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Galactosylsphingosine does not interfere with the quantitation of plasma glucosylsphingosine levels in Gaucher patients

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ABSTRACT

It has been shown that the plasma level of glucosylsphingosine (Lyso GL-1) is a useful biomarker for the diagnosis and monitoring of Gaucher disease. Potentially interfering with the quantitation of Lyso GL-1 is its isobaric structural isomer, galactosylsphingosine (psychosine). The contribution of psychosine is generally not accounted for in the determination of Lyso GL-1, due to the difficulty in separating these two isomers. Few methods have been presented in the literature to distinguish the two isomers, and those available tend to be tedious and time-consuming. Here, we developed a LC/MS/MS method able to chromatographically separate Lyso GL-1 and psychosine reproducibly and combine it with a simple, high-throughput sample preparation technique. We also show that the separation of these two isomers in the plasma of Gaucher patients is not necessary for the quantitation of Lyso GL-1 levels, as the relative psychosine level is < 3% of Lyso GL-1.

1. Introduction

Gaucher disease (GD) (OMIM #230800) is an autosomal recessive lysosomal storage disorder caused by the deficient activity of beta-glucocerebrosidase (GBA), and is the most common of the sphingolipidoses. Mutations in the *GBA* gene leads to decreased activity of this lysosomal enzyme, with > 300 *GBA* mutations having been described [1]. This reduced activity leads to the accumulation of the primary substrate, glucosylceramide (GL-1), mainly in macrophages [2].

Lyso-glycosphingolipids, the deacylated forms of glycosphingolipids, have been shown to also be elevated in many lysosomal storage disorders over the past several years [3–5]. Dekker showed that glucosylsphingosine (Lyso GL-1) is increased in 100% of GD patients, with high specificity, and that it discriminates better than glucosylceramide, as its levels are in accordance with disease severity [6]. Rolfs confirmed that Lyso GL-1 is highly specific, as only GD patients feature pathological values of Lyso GL-1 and its levels are not gender-dependent and show superior characteristics over two other biomarkers, CCL18 and chitotriosidase, in terms of diagnostic utility [7]. The levels of Lyso GL-1 decrease upon treatment with either enzyme replacement therapy or substrate reduction therapy [8].

Potentially interfering with the quantitation of Lyso GL-1 is its isobaric structural isomer, galactosylsphingosine (psychosine). The contribution of psychosine is generally not accounted for in the determination of Lyso GL-1, due to the difficulty in separating these two isomers [4,9]. However, the lack of differentiation between these two molecules could lead to impaired or incorrect decision making in terms of disease diagnosis, progression, or treatment efficacy depending on the relative ratio of psychosine to Lyso GL-1 in a sample.

Our goal was to measure Lyso GL-1 concentrations in human plasma, while also determining the relative abundance of psychosine in these same samples. Several publications have previously identified methods with the ability to differentiate glucosyl and galactosyl isomers of sphingolipids. Most publications use derivatization to identify the individual species [10–12], while another digests the purified lipid extract with recombinant glucocerebrosidase to determine the percentage of glucosylated lipid species [13]. Neither of these approaches is amenable to the clinical laboratory due to the significant time and energy needed for these extra steps in the sample preparation. A few groups have also shown the ability to separate these species using chromatography [14–16], however the sample preparation procedures employed tend to be laborious. Finally, some groups have shown the ability to separate the isomers using specialized equipment such as supercritical-fluid chromatography [9,17] or ion-mobility mass spectrometry [18,19]. However, these equipment are not readily available in most clinical laboratories. The previously published methods on Lyso GL-1 quantitation for Gaucher disease do not provide a means of identifying the individual contributions of each isomer in a format appropriate for clinical laboratories. Therefore, we have developed a high-throughput sample preparation technique and chromatography

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conditions giving separation of the two species in an LC/MS/MS format that allows for unbiased Lyso GL-1 quantitation and the determination of relative psychosine levels.

2. Materials and methods

2.1. Sample collection

Forty-one K_2EDTA plasma samples were obtained from Gaucher patients with previously confirmed clinical diagnosis who provided written, informed consent. Control K_2EDTA plasma samples from 25 healthy adults were purchased from a commercial source (BioreclamationIVT, Westbury, NY) as were bulk lots of delipidized plasma (Golden West Biologicals, Temecula, CA).

2.2. Chemicals

Lyso GL-1, psychosine, and dimethyl psychosine (internal standard) were all purchased from Avanti Polar Lipids (Alabaster, AL). Solvents used were optima grade from Fisher Scientific.

2.3. Lipid extraction

To quantify Lyso GL-1, 100 µL of plasma was placed into an Ostro SPE plate (Waters, Milford, MA) followed by 400 µL of internal standard (acetonitrile with 1% formic acid containing 25 ng/mL dimethyl-psychosine). The samples were pipet mixed and the resulting solutions were filtered through the SPE plate and collected. The main purpose of the Ostro plate is for offline removal of phospholipids, a major matrix interference in the analysis of lipids and small molecules from plasma samples. The proprietary Ostro sorbent selectively retains phospholipids within the sorbent bed, while allowing the analytes of interest to pass through and be collected for analysis. Individual calibration curves for Lyso GL-1 and psychosine were each prepared in a similar fashion, but using delipidized plasma as a matrix and spiking in concentrations of the respective isomer from 0.2–2000 ng/mL.

2.4. Liquid chromatography coupled with mass spectrometry

The collected solutions were injected (2 µL) into an LC/MS/MS system comprised of an Acquity H-Class UPLC (Waters, Milford, MA) and Qtrap6500 mass spectrometer (AB Sciex, Toronto, Canada). The chromatographic separation, depicted with pure standards of Lyso GL-1 and psychosine in Fig. 1, was achieved with an Acquity UPLC BEH HILIC column (2.1 ∗ 150 mm, 1.7 µm) using the following three mobile phases: (A) 15 mM ammonium acetate in 96% acetonitrile, 2% methanol, 1% acetic acid, and 1% water, (B) 15 mM ammonium acetate in 98% methanol, 1% acetic acid, and 1% water, and (C) 75% acetonitrile and 25% water. The gradient involved the use of mobile phases A and B to separate the isomers over 6 min, followed by using mobile phase C to flush the column before re-equilibration at the starting conditions.

As compared to the previously described methodology [4], instead of needing two 150 cm columns in series, separation is obtained using a single 150 cm column. Levels of the mobile phase additives were increased from 5 mM to 15 mM ammonium acetate and 0.5% to 1% acetic acid. The gradient was also modified. A third mobile phase was added to the current assay to flush the column after each sample analysis.

Mass spectrometry (MS) was performed in MRM mode. The transitions used were 462/282 (Q1/Q3) for Lyso GL-1 and psychosine, with 490/292 used for dimethyl psychosine. Declustering potentials were 90 V for Lyso GL-1 and psychosine, with 80 V used for dimethyl psychosine. Collision energies were 30 V and 37 V, respectively. Entrance potential and collision cell exit potentials were held constant at 10 V and 16 V with each using a dwell time of 50 ms. Source/Gas parameters were the following: CUR - 20, CAD - Medium, IS - 5400, TEM - 650, GS1 - 40, and GS2 - 40.

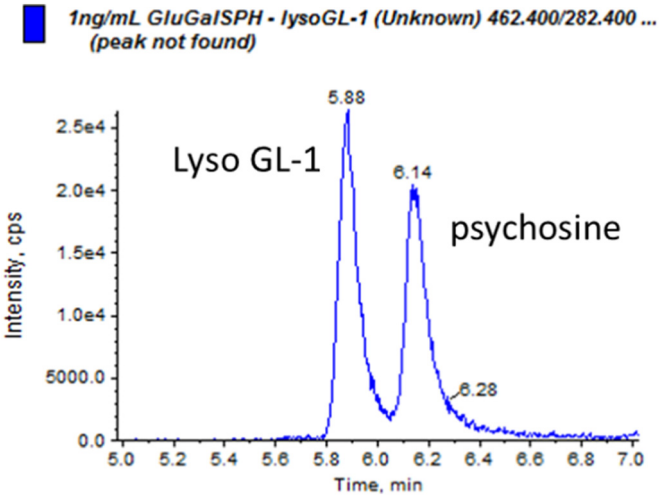

Fig. 1. Chromatographic separation of Lyso GL-1 and psychosine. Pure standards for Lyso GL-1 and psychosine were mixed and run as described in Section 2.4.

Table 1
Lyso GL-1 concentrations in human plasma samples.

Control sample ID#	Calculated concentration (ng/mL)	Gaucher sample ID#	Calculated concentration (ng/mL)
NC1	BQL	1	398.3
NC2	BQL	2	285.4
NC3	0.2	3	84.0
NC4	BQL	4	461.9
NC5	BQL	5	203.4
NC6	0.2	6	76.1
NC7	0.2	7	404.0
NC8	BQL	8	254.0
NC9	BQL	9	49.4
NC10	BQL	10	318.2
NC11	0.2	11	163.6
NC12	0.3	12	27.8
NC13	BQL	13	272.8
NC14	BQL	14	186.0
NC15	BQL	15	5.7
NC16	BQL	16	243.0
NC17	BQL	17	151.1
NC18	0.2	18	10.6
NC19	BQL	19	163.6
NC20	BQL	20	128.4
NC21	BQL	21	40.3
NC22	0.2	22	110.9
NC23	BQL	24	19.6
NC24	BQL	25	188.4
NC25	BQL	26	94.7
		27	30.4
		28	475.8
		29	343.8
		30	118.1
		31	418.3
		35	197.5
		36	55.5
		37	174.9
		39	38.3
		41	91.8
		42	24.6
		43	163.3
		44	66.2
		45	2.7
		49	85.6
		53	45.0

After initial tests were unable to identify psychosine in any sample, ten Gaucher patient samples were chosen for spiking of psychosine to determine the relative level of interference necessary to be detected.

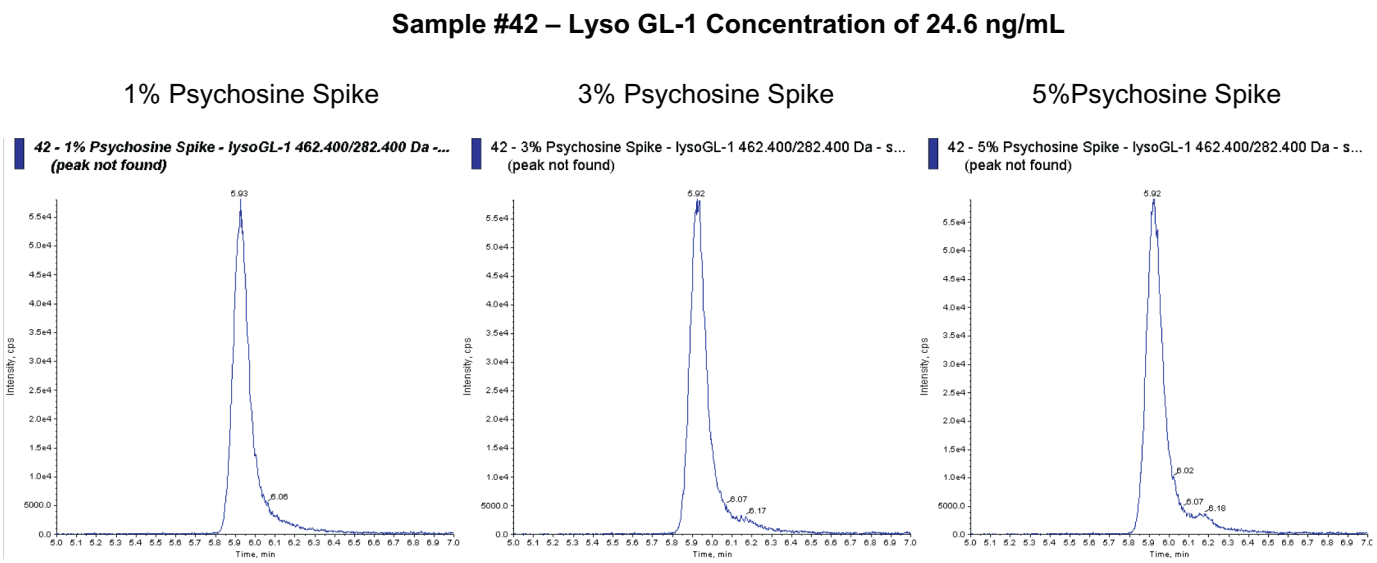

Fig. 2. Spiking of sample extracts with psychosine. Sample extracts were spiked in with three levels of psychosine and run as indicated in Section 2.4. Representative chromatograms were plotted. Sample #42 – Lyso GL-1 Concentration of 24.6 ng/mL. 1% Psychosine Spike 3% Psychosine Spike 5% Psychosine Spike.

Based on the Lyso GL-1 concentration determined for each sample, psychosine spiking levels of 1%, 3%, and 5% were calculated. These amounts of psychosine were dried into autosampler vials and reconstituted in the appropriate sample extract for analysis.

3. Results and discussion

A simple sample extraction technique and LC/MS/MS method with HILIC chromatography were developed to extract and quantify Lyso GL-1 without interference from psychosine. The method was able to efficiently separate standard Lyso GL-1 from psychosine (Fig. 1).

Dynamic range of the assay was 0.2 to 2000 ng/mL for Lyso GL-1, all points were within ± 12% bias. The Lyso GL-1 concentration of 25 normal control and 43 Gaucher patient plasma samples were measured for determining relative psychosine levels. Lyso GL-1 levels in Gaucher patient plasma (2.7–475.8 ng/mL) were all elevated compared to the normal controls (< 0.3 ng/mL). Lyso GL-1 results for each sample are shown in Table 1. No psychosine peak was observed in any of the normal controls or Gaucher patients.

Since the initial results showed no psychosine peak, ten Gaucher patient samples representing the determined Lyso GL-1 range were spiked with psychosine to prove that levels were negligible. Psychosine levels equivalent to 1, 3, and 5% of the determined Lyso GL-1 value were spiked into sample extracts. Those spiked with just a 1% relative level showed no obvious psychosine peak, indicating a relative level of 1% is either masked by the Lyso GL-1 peak or is below the detection limit of the method. For all ten of the samples though, the 3% spike was easily distinguishable at a retention time of approximately 6.2 min, and this was even more pronounced in the 5% spiked samples. Representative chromatograms showing all three spiking levels in a sample with moderately elevated Lyso GL-1 level are shown in Fig. 2. Since the relative psychosine level in human plasma is < 3% of Lyso GL-1, the interference from psychosine on the quantitation of Lyso GL-1 can be considered negligible, and future separation of the two isomers for accurate quantitation of Lyso GL-1 in human plasma is unnecessary.

In conclusion, we have developed a method to separate Lyso GL-1 and its isobaric structural isomer, psychosine. We have proven that the relative level of psychosine is low in human plasma samples first by testing of normal controls and Gaucher patients, and then by spiking of exogenous psychosine. Because relative pschosine levels are sufficiently low they can be neglected and there is no need to separate the two isomers in extracts of human plasma samples. Therefore, a more high-throughput LC/MS/MS method with short run time could be implemented in the clinical laboratory, as compared to the 16 min run time required for this HILIC separation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.03.009.

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