Glucosylceramide Quantitation in Normal and Glucocerebrosidase-Deficient Mouse Brain and Human Cell Lines

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Introduction

Gaucher disease is a lysosomal storage disease caused by mutations in the gene that encodes glucocerebrosidase (GCase). Reduced GCase activity leads to accumulation of the primary lipid substrate glucosylceramide (GlcCer). GCase deficiency leads to characteristic visceral manifestations and, in some patients, to central nervous system involvement. To provide bioanalytical support to studies of neuronopathic Gaucher disease, a liquid chromatography-mass spectrometry (LC-MS/MS) method was developed to separate GlcCer from the predominant isomer found in brain tissue (galactosylceramide,GalCer). GlcCer levels were quantitated in brain tissue from normal C57BL/6, Gaucher mice V394L/V394L (4L) and V394L/V394L prosaposin hypomorphs (4L/PS-NA). GlcCer levels were also measured in normal and Gaucher patient-derived lymphoblasts.

1. Sample Preparation and Data Acquisition

Sample Preparation

Step I: Accurately weigh 15 to 25 mg of mouse brain tissue into a FastPrep tube. Homogenize in 15 µL deionized water per mg of tissue.

Step II: Transfer 50 µL homogenate into a glass tube. Add 75 µL DMSO solution containing 0.7 µg/mL internal standard. Add 400 µL of methanol then 1.25 mL 50/50 acetone/methanol. Shake on a Multi-tube Vortexer for approximately 30 minutes. Add 300 µL water to resuspend. Centrifuge for 10 minutes at room temperature at 3220g. Transfer supernatant to a clean tube. Add 600 µL 13/87 water/methanol.

Step III: Transfer the mixture onto a pre-conditioned C18-SPE cartridge. Wash with 2 mL 67/23/10 methanol/acetone/water. Elute the cartridge with 2 mL 90/10 acetone/methanol. Evaporate eluant to dryness. Reconstitute the sample with 50 µL DMSO and 200 µL mobile phase. Inject 2 to 5 µL of the extract onto the HPLC column for LC-MS/MS analysis.

Data Acquisition Methods

HPLC Conditions:

Equipment: Shimadzu LC Pumps and Autosampler

Mobile phase: 95/2.5/2.5 acetonitrile/methanol/water + 0.5% formic acid + 5 mM ammonium formate. Elution: Isocratic. Flow Rate: 0.5 mL / min. Total run time: 9.5 minutes

Analytical column: Mac-Mod Halo HILIC Silica 2.7 µm, 4.6x150 mm

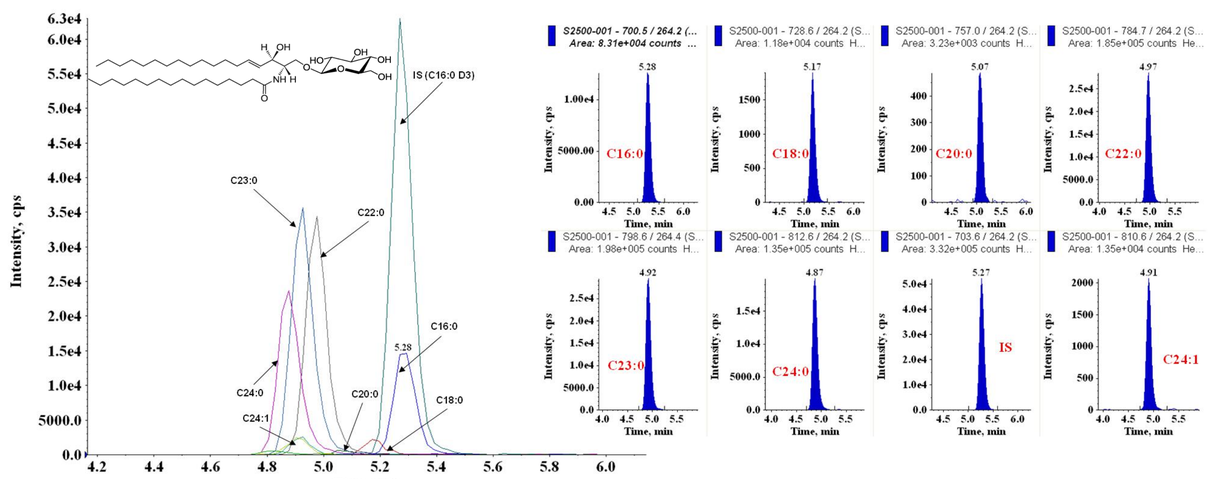
Mass Spectrometry Parameters and Conditions:

Mass Spectrometer: ABSciex 4000QTRAP LC-MS/MS system – Electrospray ionization operated in positive ion mode (ESI+)

MRM Transitions: 7 GlcCer isoforms, 700.5 / 264.2 [C16:0], 728.6 / 264.2 [C18:0], 757.0 / 264.2 [C20:0], 784.7 / 264.2 [C22:0], 798.6 / 264.2 [C23:0], 810.6 / 264.2 [C24:1], 812.6 / 264.2 [C24:0], Internal Standard (*N*-Palmitoyl-d3-Glucopsychosine) 703.6 / 264.2 [C16:0 D3]

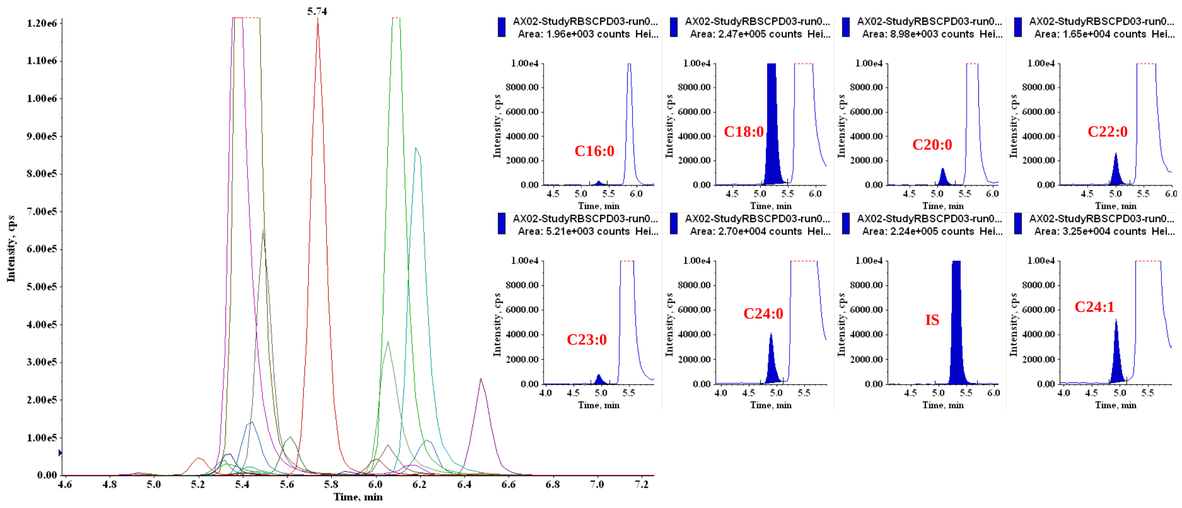
DP (volts): 76-81, CE (volts): 49-57, CXP (volts): 14-16

2. Representative Chromatograms of a GlcCer Standard [2.50 µg/mL in DMSO]



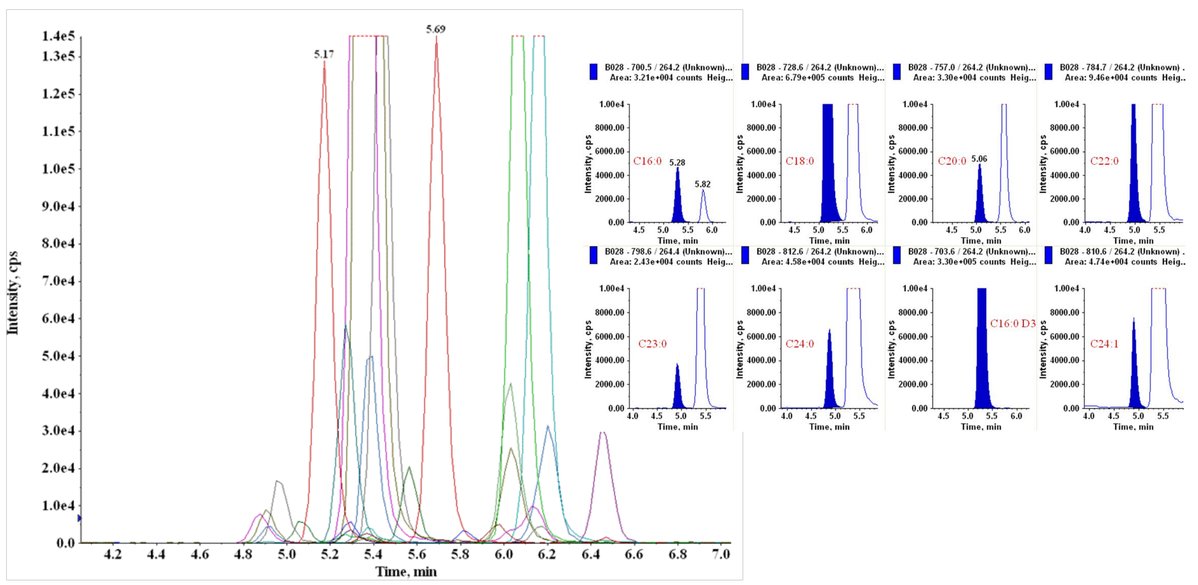
The elution profile of the seven (7) GlcCer isoforms and the internal standard (C16:0 D3) and their respective peak areas are shown in these chromatograms. The GlcCer standard (source: bovine buttermilk, supplier: Matreya LLC) was used to construct a 9-point calibration curve [0.01 to 5 µg/mL in DMSO]. GlcCer concentrations of brain tissue or lymphoblast samples were calculated based on a linear curve fitting with $1/x^2$ weighting of the ratio of the total GlcCer peak area to the peak area of the internal standard vs. nominal concentration. The acceptance criteria for standard and quality control samples were set at ± 20% of nominal at all concentration levels.

3. LC-MS/MS Chromatograms of GlcCer Extracted from a Wild-Type Mouse Brain Tissue Homogenate



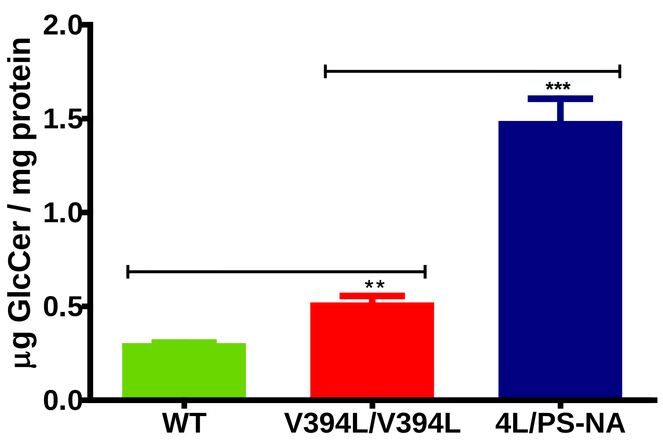
Seven (7) GlcCer isoforms were monitored and separated from GalCer isoforms based on the sugar head groups (glucose versus galactose) of the glycolipids. GlcCer and Internal standard eluted from the LC column between 4.8 and 5.3 minutes, and had lower chromatographic peak intensities than GalCer. All GlcCer isoforms were baseline resolved from their isomeric GalCer isoforms. The extraction recovery of GlcCer from brain tissue was better than or equal to 75% (data not shown). Total GlcCer measured in this wild-type mouse (20-week old) brain homogenate had a level equivalent to approximately 1.4 µg/mL of a GlcCer standard or 22 µg GlcCer per gram of tissue.

4. LC-MS/MS Chromatograms of GlcCer Extracted from a GCase-Deficient Mouse Brain Tissue Homogenate



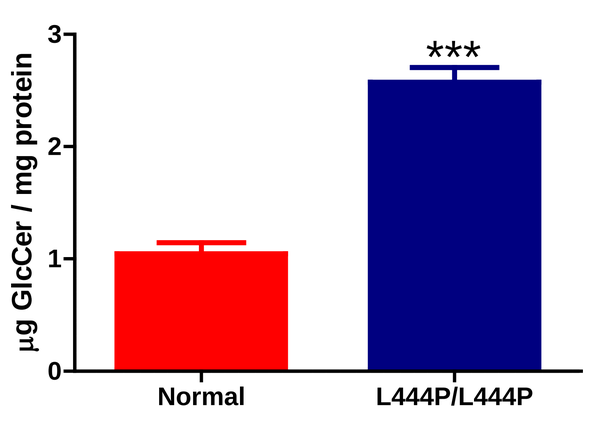
Total GlcCer in this Gcase-deficient mouse (4L/PS-NA) brain homogenate was at a level equivalent to approximately 4 µg/mL of a GlcCer standard, or 64 µg of GlcCer per gram of tissue. This GlcCer level was up to 3-fold higher than the level found in a 20-week old wild-type mouse brain homogenate.

5. GlcCer Levels in 20-Week Old C57BL/6, V394L/V394L, and 4L/PS-NA Mouse Brains



Brain tissue from 4L/PS-NA mice (n=4) contained the highest levels of GlcCer (1.47 ± 0.14 µg GlcCer per mg total protein), followed by V394L/V394L mice (n=4) (0.504 ± 0.05 µg GlcCer per mg total protein), and C57BL/6 mice (n=6) (0.287 ± 0.02 µg GlcCer per mg total protein). *** P < 0.01, ***P < 0.001*

6. GlcCer Levels in Normal and Gaucher-derived Lymphoblasts



Lymphoblast samples were analyzed under reverse phase LC conditions using a C18 analytical column (data not shown). Unlike brain homogenate, lymphoblasts (wild-type and Gaucher–derived) contain insignificant levels of GalCer. The Gaucher patient-derived lymphoblasts (n=3) contained 2.57 ± 0.22 µg GlcCer per mg total protein, 2.5-fold higher than the levels found in normal lymphoblasts (n=3) (1.05 ± 0.0.97 µg GlcCer per mg total protein). ****P < 0.001*

Conclusions

- A new and sensitive LC-MS/MS method was developed to separate GlcCer from its isomer GalCer, and to specifically quantitate GlcCer levels in brain tissue.
  - The method utilized a 50 µL sample aliquot and Solid Phase Extraction for sample clean-up and enrichment prior to detection via aqueous normal HPLC or HILIC and tandem mass spectrometry.
  - Baseline separation of the seven GlcCer isoforms from isomeric GalCer isoforms, and a lower limit of quantitation of 10 ng/mL (~0.014 µM) were achieved.
  - The method is routinely used to quantitate GlcCer in normal and GCase-deficient mouse brain and human cell lines.
- Brain tissue from 4L/PS-NA mice contains GlcCer per mg protein at a level that is:
  - Three-fold higher than the level found in brain tissue from V394L/V394L mice
  - Five-fold higher than the level found in brain tissues from wild-type mice.
- Gaucher patient-derived lymphoblasts contain 2.5-fold higher GlcCer levels than normal lymphoblasts.