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Simultaneous quantitation of sphingoid bases by UPLC-ESI-MS/MS with identical ^{13}C-encoded internal standards

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ABSTRACT

Free sphingoid bases (lysosphingolipids) of primary storage sphingolipids are increased in tissues and plasma of several sphingolipidoses. As shown earlier by us, sphingoid bases can be accurately quantified using UPLC-ESI-MS/MS, particularly in combination with identical ^{13}C-encoded internal standards. The feasibility of simultaneous quantitation of sphingoid bases in plasma specimens spiked with a mixture of such standards is here described. The sensitivity and linearity of detection is excellent for all examined sphingoid bases (sphingosine, sphinganine, hexosyl-sphingosine (glucosylsphingosine), hexosyl$_2$-sphingosine (lactosylsphingosine), hexosyl$_3$-sphingosine (globotriaosylsphingosine), phosphorylcholine-sphingosine) in the relevant concentration range and the measurements show very acceptable intra- and inter-assay variation (<10% average). Plasma samples of a series of male and female Gaucher Disease and Fabry Disease patients were analyzed with the multiplex assay. The obtained data compare well to those earlier determined for plasma globotriaosylsphingosine and glucosylsphingosine in GD and FD patients. The same approach can be also applied to measure sphingolipids in the same sample. Following extraction of sphingolipids from the same sample these can be converted to sphingoid bases by microwave exposure and subsequently quantified using ^{13}C-encoded internal standards.

1. Introduction

Previously we reported on marked increases of globotriaosylsphingosine (lysoGb3) in Fabry disease (FD) patients and glucosylsphingosine (GlcSph) in Gaucher disease (GD) patients [1–4]. Likewise, galactosylsphingosine (GalSph) is reported to be increased in Krabbe disease (KD), lysosulfatide in Metachromatic Leukodystrophy (MLD), phosphorylcholine-sphingosine (lysoSM) in Niemann Pick type A/B disease as well as lysoSM and GlcSph in Niemann Pick type C disease (NPC) [5–9]. Quantitation of elevated sphingoid bases has great diagnostic value for all these disorders. In place are validated measurements of lysoGb3 and hexosyl-sphingosine (HexSph, is GlcSph and/or GalSph) by UPLC-ESI-MS/MS with identical $^{13}C_5$-encoded standards. The use of identical internal standards in these measurements offers compensation for losses during extraction, ionization efficiency and mass spectrometric performance.

No correction for the sample matrix, such as ion suppression and in some cases ion enhancement, is required. $^{13}C_5$-encoded lysoGb3, GlcSph, sphingosine (Spho) and sphinganine (Spha) have been synthesized by us [2,10]. We now developed and validated a convenient procedure for simultaneous UPLC-ESI-MS/MS quantitation of plasma sphinganine, sphingosine, GlcSph, LacSph (lactosylsphingosine), lysoGb3 and lysoSM using isotope-encoded standards and C17-lysoSM. The sample preparation requires just 50 μL of plasma and implies lipid extraction according modified Bligh and Dyer with acidic buffer. Excellent sensitivity, high accuracy and good reproducibility are obtained with the reported procedure. We further demonstrate that from the same sample sphingolipids (ceramide, hexosyl-ceramide, hexosyl$_2$-ceramide, and hexosyl$_3$-ceramide) can be quantified with the same procedure. For this, sphingolipids are extracted, deacylated by microwave exposure and the obtained sphingoid bases are quantified with corresponding standards.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS–grade methanol, 2-propanol, water, formic acid and HPLC-grade chloroform was purchased from Biosolve (Valkenswaard, The Netherlands). LC-MS grade ammonium formate and sodium hydroxide

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were from Sigma-Aldrich Chemie GmbH (St Louis, USA), butanol and hydrochloric acid were from Merck Millipore (Billerica, USA). C18-lysoSM and C18-lysoGb3 were from Avanti (Albaster, USA). $^{13}C_5$-sphinganine, $^{13}C_5$-sphingosine, $^{13}C_5$-GlcSph and $^{13}C_5$-lysoGb3 were synthesized at the Department of Bio-organic Synthesis of the Leiden Institute of Chemistry, Leiden University [10]. The label was incorporated in C5, 6, 7, 8, and 9 of the sphingosine backbone (see Fig. 1). C18-sphingosine, C18-sphinganine, C18-GlcSph, C18-lactosylsphingosine and C17-lysoSM were obtained from Avanti. C17-dihydroceramide (C17-dhCer) was synthesized in house (see Scheme 1); C17-sphinganine, C18-ceramide (Cer), C18-glucosylceramide (GlcCer) and Lactosylceramide (LacCer) were purchased from Avanti and C18-globotriaosylceramide (Gb3) from Matreya (State College, USA).

2.2. Synthesis of C17-dihydroceramide

The synthesis of C17-dhCer starts with acetylation of commercial C17-sphinganine. C17-sphinganine (5 mg, 0.016 mmol, 1 eq) was dissolved in tetrahydrofuran (THF) (0.2 mL) and sat. aq. NaOAc (0.2 mL), was added. Palmitoyl chloride (6.6 μL, 0.022 mmol, 1.3 eq) was added and the reaction mixture was stirred vigorously at room temperature for 3 h. The mixture was diluted with THF (5 mL) and washed with water (5 mL). The water layer was extracted with THF (3 × 5 mL) and the combined organics were dried ($MgSO_4$), filtered and concentrated *in vacuo*. The C17-dhCer was purified by silica column chromatography (chloroform to 5% MeOH in chloroform) giving a white solid (6 mg, 0.011 mmol, 72%). R_f = 0.5 (5% MeOH in chloroform), ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.36 (d, 1H, J = 7.6 Hz), 4.01 (d, 1H, J = 11.3 Hz), 3.83 (m, 1H), 3.80–3.72 (m, 2H), 2.90–2.50 (bs, 2H), 2.23 (t, 2H, J = 7.4Hz), 1.68–1.59 (m, 4H), 1.59–1.45 (m, 2H), 1.38–1.19 (m, 44H), 0.88 (t, 6H, J = 7.2 Hz); ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.6, 74.4, 62.7, 54.2, 37.1, 34.7, 32.10, 29.86 × 4, 29.84 × 3, 29.82 × 3, 29.79 × 2, 29.75 × 3, 29.72, 29.71, 29.67, 29.66, 29.52, 29.51, 29.48, 22.84, 14.2.

2.3. Samples

All plasma samples were prepared from EDTA-anticoagulated blood by centrifugation and stored at −20 °C until analysis. Approval had been obtained from the institutional ethics committee and informed consent according to the Declaration of Helsinki.

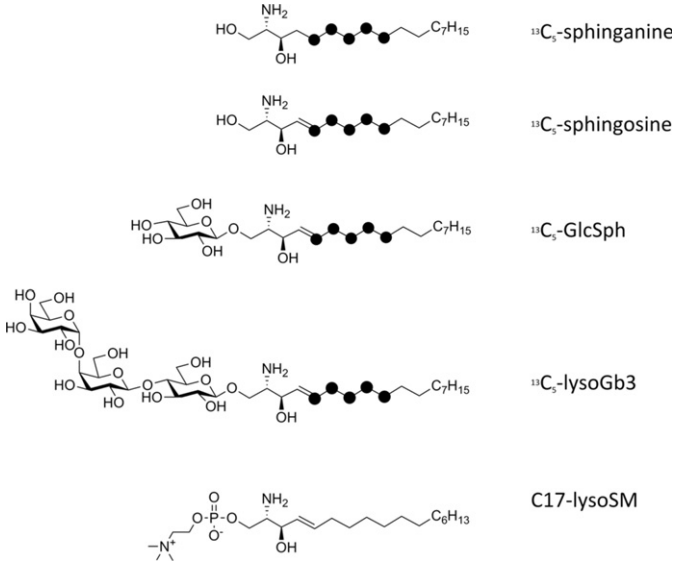


Fig. 1. Structures of internal standards.

2.4. Internal standard mixture

An internal standard mixture (ISM) of isotope-labeled and C17-lysoSM standards was made in methanol containing an equal concentration of each internal standard with the concentration of 0.1 pmol/μL (0.1 μM). To 50 μL of plasma sample 25 μL of ISM was added as spike. This is 50 nmol/L plasma.

2.5. Assay validation

Calibration curves for C18-sphingosine, C18-sphinganine, C18-lysoGb3, C18-LacSph, C18-GlcSph and C18-lysoSM were constructed in plasma of healthy controls: 0-1-2-5-10-20-50-100-200-500-1000 nmol/L. As internal standard ISM (25 μL of 0.1 pmol/μL) was used. Plasma was spiked with equal amounts of C18 sphingoid bases. Added to plasma were equimolar amounts of C18-sphingosine, C18-sphinganine, C18-lysoGb3, C18-LacSph, C18-GlcSph and C18-lysoSM.

2.6. Intra and inter-assay variation

For the determination of intra-assay variation control plasma was spiked with three different concentrations of all C18 bases (A: added 20 pmol/mL, B: added 50 pmol/mL and C: added 100 pmol/mL) and extracted in 6-fold, one without internal standards and five with internal standards. The same control plasma specimen without any spike was also extracted in 6-fold one without internal standards and five with internal standards. Extraction and measurement of samples was repeated on a subsequent day to determine inter-assay variation.

2.7. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD is defined as signal-to-noise ratio of 3 (peak to peak). The LOQ is defined as signal-to-noise ratio of 10 (peak to peak).

2.8. Stability assessment

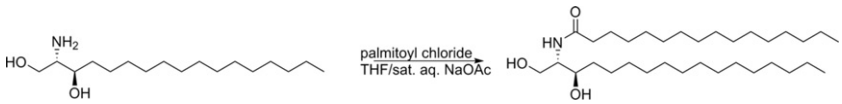
Samples were tested under three different conditions: freezer (−20 °C), fridge (4 °C) and room temperature. After preparation of plasma samples, the samples were stored in different conditions for one week. The samples were extracted and measured in the same day. For each condition, three differently spiked plasma controls (A, B and C) were extracted in 6-fold, one without internal standards and five with internal standards. Same control plasma without any spike was also extracted in 6-fold, one without internal standards and five with internal standards.

2.9. Studied plasma specimens of healthy volunteers (CTRLs), GD and FD patients

Plasma lyso-sphingolipids were measured for male (n = 21) and female CTRLs (n = 21), GD (n = 10 each gender) and FD patients (n = 10 each gender). Of the female CTRLs 10 were using oral contraceptives, and 11 were not.

2.10. Lipid extraction

Sphingoid bases were extracted from plasma by a modified Bligh and Dyer extraction [11] using acidic buffer (100 mM ammonium formate buffer pH 3.1). Plasma (50 μL) was pipetted in a 1.5 mL Eppendorf tube with a screw cap. Next 25 μL of ISM was added. To this, 350 μL methanol (or 325 μL methanol + 25 μL ISM) and 175 μL chloroform were added and stirred. The sample was next incubated for 10 min at room temperature and stirred occasionally, then centrifuged for 10 min at 15,700 × *g* to precipitate protein. The supernatant was transferred to a clean tube, 175 μL chloroform and 265 μL buffer were added. After stirring the sample for 1 min, the tube was centrifuged for 5 min at



Scheme 1. Synthesis of C17-dihydroceramide.

15,700 × *g*. The upper phase was transferred to a clean tube (extract A). To the lower phase (chloroform phase) 350 μL methanol and 315 μL buffer were added and stirred for 1 min. The sample was then centrifuged for 5 min at 15,700 × *g* and the upper phase was removed and pooled with extract A. This was dried at 45 °C in an Eppendorf Concentrator Plus. The residue was further extracted with 500 μL butanol and 500 μL H_2O stirred for 1 min and centrifuged for 5 min at 15,700 × *g*. The upper phase (butanol phase) was transferred to a clean tube and taken to dryness in Eppendorf Concentrator Plus at 45 °C. The residue was dissolved in 100 μL methanol, stirred and sonicated in a bath for 30 s and centrifuged for 5 min at 15,700 × *g*. Finally, 10 μL of the solution was applied to the UPLC-MS.

2.11. LC-MS/MS

Measurements were performed by reverse-phase liquid chromatography using a Waters UPLC-Xevo-TQS micro and a BEH C18 column, 2.1 × 50 mm with 1.7 μm particle size (Waters, USA) using the following eluents: eluent A was 1 mM ammonium formate and 0.5% formic acid in water. Eluent B was 1 mM ammonium formate and 0.5% formic acid in methanol. A mobile-phase gradient was used during a 6.50 min run: 0.00 min 0% B; 2.50 min 100% B; 5.05 min 100% B; 6.00 min 0% B; 6.50 min 0% B. The flow rate was 0.25 mL/min. The eluent was diverted to waste between 0.00 and 2.00 min to keep the source free of contaminants; data were collected between 2.00 and 5.05 min, and after 5.05 min the eluent was again diverted to waste. Mass spectrometry detection in positive mode using an electrospray ionization (ESI) source was carried out with a Xevo TQS micro instrument. Data were analyzed with Masslynx 4.1 Software (Waters Corporation; Milford MA). All LC and MS/MS parameters are presented in Table 1 and Table 2.

In Fig. 2 chromatograms of various sphingoid bases and the $^{13}C_5$ isotope analogues are shown. Of note, the chromatography used is not able to separate GlcSph and GalSph. Therefore these (HexSph) lipids in biological samples will not be separately quantified. Likewise, the chromatography not necessarily separates Glu-Gal-Sph (LacSph) from Gal-Gal-Sph (Gal_2-Sph). Again, these (Hex_2Sph) lipids are *a priori* not separately quantified. Although lysoSM and GlcSph are not separated by the chromatography, their separate quantitation is feasible by their distinct parent and daughter *m/z* values (see Table 2).

Table 1
LC parameters.

UPLC				
Column	Acquity BEH C18 column, 2.1 × 50 mm with 1.7 μm			
Column temperature	23 °C			
Wash	MeOH:H_2O (50:50)			
Mobile phase A	H_2O, 1 mM ammonium formate, 0.5% Formic acid			
Mobile phase B	MeOH, 1 mM ammonium formate, 0.5% Formic acid			
Gradient				
Time (min)	Flow (mL/min)	% A	% B	Curve
Initial	0.25	100	0	Initial
2.5	0.25	0	100	6
5.05	0.25	0	100	6
6.00	0.25	100	0	6
6.50	0.25	100	0	6

2.12. Determination of neutral (glyco)sphingolipids (NGSL) in the same sample

Plasma was subjected to the modified Bligh and Dyer extraction exactly as described above. In some experiments, 50 μL of C17-dhCer (20 μM) was added to the sample to establish efficiency of deacylation, generate calibration curves and for comparison with the use of ^{13}C-encoded lyso-lipids as internal standards. The lower phase of the extraction procedure was transferred to a Pyrex tube, dried at 45 °C under the gentle stream of nitrogen gas. The residue was dissolved in 500 μL of methanolic NaOH (0.1 M) by stirring. After putting the screw cap tightly on the tube, the sample was placed in the microwave for 1 h for deacylation [17]. The sample was cooled down and stirred well. Next, 200 μL of deacylated sample was transferred to a 1.5 mL Eppendorf tube with a screw cap and 20 μL of methanolic HCl (1 M) was added to neutralize the sample. 80 μL of $^{13}C_5$ lyso-GSL ISM (5 pmol/μL of each standard) was added to the sample. The sample was dried in an Eppendorf concentrator Plus at 45 °C. Next the samples were processed exactly as described above by butanol extraction and 10 μL of the solution was applied to the UPLC-MS. All plasma samples were extracted in duplicate. Studied was pooled plasma of normal males (two sets of 4 individuals), pooled plasma of 4 healthy females using a contraceptive and that of 4 females not using a contraceptive. Furthermore, plasma of 2 male GD patients, 2 female GD patients, 2 male FD patients and 2 female FD patients were analyzed (see supplemental information Fig. 1).

Calibration curves of NGSL in normal control plasma were constructed as follows. Normal plasma was spiked with a mixture of C18-Cer, C18-GlcCer, C18-LacCer, C18-Gb3 (1 pmol/μL). The concentrations of each NGSL were 0-0.2-0.4-1-2-5-10-20 nmol/mL plasma. NGSL and C17-dhCer were added before acidic extraction. Deacylation of C17-dhCer to C17-Spha was monitored by detection of *m/z* 288.3 > 270.3

Table 2
MS/MS parameters.

MS/MS parameters					
Mass spectrometer	Xevo-TQS-Micro (waters)				
Ionization mode	ESI^+				
Capillary voltage	3.50 kV				
Source temperature	150 °C				
Desolvation temperature	450 °C				
Cone gas flow	50 L/h				
Desolvation gas flow	950 L/h				
Dwell time	0.05 s				
MS Inter-Scan	0.005 s				
Inter-channel delay	0.005 s				
Compound	Parent (*m/z*)	Daughter (*m/z*)	Cone voltage (V)	Collision energy (V)	Retention time (min)
Sphingosine	300.3	282.3	20	15	3.39
Sphinganine	302.3	284.3	20	15	3.42
$^{13}C_5$-sphingosine (IS)	305.3	287.3	20	15	3.37
$^{13}C_5$-sphinganine (IS)	307.3	289.3	20	15	3.42
C17-lysoSM (IS)	451.4	184.4	20	25	3.24
lysoSM	465.4	184.4	20	25	3.31
GlcSph	462.3	282.3	30	20	3.33
$^{13}C_5$-GlcSph (IS)	467.3	287.3	30	20	3.33
LacSph	624.4	282.3	43	30	3.28
lysoGb3	786.4	282.3	55	40	3.29
$^{13}C_5$-lysoGb3 (IS)	791.4	287.4	55	40	3.27

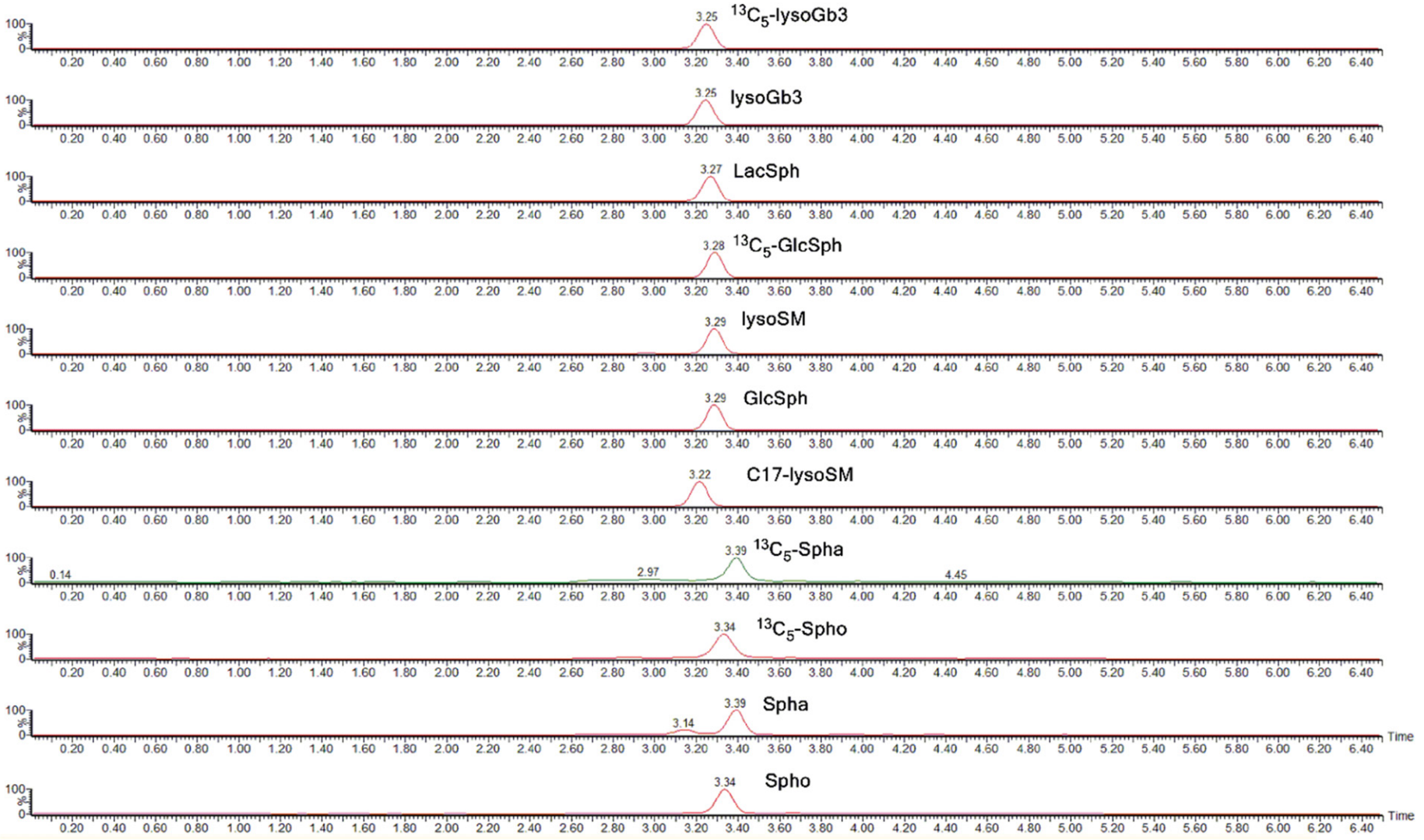


Fig. 2. Chromatogram of lyso(glyco)sphingolipids and their internal standards.

(cone (v) 20, Collision(v) 15). The mixture of $^{13}C_5$ lyso-GSL ISM was added after the microwave step.

2.13. Statistical analysis

Values in figures are presented as mean ± S.D. Data were analyzed by unpaired Student's *t*-test using GraphPad Prism. *P* values < 0.05 were considered significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

3. Results

3.1. Assay validation

3.1.1. Calibration curves

A linear response was obtained over the entire concentration range (M&M), R^2 for each sphingoid bases exceeded 0.995 (data not shown).

3.1.2. Intra- and inter-assay validation

Different concentrations of non-labeled C18 bases (M&M) were added to control plasma from a healthy subject. Samples were six times separately extracted to test the intra-assay variation. To test inter-assay variation the same plasma specimens were extracted and measured six times on subsequent days. Fig. 3 shows the inter- and intra-assay variations for the individual sphingoid bases. The intra- and inter-assay variation was on average 4.58% and 9.83%, respectively (ranges 1.34–14.54% and 1.28–19.28%, respectively), (see also Table 1 in supplemental information). The LOD for GlcSph, LacSph, lysoGb3 and lysoSM was 0.05 nmol/L, and for sphingosine and sphinganine 0.08 nmol/L. The LOQ for GlcSph, LacSph, lysoGb3 and lysoSM was 0.15 nmol/L, and for sphingosine and sphinganine 0.45 nmol/L.

3.1.3. Effect of different storage conditions and stability of sphingoid bases

Plasma samples, identical to the ones described above, were stored for 1 wk. at −20 °C, 4 °C and RT. As shown in Fig. 4, no significant differences in levels of various bases when stored at different conditions were noted. The levels of bases were similar to those in the directly used same plasma sample (Fig. 3 and Table 2 in supplemental information), pointing to acceptable stability. Excellent stability of the sphingoid bases lysoGb3 and GlcSph was earlier observed by us and others [3,12].

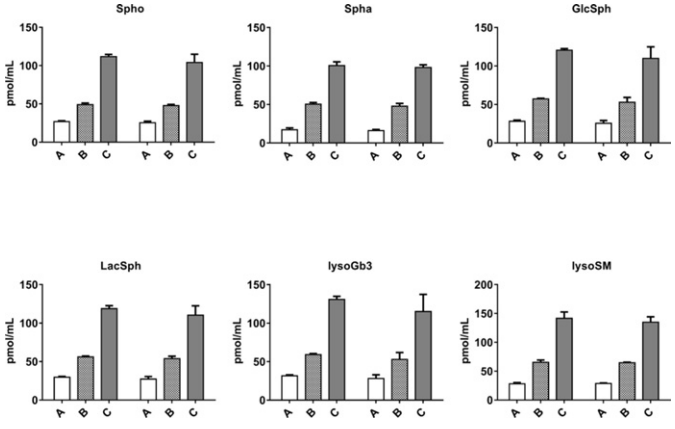


Fig. 3. Intra-assay and inter-assay validation. Left bars represent intra-assay and right bars represent inter-assay validation. A, B and C are different concentrations of spike of equal amounts of lyso-glycosphingolipids in control plasma, 20, 50 and 100 pmol/mL respectively.

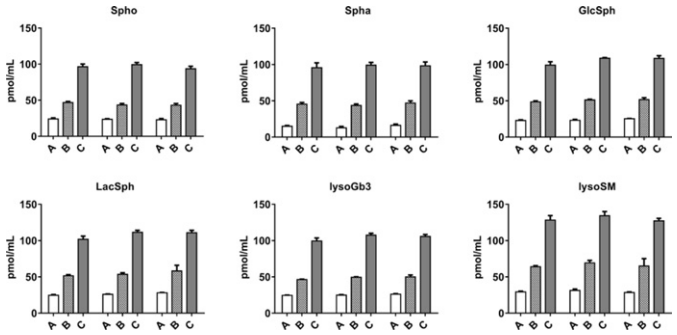


Fig. 4. Sphingoid bases in plasma stored for 1 week at different conditions. From left to right bars, samples kept in freezer (−20 °C), fridge (4 °C) and room temperature. A, B and C are different concentrations of spiked equal amounts of lyso-GSLs in control plasma, 20, 50 and 100 pmol/mL respectively. Presented data were calculated with correction for the appropriate blanks.

3.2. UPLC-ESI-MS/MS quantitation of sphingoid bases in female and male control, GD and FD plasma specimens

We next simultaneously quantified sphingoid bases in plasma specimens of control subjects (males n = 21, females n = 21) by a single extraction, in plasma of GD patients (males n = 10, females n = 10) in duplicate and in plasma of FD patients (males n = 10, females n = 10) in duplicate (see Fig. 5). Significant elevations of Hex_3Sph (=lysoGb3) in male FD patients and to a more modest extent in female FD patients were detected, exactly as observed previously [1,13]. Of note, plasma lysoGb3 tended to be increased in male and female GD patients, again reported earlier [13]. As expected, plasma HexSph (=GlcSph) was found to be clearly increased in all GD patients [3]. Plasma sphingosine in GD patients was higher than in gender matched controls or FD patients, except for FD females. Plasma sphinganine levels were similar in the case of controls and patients, except for FD males showing a significantly lower level. Of note, plasma lysoSM and presumed LacSph were significantly increased in GD patients. The plasma Hex_2Sph was significantly increased in GD males and females. A much smaller, but significant, elevation was noted for FD males and females. The plasma Hex_2Sph (at least in GD patients) seems to be therefore largely LacSph and not the indistuinguishable Gal_2-Sph; Gal_2-Cer and its base are expected to be increased in FD since they are both substrates of the deficient α-galactosidase A in FD.

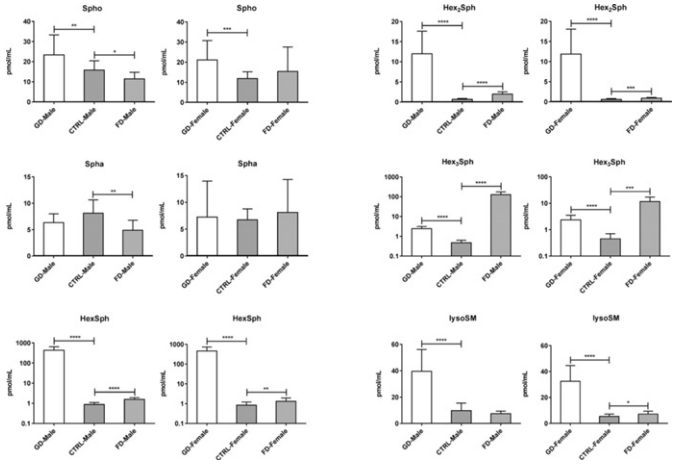


Fig. 5. Levels of various sphingoid bases in plasma specimens of male and female control subjects, symptomatic GD and FD patients. Sphingoid bases were simultaneously quantified as described in Materials and Methods. To assist comparison the levels of sphingoid bases in control plasma are depicted as the central bar. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

3.3. Effect of contraceptives in females

We investigated the impact of the oral use of contraceptives (OC) by females on their plasma sphingoid bases. Significantly lower levels of plasma GlcSph were detected in OC females (see Fig. 6). This difference could not be attributed to a difference in age (see supplemental information Fig. 2).

3.4. Concomitant determination of neutral (glyco)sphingolipids in same plasma sample following their deacylation to sphingoid bases

We next investigated whether the described analysis of sphingoid bases can be used to develop a convenient method for accurate quantitation of sphingolipids in the same sample. It is possible to separate sphingolipids and sphingoid bases during extraction, the bases partitioning in the upper water phase and sphingolipids in the lower chloroform phase. Sphingolipids can be subsequently microwave-assisted deacylated [17,18] and the resulting sphingoid bases can be next analyzed exactly like the sphingoid bases collected from the upper phase of the extraction. In this way parallel data could be generated for sphingolipid and sphingoid bases in the same sample.

To examine the feasibility of this approach, we first spiked C18-Cer, C18-GlcCer, C18-LacCer and C18-Gb3 into normal plasma. Also added was C17-dhCer to be used as alternative internal standard. Following the procedure exactly as described in M&M, the recoveries of all spiked lipids were high, either when using $^{13}C_5$ lyso-GSL as internal standard or C17-dhCer as internal standard to calculate absolute amounts of lipid (see Table 3). The linearity of detection was high for Cer, dhCer, GlcCer, LacCer and Gb3, exceeding in all cases 0.96 (see Table 3 supplemental information).

Next, we determined the concentrations of endogenous sphingolipids (Cer, dhCer, HexCer, Hex_2Cer and Hex_3Cer) in plasma using $^{13}C_5$-sphingoid bases as standards in a variety of plasma samples, see Table 4. Quite comparable data were obtained using C17-dhCer as internal standard (see supplemental information table 4 and Fig. 3 supplemental information).

4. Discussion

We here demonstrate the feasibility of simultaneous quantitation of the sphingoid bases sphinganine, sphingosine, HexSph (GlcSph), Hex_2Sph (LacSph), Hex_3Sph (lysoGb3) and lysoSM in plasma specimens

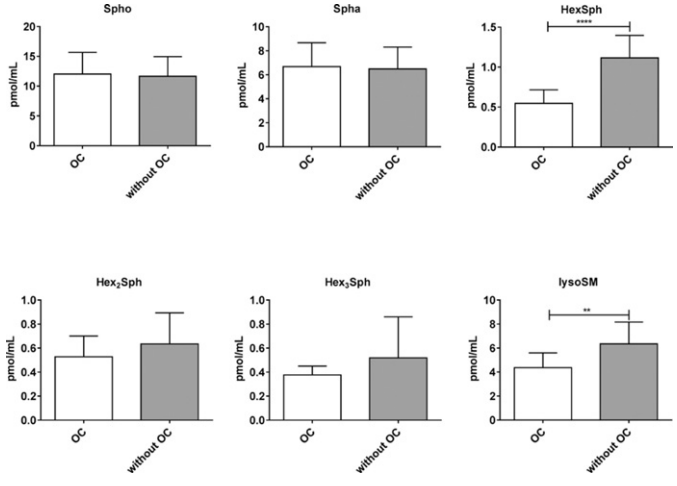


Fig. 6. Levels of various sphingoid bases in plasma specimens of female using oral contraceptives (OC) or not. Sphingoid bases were simultaneously quantified as described in Materials and methods. To assist comparison the levels sphingoid bases in control plasma are depicted as the central bar. Significance of differences: $^{**}P < 0.01$ and $^{****}P < 0.0001$.

Table 3
Recovery of spiked sphingolipids lipids.

%	C18-Cer	C18-GlcCer	C18-LacCer	C18-Gb3
$^{13}C_5$ lyso-GSL	96.9	84.9	98.8	92.9
C17-dhcer	108.7	99.2	110.7	104.5

with UPLC-ESI-MS/MS and identical $^{13}C_5$-encoded internal standards, except for LacSph and lysoSM. In the case of LacSph no isotope-encoded standard was available. In the case of $^{13}C_5$-lysoSM there was background in plasma and superior quantitation was obtained with C17-lysoSM as standard. The sensitivity and linearity of detection is excellent for all examined sphingoid bases in the relevant concentration range and the measurements show very acceptable intra- and inter-assay variation. Plasma samples of a series of male and female GD and FD patients were analyzed with this multiplex assay. The obtained data compare well to those earlier determined for plasma lysoGb3 and GlcSph in GD and FD patients [2,3,13]. Very recently Polo and coworkers reported a procedure for simultaneous measurements of HexSph, lysoGb3, lysoSM and lysoSM-509 [12]. Their method makes use of the same equipment as our study, but differs in the employed extraction procedure and the use of a single internal standard (GlcSph from plant source). Polo et al. describe quite comparable accuracy, linearity of detection and inter- and intra-assay variation as reported here. They demonstrated the value of their method by analysis of plasma from 194 control subjects, 16 FD patients, 10 GD patients, 3 KD patients and 11 NPC patients. The reported values for lysoGb3 and GlcSph in control subjects, GD and FD patients slightly differ from ours [12]. For example, in control plasma the median value for lysoGb3 was 0.29 nmol/L and for HexSph 2.09 nmol/L. In our analysis, in male control plasma the median value for lysoGb3 (Hex_3Sph) was 0.47 nmol/L and for HexSph 0.87 nmol/L; in female control plasma these were 0.45 nmol/L and 0.84 nmol/L, respectively. Thus, we quantified higher amounts of lysoGb3 but smaller ones for HexSph. These differences might be due to differences in the internal standards used. In principle the use of a non-identical internal standard is less attractive because of possibly required corrections for sample matrix effects such as ion suppression and in some cases ion enhancement.

Our study confirms the occurrence of modestly elevated levels of other sphingoid bases (LacSph, lysoGb3 and lysoSM) in plasma of symptomatic GD patients. The levels of lysoGb3 in GD females actually overlap with those in female FD patients. We earlier pointed out that caution

Table 4
Quantitation of plasma (glyco)sphingolipids (Cer, dhCer, HexCer, Hex_2Cer and Hex_3Cer).

Plasma sample $^{13}C_5$ lyso-GSLs (IS)		Cer nmol/mL	dhCer nmol/mL	HexCer nmol/mL	Hex_2Cer nmol/mL	Hex_3Cer nmol/mL
CTRL male	Mean	5.30	0.57	3.28	1.86	0.63
	SD	0.26	0.04	0.59	0.51	0.32
FD male 1	Mean	3.18	0.33	2.54	1.71	2.77
	SD	0.14	0.00	0.24	0.15	0.11
FD male 2	Mean	4.13	0.48	3.51	1.87	0.93
	SD	0.16	0.02	0.43	0.24	0.14
GD male 1	Mean	2.61	0.42	7.47	1.69	0.50
	SD	0.13	0.01	1.46	0.21	0.04
GD male 2	Mean	2.59	0.40	8.96	1.77	0.44
	SD	0.05	0.01	1.60	0.33	0.08
CTRL female	Mean	4.78	0.69	3.82	1.73	0.66
	SD	0.84	0.02	0.43	0.26	0.12
FD female 1	Mean	4.30	0.50	4.01	2.49	0.94
	SD	0.01	0.00	0.22	0.11	0.03
FD female 2	Mean	6.28	0.92	4.87	2.13	0.77
	SD	0.21	0.02	0.31	0.36	0.09
GD female 1	Mean	4.99	0.63	13.61	2.53	0.85
	SD	0.02	0.06	0.18	0.19	0.21
GD female 2	Mean	2.99	0.51	6.45	1.27	0.36
	SD	0.26	0.00	0.74	0.29	0.08

is needed when interpreting modest increases in plasma lysoGb3 as confirmative for (atypical) FD [13,14]. The non-specific modest elevations in lysoGb3, LacSph and lysoSM in plasma of GD patients are most likely caused by activation of acid ceramidase in lysosomes of lipid-laden macrophages (Gaucher cells) resulting in deacylation of various sphingolipids [13]. Another interesting finding made during the study is the noted lowering effect of oral contraceptives on plasma GlcSph level. It has been earlier described that OC use is associated with reduction of plasma GlcCer [15]. This glycosphingolipid functions as a cofactor for activated protein C (APC), an anti-coagulant. Reduced GlcCer has therefore been proposed to promote the increased venous thrombosis risk in females using OCs [15]. It has earlier been reported that estradiol reduces GlcCer by inhibiting its synthesis, whereas testosterone has opposite effects [16].

Since it is possible to separate sphingolipids and sphingoid bases by differential extraction and the obtained sphingolipids can be subsequently de-acylated, either microwave-assisted or by enzymatic digestion with ceramide *N*-deacylase [17–20], we looked into the possibility to develop a procedure for convenient simultaneous quantitation of sphingolipids and sphingoid bases in the same plasma sample. We used microwave exposure for the de-acylation of extracted sphingolipids. We concluded from the extremely poor recovery of spiked sphingomyelin standard that this procedure cannot be applied for this sphingolipid. However for the other (glyco)sphingolipids tested (Cer, GlcCer, LacCer and Gb3) the recovery was excellent and quantitation was linear. Obviously, no information is obtained on the fatty acyl composition of the sphingolipids with this procedure, but the offered absolute quantitation of sphingolipids is likely superior to that reached by shot gun lipidomics or other procedures based on scanning for isoforms of a sphingolipid.

In conclusion, sensitive simultaneous quantitation of (glyco)sphingoid bases is feasible with LC-MS/MS using ^{13}C-encoded internal standards requiring a small amount of plasma. Potentially an extended procedure can be developed to determine also absolute amounts of specific (glyco)sphingolipids following their deacylation and quantitation of the corresponding bases.

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