1,2	na	2,5	1,3	1,6	3,7	2,8
Mean curve R^2 (n =5)	0,994	na	na	na	na	na	na

GluCer Isoform Levels in Parkinson Disease Brain Tissue Samples

Supplementary Table S-2 shows the concentrations of five different GluCer isoforms, and their total measures, in every sample analyzed in this study. These results are summarized in Figure 5a where the mean concentrations measured for different GluCer isoforms and the sums are shown for five human brain tissue sample groups as a bar chart. The whiskers correspond to ± one standard deviation. A non-parametric Kruskall-Wallis test was performed to determine if the biomarkers analyzed are discriminated between the sample groups. The non-parametric test was preferred over the parametric ANOVA test owing to the small sample sizes (between 6 and 12 samples per group). The Kruskall-Wallis *p*-values measured for different GluCer isoforms and their total sums are presented in Figure 3a. These values ranged between 0.44 and 0.97. With our actual sample size, the sample groups thus cannot be discriminated by the biomarkers analyzed since all the *p*-values are superior to 0.05.

Normalization of GluCer isoforms with GalCer isoforms

Since substrate of the GCase enzyme suspected to be involved in Parkinson disease is GluCer, not GalCer, the GluCer isoform levels measured in the samples were normalized to the levels of their GalCer isoform counterparts. The differences in the GluCer/GalCer ratios between the different sample groups are likely to be more relevant than differences in the GluCer levels normalize to protein concentration since GalCer is an endogenous molecule with similar chemical properties. Supplementary Table S-3 shows the peak area ratios between the different GluCer isoforms and their matching GalCer

isoforms. These results are summarized in Figure 5b. For the GluCer(C18:0)/GalCer(C18:0), GluCer(C20:0)/GalCer(C20:0), and GluCer(C22:0)/GalCer(C22:0) ratios, a slight increasing trend with PD severity was observed. However, owing to the large standard deviations measured for the GluCer/GalCer ratios in the different sample groups, the increasing trend was not statistically significant. This was confirmed by the calculated Kruskall-Wallis *p*-values which were higher than 0.05 (between 0.29 and 0.94). A sample size of 96 (48 samples per group) would be necessary to statistically confirm the GluCer(Total)/GalCer(Total) ratio differences observed between the PD (Stage IV) and control groups, according to the Mann-Whitney non-parametric statistical test. Since PD alters lipid pathways,[25] the slightly increased GluCer/GalCer ratios observed for PD patients (Stage IV) compared to controls might be due to a GluCer increase as well as to a GalCer decrease. Supplementary Figure S-2 shows the levels of GalCer isoforms in the sample groups. As for GluCer isoforms, the standard deviation within the groups was very high. GalCer isoform levels were slightly higher in the control group compared to the 4 other groups.

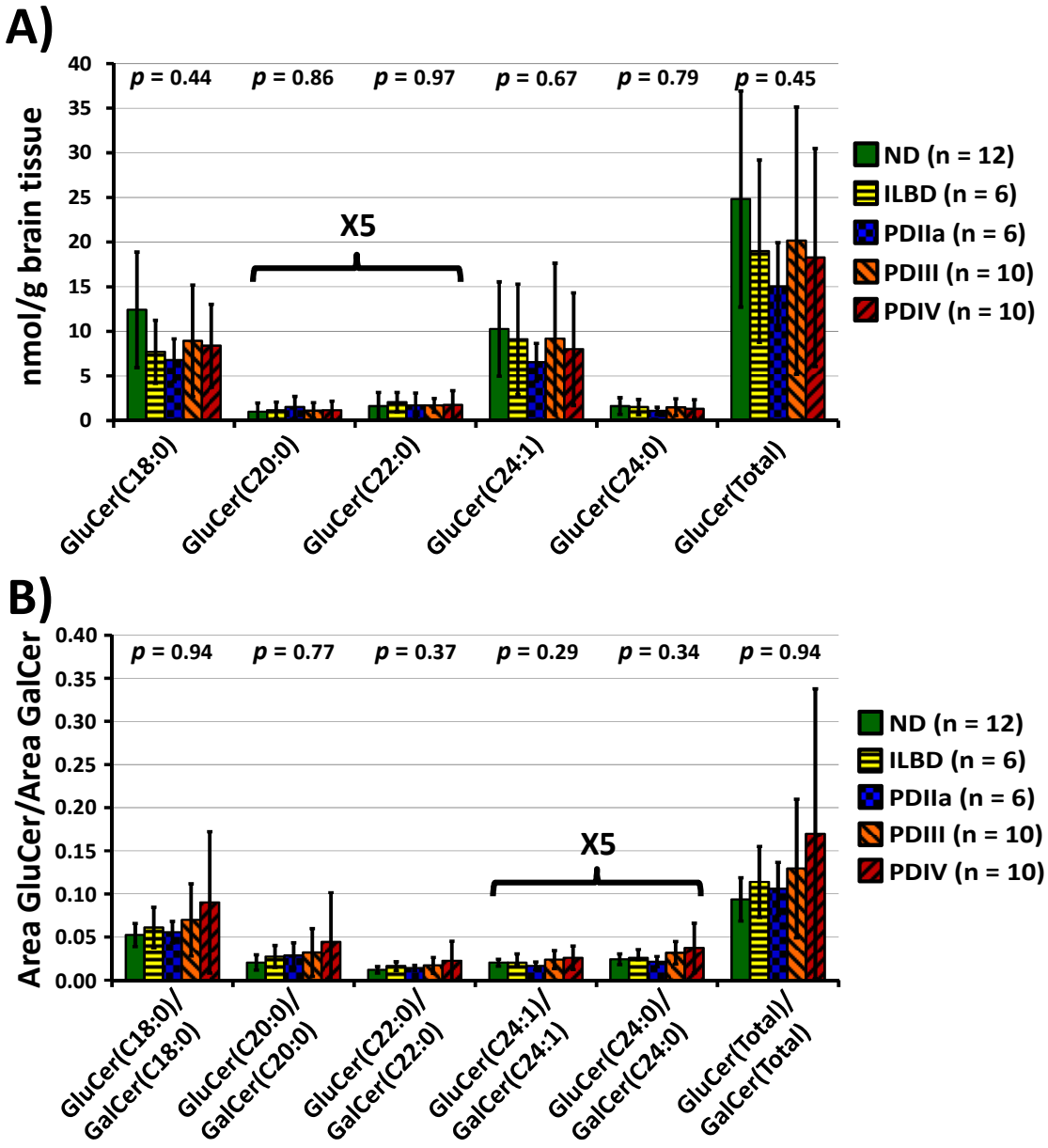

Figure 5. (a) Mean concentrations of GluCer isoforms in different groups of brain tissue samples. (b) Mean areas of GluCer isoforms normalized with the mean areas of their GalCer counterparts in different groups of brain tissue samples. ND = controls, ILPD = Incidental Lewy Body Disease patients, PDIIa = Parkinson Disease stage IIa patients, PDIII = Parkinson Disease stage III patients, and PDIV = Parkinson disease stage IV patients. The whiskers correspond to ± one standard deviation. The non-parametric Kruskall-Wallis *p*-values ($p < 0.05$ for statistically significant differences between sample groups) are indicated for each GluCer isoform.

CONCLUSIONS

A UPLC-MS/MS method was developed and validated for the simultaneous analysis of GluCer and GalCer isoforms in human brain tissue samples. A baseline separation was achieved between GluCer and GalCer isoforms, which are differentiated only by the axial and equatorial conformation of one hydroxyl group. A high UPLC performance separation was needed owing to the GalCer isoform concentrations, which were up to 300 times more abundant than their GluCer counterparts. Five GluCer isoforms (C18:0, C20:0, C22:0, C24:1, and C24:0) were detected and analyzed in human brain tissue samples. To our knowledge, it is the first time that GluCer isoforms are analyzed in temporal cortex human brain tissue samples from PD patients at different stages (IIa, III, and IV) and compared with two control groups (Controls (ND), and Incidental Lewy Body Disease (ILBD) patients). The possibility that the decreased GCase activity observed in Parkinson brain tissue samples was accompanied with the increase of its GluCer substrate was not confirmed. No statistically significant differences of GluCer isoform levels were observed between the Parkinson disease sample groups and the control groups, even when the GluCer isoform levels were normalized with their endogenous GalCer counterparts. Perspective studies with larger sample sizes for PD Stage IV and control groups would be necessary to statistically confirm the GluCer(Total)/GalCer(Total) ratio differences observed between these two groups.

ASSOCIATED CONTENT

Supporting Information

Figure S-1 shows GluCer and GalCer(C24:1) fragmentation studies in a human brain tissue sample. Figure S-2 shows the histogram of the response of GluCer isoforms in

brain tissue samples. Table S-1 shows instrument parameters for fragmentation studies on the QTof mass spectrometer. Table S-2 provides levels of GluCer isoforms in brain tissue samples. Table S-3 shows the area of each GluCer isoform normalized to the area of its GalCer counterpart in different brain tissue sample groups. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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