

Article

Long-Chain Base (LCB)-Targeted Lipidomics Study Uncovering the Presence of a Variety of LCBs in Mammalian Blood

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Abstract: Globotriaosylsphingosine (LysoGb₃) is a biomarker for Fabry disease (OMIM 301500) that contains long-chain bases (LCBs) as a building block. There have been several studies proposing that LysoGb₃ forms with distinct LCBs could be putative disease subtype-related biomarkers for this congenital disorder; however, there have been no detailed multiple reaction monitoring-based studies examining the LCB distribution in this lysosphingolipid. To achieve this, we established an assay procedure that aimed at elucidating the LCB-targeted lipidome using liquid chromatography–tandem mass spectrometry. Consistent with previous studies, we found d18:1 to be the major LCB species of the LysoGb₃ in pooled human plasma, while some atypical LCBs, such as d18:2, d18:0, t18:1, d16:1, and d17:1, were detected as minor fractions. When the same methodology was applied to fetal bovine serum (FBS) as a positive control, we identified additional unique LCB species, such as t18:0, d20:1, t19:1, and t21:1, in herbivore LysoGb₃. Furthermore, we found an elevation of sphingosine and LysoGb₃, which are *N*-deacylated forms of ceramide and Gb₃, respectively, in FBS, suggesting that ceramidase activity may be involved in this process. Thus, our LCB-targeted lipidomics data revealed that mammalian LCBs in glycosphingolipids have a greater variety of molecular species than previously expected.

Keywords: long-chain base; sphingolipid; mass spectrometry

1. Introduction

Fabry disease (OMIM 301500) is caused by a pathogenic deficiency in α -galactosidase A (GLA, EC 3.2.1.22) enzyme activity linked to an accumulation of globotriaosylceramide [1]. Recent studies have suggested that there are two disease subtypes for Fabry disease. Classic Fabry disease shows angiokeratoma, acroparesthesias, hypohidrosis, and corneal opacity in childhood [2]. In contrast, late-onset Fabry disease shows three disease subtypes with cardiac, renal, and neurological manifestations. The overall prevalence of Fabry disease may vary depending on the frequency of the late-onset form. An earlier study indicated that the prevalence of Fabry disease was reported as 1 in 50,000–100,000 [3]. Subsequently, a newborn screening program in Taiwan reported that approximately 0.03% (1:3000) of the population has the *GLA* IVS4 + 919 G > A (where IVS stands for intervening sequence) genetic mutation that leads to the cardiac phenotype after four decades of life [4]. Apart from this extreme example, Fabry disease has been considered as a more common lysosomal storage disorder (LSD). In New York State newborn screening, various *GLA* mutations with potential pathogenicity have been found [5]. For the treatment of Fabry disease, enzyme replacement therapy has been used for more than a decade with satisfactory results [6]. Furthermore, pharmacological chaperon therapy, which increases the half-life of the GLA enzyme with a missense mutation through

protein–small molecule interaction, has been commercialized with good outcomes [7]. In these cases, the efficacy of their therapeutic effect is usually assessed by a decrease in globotriaosylsphingosine (LysoGb₃) concentration as a biomarker. Usually, individuals with classic Fabry disease exhibit two orders of magnitude higher LysoGb₃ concentrations in the plasma than those with healthy control; however, patients with late-onset Fabry disease show one order of magnitude higher LysoGb₃ levels [8–10]. Generally, GLA enzyme activity and plasma LysoGb₃ concentration are inversely correlated, particularly in classic Fabry disease-affected male individuals [11].

A long-chain base (LCB) is an essential component for sphingolipids, which include ceramide, sphingomyelin, and other glycosphingolipids involving LysoGb₃. Its biosynthesis stems from the condensation of serine and palmitoyl-CoA catalyzed by serine:palmitoyl-CoA transferase (SPT, EC 2.3.1.50) [12]. Subsequently produced sphinganine (dihydrosphingosine) reacts with ceramide synthase followed by an insertion of a double bond between C4 and C5, leading to ceramide [13]. Thus, the major LCB species biosynthesized in mammals have a sphingosine backbone (d18:1, where d is the number of the hydroxyl group (either di or tri), X is the number of carbons, and 1 is the number of double bonds). During this process, it is also unknown which of several LCB species with different numbers of aliphatic carbons may be generated from the altered substrate specificity of the SPT enzyme, particularly in those of non-mammalian origin. Separately, some minor LCB species with two or more double bonds and/or hydroxyl groups have occasionally been detected in animals, possibly due to LCB-metabolizing enzymes found in both mammalian and non-mammalian species. Thus, based on this evidence, the molecular species of LCBs found in animals have a rather broad spectrum.

Accumulating evidence argues for the benefit of considering the LysoGb₃ species found in Fabry patients for diagnosis. Specifically, these biomarkers have been detected using mass spectrometry. Based on the combination of MS1 and MS2 used in multiple reaction monitoring (MRM)-based detection, some studies have identified LysoGb₃ with LCBs other than d18:1 [14–20]. In this study, (a) to examine the accumulation of a variety of LysoGb₃ species found in plasma, we first defined the particular LysoGb₃ species that we observed; (b) then, to understand better the molecular species of the LCBs that were detectable, we examined the relative quantities of the LysoGb₃ of different LCB species through an LCB-targeted lipidomics strategy; (c) next, to ensure whether the elevation of authentic LysoGb₃ correlated with the other discovered species of LysoGb₃ with different LCBs, we performed a validation study for LysoGb₃ quantification; and finally, (d) to compare the alterations in the sphingolipid species using a sphingolipid-targeting lipidomics technique, we compared the levels of Gb₃ and LysoGb₃ together with ceramide and sphingosine.

2. Materials and Methods

2.1. Materials

LysoGb₃ (cat #1520; C₃₆H₆₇NO₁₇, exact mass 785.440904, CAS#126550-86-5), *N*-glycinated globotriaosylsphingosine (LysoGb₃-Gly, cat #1530), and *N*-C18:0-globotriaosylceramide (C18-Gb₃, cat #1520) were purchased from Matreya (Pleasant Gap, PA, USA). *N*-C17:0-ceramide (C17-ceramide, cat# 22532) and sphingosine (d17:1, cat 10007902) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Sphingosine (S0874) and phytosphingosine (P1765) were purchased from Tokyo Chemical Industries (Tokyo, Japan). Acetonitrile was purchased from Fischer Scientific (Tokyo, Japan). Methanol and ammonium were purchased from Wako Pure Chemicals (Tokyo, Japan). Deionized water was obtained from a Milli-Q water system from Millipore (Milford, MA, USA). Formic acid and phosphoric acid were purchased from Kanto Chemical (Tokyo, Japan). Pooled human plasma was purchased from Kohjin Bio (Tokyo, Japan). FBS was purchased from Nichirei (Tokyo, Japan), Gibco (Tokyo, Japan), and Biowest (Riverside, MO, USA) (Supplementary Table S1). The other reagents used in this study were of the highest grade commercially available.

2.2. Ethical Approval

This study was approved by the Institutional Research Board of the National Center for Child Health and Development, Tokyo, Japan.

2.3. Preparation of the Standard Solution of LysoGb₃

To generate a calibration curve for LysoGb₃, 0, 0.1, 0.2, 0.5, 1, 10, 50, 100, and 200 nM of methanolic LysoGb₃ solutions were prepared by serial dilution. These solutions were stored at −20 °C prior to use.

2.4. Sample Preparation for the LysoGb₃ Assay

The LysoGb₃ in the human plasma was extracted in accordance with a previous study with a slight modification [11]. In brief, an aliquot of a plasma sample (0.1 mL) in a 1.5 mL microtube was mixed with 2% phosphoric acid (0.5 mL) and methanol (0.5 mL) containing 10 nM LysoGb₃-Gly as an internal standard. Each supernatant was transferred to a well in a 96-well plate for MCX solid-phase extraction (OASIS, Waters, Milford, MA, USA, 30 μm, 30 mg), which was equilibrated with methanol (1 mL) and 2% phosphoric acid in water (1 mL). After washing with 2% formic acid in water (1 mL) and 0.2% formic acid in methanol (1 mL), the LysoGb₃ and related species were eluted using 2% ammonium in methanol (0.6 mL). The solvent was evaporated using a nitrogen generator (model ANW3-009TMM-PCO, Nihonseiki Co., Ltd., Osaka, Japan) and a 96 well plate temperature controller (model NDG962, Nissin Rika, Tokyo, Japan), and the dried residue was reconstituted with 0.2% formic acid in acetonitrile/water (50/50, 0.1 mL).

2.5. Sample Preparation for the Gb₃ and Ceramide Assay

The lipids, including Gb₃ and ceramide, in the human plasma and FBS were extracted in accordance with a previous study with a slight modification [21]. In brief, an aliquot of a plasma sample (0.05 mL) in a 1.5-mL microtube was mixed with methanol containing 10 nM C17:0-ceramide (0.2 mL). After centrifugation at 12,000 rpm for 5 min at room temperature, the supernatant was transferred to a polypropylene sample vial (#9-425, GL Sciences, Tokyo, Japan).

2.6. Sample Preparation for the Characterization of the Minor LCBs in Pooled Healthy Human Plasma

Due to lower concentrations of the various LysoGb₃ species in the pooled human plasma, we used a larger amount (1 mL) as the starting material. In brief, an aliquot of a plasma sample (1 mL) in a 15 mL polypropylene tube was mixed with 2% phosphoric acid (5 mL) and methanol (5 mL) containing 10 nM LysoGb₃-Gly. After centrifugation at 3000 rpm for 5 min at room temperature (Allegra® X-12, Beckman, Indianapolis, IN, USA), the supernatant (approximately 10 mL) was collected. This supernatant was transferred to an in-house made MCX solid-phase extraction column (OASIS, Waters, 30 μm, 300 mg, 6 mL), which was equilibrated with methanol (6 mL) and 2% phosphoric acid in water (6 mL). After washing with 2% formic acid in water (6 mL) and 0.2% formic acid in methanol (6 mL), the LysoGb₃ was eluted using 2% ammonium in methanol (6 mL). The solvent was evaporated under a nitrogen stream as described above, and the dried residue was reconstituted with 0.2% formic acid in acetonitrile/water (50/50, 0.2 mL).

2.7. LC-MS/MS Assay for the LCBs of LysoGb₃

The extracted LysoGb₃ from the plasma was separated on an ACQUITY BEH C18 column (Waters, 2.1 × 50 mm, 1.7 μm, Milford, MA, USA) over 9 min at a flow rate of 0.5 mL/min with gradient elution. Chromatography was performed using binary mobile phases; mobile phase A contained 0.2% formic acid in water/acetonitrile (95/5) and mobile phase B contained 0.2% formic acid in acetonitrile. For the separation of the LysoGb₃, the percentage of mobile phase B was programmed as 0% for 0.0–1.0 min, 0–35% for 1.0–3.0 min, 35–50% for 3.0–5.5 min, 90% for 5.51–7.0 min, and 0% for 7.01–9.0 min. An aliquot (5 μL) of the extracted sample was injected onto the LC-MS/MS using

a partial needle-fill method. LysoGb₃ and its related species were detected on a Xevo TQ-S micro mass spectrometer (Waters) using ESI-positive mode and equipped with an ACQUITY H-class UPLC (Waters). The data were analyzed using the data analysis software MassLynx (V4.1, Waters). For MRM detection, a combination of MS1 for [M + H]⁺ and MS2 for [LCB – H₂O + H]⁺, [LCB – 2 × H₂O + H]⁺, [LCB – 3 × H₂O + H]⁺, and [LCB – 4 × H₂O + H]⁺ was used. Detailed analytical conditions, including instrumental settings and a list of MRM tables, are described in Supplementary Tables S2–S4.

2.8. LC–MS/MS Assay for the Gb₃, Ceramide, and Sphingosine

The extracted Gb₃ and ceramide from the plasma were separated on an ACQUITY BEH C18 column (Waters, 2.1 × 50 mm, 1.7 μm) over 9 min at a flow rate of 0.5 mL/min with gradient elution. Chromatography was performed using binary mobile phases; mobile phase A contained 0.2% formic acid in water/acetonitrile (95/5), and mobile phase B contained 0.2% formic acid in acetonitrile. For the separation of Gb₃, the percentage of mobile phase B was programmed as follows: 0% for 0.0–1.0 min, 0–35% for 1.0–3.0 min, 35–50% for 3.0–5.5 min, 90% for 5.51–7.0 min, and 0% for 7.01–9.0 min. An aliquot (5 μL) of the extracted sample was injected onto the LC–MS/MS using the partial needle-fill method. The concentrations of Gb₃ and ceramide and sphingosine were quantified using C18-Gb₃, C17-ceramide, and C17-sphingosine as an internal standard as described above. Detailed analytical conditions are described in Supplementary Tables S5 and S6.

2.9. Statistics

The data were expressed as mean ± standard deviation as indicated. The statistical significance of the differences in the mean values between the two groups was determined by Student's *t*-tests. A difference of *P* < 0.05 was considered as statistically significant. Because a limited number of clinical specimens were examined, a 95% confidence interval was calculated for the plasma LysoGb₃ concentrations of the healthy controls.

3. Results

LysoGb₃ contains an LCB moiety linked to a globotriaosyl moiety through a Glc(β1-1')Cer linkage (Figure 1A). LysoGb₃ has a C18-LCB with a double bond between C4 and C5 of *E* geometry (d18:1, Figure 1B). Reported LysoGb₃ species include LysoGb₃(–2) for d18:2 (4*E*, 14*E*), LysoGb₃(+2) for d18:0, LysoGb₃(–28) for d16:1, LysoGb₃(+18) for t18:0 or phytosphingosine, and LysoGb₃(+16) for t18:1 or dehydrophytosphingosine.

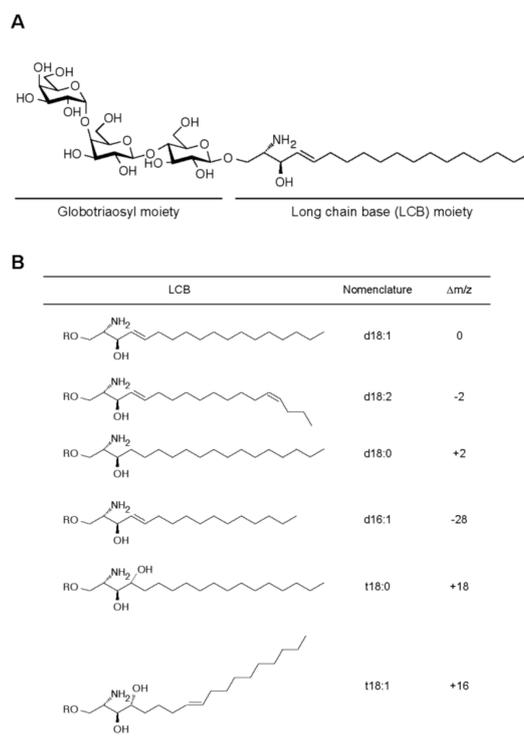


Figure 1. Long-chain bases (LCBs) of globotriaosylsphingosine (LysoGb₃). **(A)** Chemical structure of LysoGb₃. sphingosine, (2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol, is linked to Gb₃ through the Glc(β1-1')Cer moiety. **(B)** The chemical structures of LCBs occasionally found in sphingolipids. In most cases, LysoGb₃ contains d18:1 for LCB, whereas LysoGb₃(-2), LysoGb₃(+2), LysoGb₃(-28), LysoGb₃(+18), and LysoGb₃(+16) contain d18:2 (4*E*, 14*E*), d18:0, d16:1, t18:0 or phytosphingosine, and t18:1 or dehydrophytosphingosine, respectively.

To explore the biochemical basis for LysoGb₃ accumulation in human plasma further, we hypothesized whether any animal-derived material with a similar LCB distribution might be available as a positive control. Based on the preliminary screening results, we found that FBS contains a detectable amount of LysoGb₃ similar to that of human plasma. Thus, we first examined the distribution of the LCB species in the LysoGb₃ in FBS using a combination of MS1 and MS2 for MRM detection (Supplementary Table S3). In this examination, the profile of an authentic LysoGb₃ that contains d18:1, where d, 18, and 1 stand for the number of the double bond (i.e., in this case, dihydroxy), carbon atom (C18), and double bond, respectively, showed the largest peak area derived from [LCB - H₂O + H]⁺ at 30V (Figure 2A top and Figure 2B top, green, closed circle). Consistently, a putative LysoGb₃ from the FBS exhibited nearly the same profile (Figure 2B top, blue). The other peaks for the di-hydroxy (dX:Y, X = 16 or 18, Y = 0–2) exhibited a similar profile (data not shown). In sharp contrast, a putative peak with t18:1 showed a substantial peak derived from [LCB - 3H₂O + H]⁺ at 40–50V (Figure 2A bottom and Figure 2B bottom, open circle, blue), which was undetectable in di-hydroxylated LCB species such as d18:1 (Figure 2B top, open circle, blue). Under this assay condition, small amounts of d16:1 (5% of d18:1), d17:1 (5%), d20:1 (5%), t19:1 (20–40% of t18:1), and t21:1 (5–10%) were detected in the FBS (Figure 2C). Consistent with cases for FBS, we similarly found that d18:1 was the predominant LCB within the series of dX:1 in the pooled plasma (Figure 2C dX:1, white bar). Under this assay condition, a small amount of d16:1 (10% of d18:1) and d17:1 (10%) were detected in the pooled human plasma (Figure 2C white). Interestingly, although t18:0 accumulated remarkably in the FBS, this species was undetectable in the pooled human plasma (Figure 2D and Supplementary Figure S1). Essentially, all of the LCBs found in the LysoGb₃ from humans were also identified in the FBS (Figure 2E). Furthermore, some putative microbial-derived LCBs, such as t19:1 and t21:1, were also found in the FBS. Finally, we also examined

the concentrations of LysoGb₃ with atypical LCBs in Fabry patients (Supplementary Figure S2). In fact, we were able to observe an elevation of the relative concentration of LysoGb₃(−28), a putative LysoGb₃ with d16:1 LCB, in Fabry males.

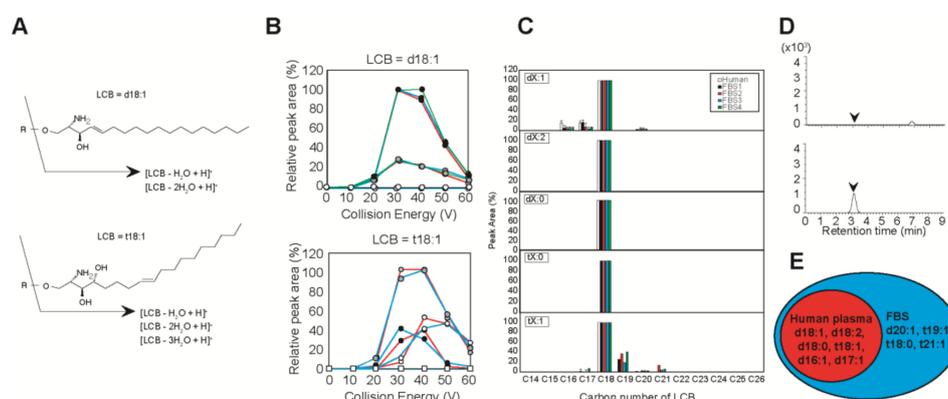


Figure 2. LCB-targeted lipidomics analysis of LysoGb₃. (A) A representative fragmentation pattern of LysoGb₃ with d18:1 (top) and t18:1 (bottom) as the LCB. (B) Relative peak area of LysoGb₃ (LCB = d18:1, top) and LysoGb₃(+16) (LCB = t18:1, bottom) across collision energy. MS2 for [LCB − H₂O + H]⁺ (closed circle), [LCB − 2H₂O + H]⁺ (gray circle), [LCB − 3H₂O + H]⁺ (open circle), and [LCB − 4H₂O + H]⁺ (open square) are shown. (C) Relative peak area of LysoGb₃ with dX:1, dX:2, dX:0, tX:0, and tX:1. X, number of carbons in LCB; d/t, number of the hydroxyl group (either di or tri); 0–2, number of double bonds in LCB. (D) MRM-based chromatograms for LysoGb₃ with t18:0 as the LCB in human plasma (top) and in FBS (bottom). (E) Venn diagram of molecular species detected in pooled human plasma (red) and FBS (blue).

Then, to quantify the plasma LysoGb₃, we ensured that the linear range for the LysoGb₃ measurement was 0.2–100 nM under this experimental condition, with LysoGb₃-Gly used as an internal standard (Figure 3A). Based on the validation study, we found that the limit of detection and quantification was 0.05 and 0.15 nM, respectively. The intraday coefficient of variance (CV) values of quality control (QC) Low (2.5 nM), QC Middle (17.5 nM), and QC High (79.3 nM) of LysoGb₃ in pooled human plasma were 8.1%, 1.3%, and 3.0%, respectively (*n* = 5) (Table 1). The interday CV values of QC Low, QC Middle, and QC High of LysoGb₃ were 9.1%, 8.4%, and 12.4%, respectively (*n* = 4). Using pooled human as the matrix, we found the recovery of 92.0% for QC Low, 94.9% for QC Middle, and 92.7% for QC High samples. We were able to obtain 49.9 nM of LysoGb₃ in methanol and 49.7 nM in pooled human plasma when 50 nM LysoGb₃ was added to these matrices, demonstrating that the matrix effect of human pooled plasma was calculated as 0.6% when an MCX-mediated sample preparation has been performed as described in the Materials and Methods section. When we examined the LysoGb₃ concentrations in the plasma of the Fabry patients, as reported previously, Fabry males had elevated LysoGb₃, whereas healthy males and females had low plasma LysoGb₃ (Figure 3B). Overall, the concentrations of LysoGb₃ in Fabry males and females (mean ± SD) were 49.3 ± 58.4 nM (*n* = 4; median = 25.9, min = 9.6, max = 135.6) and 7.5 ± 0.1 nM (*n* = 2; median = 7.4, min = 7.3, max = 7.5), respectively, whereas in healthy males and females, they were 0.6 ± 0.1 nM (*n* = 3; median = 0.5, min = 0.5, max = 0.7, 95% confidence interval = 0.41–0.72) and 0.5 ± 0.1 (*n* = 5; median = 0.5, min = 0.4, max = 0.6, 95% confidence interval = 0.38–0.54) nM, respectively (Figure 3C). LysoGb₃ was stable in human plasma at 37 °C for 24 h (Supplementary Figure S3).

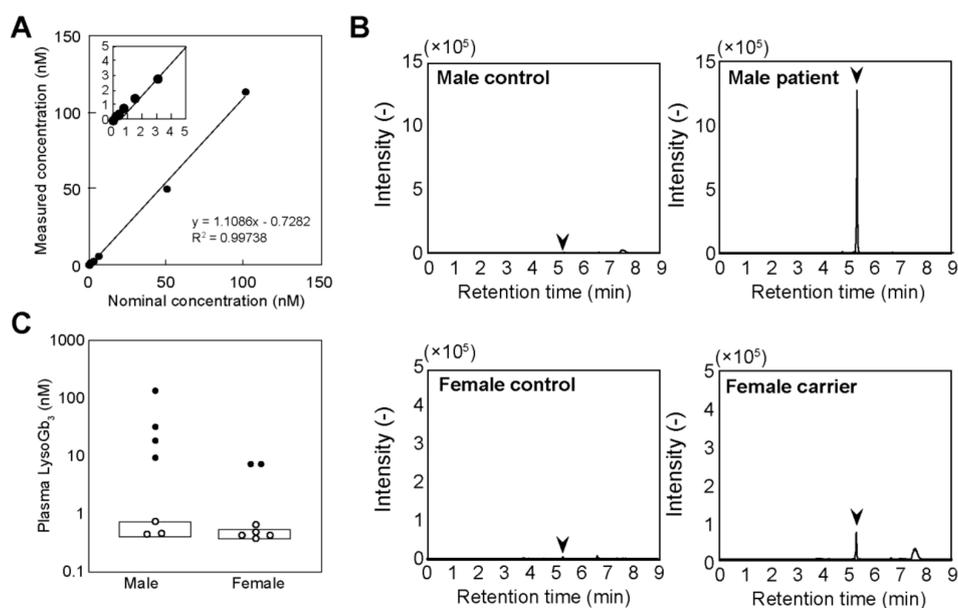


Figure 3. Accumulation of LysoGb₃ in healthy controls and Fabry patients. (A) Calibration curve for LysoGb₃. (B) Chromatograms for LysoGb₃ of male controls (upper left), male patients (upper right), female controls (lower left), and female carriers (lower right). (C) The concentration of LysoGb₃ in affected males ($n = 4$, closed circle), control males ($n = 3$, open circle), carrier females ($n = 2$, closed circle), and control females ($n = 5$, open circle). Box indicates 95% confidence interval.

Table 1. CV values for LysoGb₃ assay.

QC Samples	Low	Middle	High
Concentration (mean \pm SD, nM)	2.5 \pm 0.2	17.5 \pm 0.2	79.3 \pm 2.4
Intraday CV (%)	8.1	1.3	3.0
Interday CV (%)	9.1	8.4	12.4
Mean recovery (%)	92.0	94.9	92.7

When the concentration of LysoGb₃ in the FBS was examined, we, by chance, noticed a marked and consistent elevation of LysoGb₃ in the FBS (Figure 4A top). To explore further why LysoGb₃ accumulated at such a higher concentration in the FBS than in the human plasma, we examined the concentration of Gb₃, a putative parent compound of LysoGb₃. In contrast to initial expectations, the levels of Gb₃, an *N*-acylated form of LysoGb₃, in the FBS were somewhat lower than in the pooled human plasma (Figure 4A bottom). When we measured the LysoGb₃ levels in four batches of FBS, all of them exhibited an elevated LysoGb₃ concentration (mean \pm SD) at 37.0 ± 16.5 nM ($n = 4$; median = 32.2, min = 23.0, max = 60.8) (Figure 4B top right). Thus, we decided to quantify the associated sphingolipids, such as sphinganine (dihydroxysphingosine), ceramide, and sphingosine together with Gb₃ and LysoGb₃. As with LysoGb₃, we found a markedly higher accumulation of sphingosine, an *N*-deacylated form of ceramide, in the FBS as compared to the pooled human plasma (Figure 4B center).

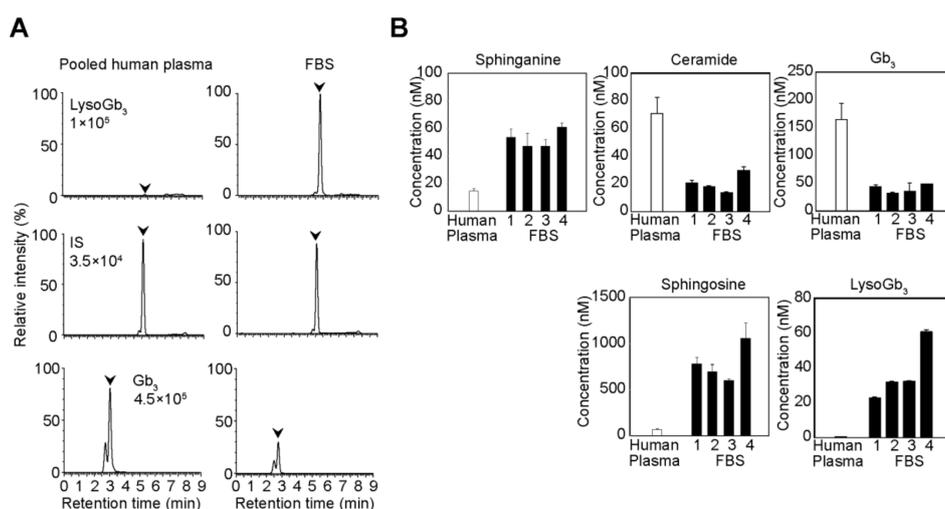


Figure 4. Accumulation of LysoGb₃ and its associating sphingolipids. **(A)** Chromatograms for LysoGb₃ (top), internal standard (IS) (LysoGb₃-Gly, center), and Gb₃ (bottom) are shown. An arrowhead indicates the position of the migration for LysoGb₃, IS, and Gb₃. **(B)** Concentrations of sphinganine, ceramide, Gb₃, sphingosine, and LysoGb₃. The concentration of ceramide was calculated as the sum of C14–C26 ceramide species, as described in Supplementary Table S5. Data are presented as mean ± SD (*n* = 3).

4. Discussion

Plasma LysoGb₃ is an established biomarker for Fabry disease [11]. Although unaffected individuals show less than 1 nM LysoGb₃ in plasma, Fabry males and females show 10–200 nM and 1–10 nM LysoGb₃ in plasma, respectively [3,22]. Such an elevation of LysoGb₃ in individuals with Fabry disease decreases in response to enzyme replacement therapy, demonstrating clearly that the concentration of LysoGb₃ correlates with the effectiveness of the therapy. In this study, we explored the putative mechanism of LysoGb₃ biosynthesis in animals. Consistent with previous studies, we were able to detect nearly the same levels of LysoGb₃ (d18:1, in this case) in the Fabry patients as previously reported (Figure 3). Similarly, the elevation of other LysoGb₃ species was detectable in our LCB-targeted lipidomics assay conditions for LysoGb₃ (Figure 2 and Supplementary Figure S2). Finally, because the levels of sphingosine, a unique ceramidase-derived *N*-deacylated form of ceramide, were also elevated in FBS, we thus concluded that ceramidase activity could be linked to the accumulation of lysosphingolipids, such as LysoGb₃ and sphingosine, in human plasma and FBS [23]. The reason why FBS contains such high levels of LysoGb₃ remains unclear. However, several biomarkers for LSDs are known to show high levels in neonates as compared to adults. For example, in mucopolysaccharidosis, glycosaminoglycans are known to be more highly elevated in neonates than in adults [24]. Some free oligosaccharides, such as glucose tetrasaccharide, are also known to be elevated in neonates with Pompe disease [25].

Previous studies have suggested that there is a substantial pool of ceramide that manages both endogenously produced ceramide and exogenously derived ceramide mainly from dietary intake. The biosynthesis of ceramide is linked to the generation of various LCBs due to the substrate specificity of SPT. In animals, the SPT enzyme reaction involves a condensation of serine and palmitoyl-CoA [12]. In fact, several biochemical studies have established that mammalian SPT favors palmitoyl-CoA as a substrate, while bacterial SPT exhibits more broad specificity [13,26]. Furthermore, LCBs in plants and fungi have a broad spectrum with regard to the number of double bonds, their position, the geometry of the double bond (i.e., *Z* or *E*), and the number of the hydroxyl group. More uniquely, fungal sphingolipids contain C9-methylated LCBs, a population of odd-numbered LCBs. In fact, this is consistent with our observation of a small amount of d17:1 in pooled human plasma and relatively large amounts of d17:1, t19:1, and t21:1 in FBS (Figure 2C,E).

The analytical procedure for LysoGb₃ in clinical laboratory medicine has been improving in the last decade. Historically, the first assay uses HPLC with fluorometric detection using OPA as the derivatization reagent [11]. In contrast, currently, almost all LysoGb₃ assays, including this study, use LC–MS/MS-based technique, due to an improved sensitivity [15]. The variety of assays arises from the choice of an internal standard and an extraction procedure. Most of the clinical laboratories, including our, prefer to use a commercially available internal standard [15]. In contrast, some research groups capable of synthesizing ¹³C₅- and ¹³C, D₃-labeled LysoGb₃ are allowed to use such labeled material for an internal standard [27,28]. The other variation of assay relates to the extraction procedure of LysoGb₃ from blood plasma. Due to low (i.e., nM) concentration of LysoGb₃ in control human plasma, most of the studies choose the use of solid phase extraction rather than simple liquid–liquid extraction. This does not appear to be a prerequisite as far as the matrix effect is eliminated before quantification of the analyte. For example, there is a successful study using a combination of a semi-micro-sized analytical column and a smaller amount of sample [29]. These differences in assay procedure, however, seem to be a rather minor issue for proper measurement of LysoGb₃ in clinical chemistry, when assay validation has been performed at each laboratory. In fact, the concentration of LysoGb₃ under pathogenic conditions appears unaltered in all these studies [11,15,27–29].

In conclusion, we examined the molecular species of LysoGb₃ with various LCBs in pooled human plasma and FBS, and we further performed a validation study of the LysoGb₃ quantification in human plasma. The pooled human plasma mainly contained d18:1 as the major LCB, while d18:2, d18:0, t18:1, d16:1, and d17:1 were detected as minor LCBs. All four examined batches of FBS contained these species together with t18:0, d20:1, t19:1, and t21:1. Our data also demonstrated an accumulation of sphingosine, as similarly observed in LysoGb₃, suggesting that ceramidase activity could play an important role in the fetal bovine [23]. Apart from a biosynthesis mechanism, we do not know the physiological roles of elevated LysoGb₃ in FBS. Therefore, further studies are needed to address the mechanism of biosynthesis in these minor species along with their physiological relevance in animals.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2297-8739/7/4/57/s1>, Figure S1: Chromatograms for LysoGb₃(+18) (LCB = t18:0) in FBS of separate lot.; Figure S2: Accumulation of LysoGb₃ species in human plasma.; Figure S3: Stability of LysoGb₃ in pooled human plasma under various storage conditions at 37 °C. Table S1: Source and origin of country for pooled human plasma and FBS used in this study; Table S2: MS1, MS2, cone energy and collision energy for the quantification of LysoGb₃ and its analogues in human plasma using LC-MS/MS; Table S3: MRM table for LysoGb₃ species with C14-C26 LCB; Table S4: Instrument parameters for the quantification of LysoGb₃ and other compounds in this study using reversed-phase chromatography; Table S5: MRM table for ceramide and Gb₃; Table S6: MRM table for sphinganine and sphingosine.

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