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Quantification of Globotriaosylsphingosine in Plasma and Urine of Fabry Patients by Stable Isotope Ultraperformance Liquid Chromatography–Tandem Mass Spectrometry

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BACKGROUND: Biochemical markers that accurately reflect the severity and progression of disease in patients with Fabry disease and their response to treatment are urgently needed. Globotriaosylsphingosine, also called lysoglobotriaosylceramide (lysoGb3), is a promising candidate biomarker.

METHODS: We synthesized lysoGb3 and isotope-labeled [5,6,7,8,9] $^{13}C_5$-lysoGb3 (internal standard). After addition of the internal standard to 25 μL plasma or 400 μL urine from patients with Fabry disease and healthy controls, samples were extracted with organic solvents and the lysoGb3 concentration was quantified by UPLC-ESI-MS/MS (ultraperformance liquid chromatography–electrospray ionization–tandem mass spectrometry). Calibration curves were constructed with control plasma and urine supplemented with lysoGb3. In addition to lysoGb3, lyso-ene-Gb3 was quantified. Quantification was achieved by multiple reaction monitoring of the transitions m/z 786.4 > 282.3 $[M+H]^+$ for lysoGb3, m/z 791.4 > 287.3 $[M+H]^+$ for [5,6,7,8,9] $^{13}C_5$-lysoGb3, and 784.4 > 280.3 $[M+H]^+$ for lyso-ene-Gb3.

RESULTS: The mean (SD) plasma lysoGb3 concentration from 10 classically affected Fabry hemizygotes was 94.4 (25.8) pmol/mL (range 52.7–136.8 pmol/mL), from 10 classically affected Fabry heterozygotes 9.6 (5.8) pmol/mL (range 4.1–23.5 pmol/mL), and from 20 healthy controls 0.4 (0.1) pmol/mL (range 0.3–0.5 pmol/mL). Lyso-ene-Gb3 concentrations were 10%–25% of total lysoGb3. The urine concentration of lysoGb3 was 40–480 times lower than in corresponding plasma samples. Lyso-ene-Gb3 concentrations in urine were comparable or even higher than the corresponding lysoGb3 concentrations.

CONCLUSIONS: This assay for the quantification of lysoGb3 and lyso-ene-Gb3 in human plasma and urine samples will be an important tool in the diagnosis of Fabry disease and for monitoring the effect of enzyme replacement therapy in patients with Fabry disease.
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Fabry disease is an X-linked hereditary disorder caused by a deficiency of the lysosomal enzyme α-galactosidase A. The defect results in the accumulation of globotriaosylceramide (Gb3),[4] galabiaosylceramide, and blood group B glycolipids *(1)*. Clinically, patients manifest angiokeratoma, anhidrosis, and acroparesthesias, and in later stages cardiomyopathy, cerebrovascular disease, and renal insufficiency. Both men and women are affected by the disease, although the clinical manifestations are generally less severe in heterozygote women *(1)*. Two different recombinant α-galactosidase A preparations, algalsidase alfa (Replagal, Shire HGT) and agalsidase β (Fabrazyme, Genzyme Corp), are approved for the treatment of Fabry disease. The enzyme therapies involve 2 weekly intravenous infusions of an enzyme preparation *(2)*. Laboratory monitoring of disease progression in Fabry patients has been based on measurement of plasma and urinary Gb3 *(3)*. However, the concentration of this lipid is not abnormal in the plasma of most female Fabry heterozygotes, even when symptomatic *(4)*. We previously reported on a much more prominent lipid

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[4] Nonstandard abbreviations: Gb3, globotriaosylceramide; lysoGb3, globotriaosylsphingosine; OPA, orthophthaldialdehyde; UPLC-ESI-MS/MS, ultraperformance liquid chromatography–electrospray ionization–tandem mass spectrometry.

abnormality in plasma of Fabry patients *(5)*. The deacylated form of globotriaosylceramide, globotriaosylsphingosine (lysoGb3), is increased approximately 100-fold in the plasma of symptomatic Fabry hemizygotes and it is also increased in the plasma of symptomatic Fabry females *(5)*, a finding that has been confirmed by other studies *(6–9)*. Quantification of plasma lysoGb3 is of value for confirmation of the diagnosis of Fabry disease, especially in the case of female patients. Moreover, monitoring of the changes in plasma lysoGb3 concentrations in Fabry patients following enzyme therapy renders insight into the biochemical efficacy of the costly therapeutic intervention. We previously employed a procedure for quantifying lysoGb3 that was based on derivatization of the amine in the lysoglycolipid with orthophthaldialdehyde (OPA), followed by HPLC separation and fluorescence detection *(5, 10, 11)*. This method is able to quantify the increased concentrations of lysoGb3 in plasma of classically affected male Fabry patients. However, with a detection limit of about 3 nmol/L, the method is not analytically sensitive enough to accurately quantify lipid concentrations in plasma obtained from healthy individuals, nor in plasma samples from a large proportion of female heterozygous Fabry patients. Moreover, the original procedure is hampered by the fact that plasma samples may contain other OPA-reactive compounds that can interfere with detection of lysoGb3. This procedure thus requires multiple extractions and a mild alkaline hydrolysis step to obtain sufficiently pure preparations of lysoGb3 to allow reliable detection by the HPLC-based procedure *(10, 11)*.

Here we present a new method for the quantification of lysoGb3 based on ultraperformance liquid chromatography–electrospray ionization–tandem mass spectrometry (UPLC-ESI-MS/MS) and the use of [5,6,7,8,9] $^{13}C_5$-labeled lysoGb3 as the internal standard, which was synthesized for this particular purpose. In addition, we quantify a compound closely related to lysoGb3, lyso-ene-Gb3.

Material and Methods

PATIENT SAMPLES

Plasma and urine samples were obtained from men and women affected by classical Fabry disease *(1)* referred to the Lysosomal Outpatient Clinic of the Academic Medical Center in Amsterdam. The diagnosis had been confirmed by demonstrating a deficiency of α-galactosidase A in leukocytes and a mutation in the galactosidase, alpha (*GLA*) gene in male patients. Classically affected female patients were from families with classically affected males and their diagnosis had been confirmed by mutation analysis of the *GLA* gene. Control samples were obtained from age-matched healthy volunteers (21–60 years old). Informed consent was obtained according to the Declaration of Helsinki. Plasma was obtained from 5 mL EDTA-anticoagulated blood after centrifugation at 1750*g* for 10 min and stored at −20 °C until use. Urine samples from 24-h collections or early morning voids were stored at −20 °C until use. On thawing the urine samples were vigorously mixed to ensure that the sample was homogeneous. LysoGb3 is stable in plasma and urine for at least 5 days at room temperature and at 4 °C and for at least 1 year at −20 °C (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue3).

SYNTHESIS OF UNLABELED AND [5,6,7,8,9] $^{13}C_5$-LABELED LysoGb3

Nonlabeled lysoGb3 was synthesized as previously described *(12)*. The synthesis of [5,6,7,8,9] $^{13}C_5$-labeled lysoGb3 was accomplished as depicted in the scheme in the online Supplemental Data file. Key to the synthetic scheme was the preparation of [5,6,7,8,9]-$^{13}C_5$-*erythro*-sphingosine 10, which was accomplished as follows (for full experimental and analytical details see the online Supplemental Data). The stable isotopes were introduced in 3 consecutive additions of ^{13}C-labeled reagents starting from 1-bromononane 1. Displacement of the bromine with $^{13}C_1$-potassium cyanide yielded 1-$^{13}C_1$-decanitrile 2. Partial reduction of the nitrile gave 1-$^{13}C_1$-decanal 3, which was elongated using $^{13}C_2$-labeled Horner-Wadsworth-Emmons reagent 4. Reduction of the double bond in the resulting $^{13}C_3$-Weinreb amide 5 was followed by partial reduction to the aldehyde and a second condensation with HWE reagent 4 to give $^{13}C_5$-Weinreb amide 6. Reduction of the double bond and partial reduction of the Weinreb amide to the aldehyde provided an intermediate $^{13}C_5$-tetradecanal, which was homologated with methyltriphenylphosphonium bromide to give $^{13}C_5$-pentadec-1-ene 7. The $^{13}C_5$-labeled sphingosine 9 was then prepared using a cross-metathesis methodology in which the known chiral allylic alcohol 8 served as the second olefin reaction partner, next to $^{13}C_5$-olefin 7.

Benzoylation of the secondary alcohol in 9 was followed by selective removal of the isopropylidene group to give primary alcohol 10. In the final stages of the synthesis, compound 10 was condensed with protected globotriaosyl donor 11. The resulting $^{13}C_5$-labeled lysoGb3 construct was globally deprotected in a 4-step sequence and purified to homogeneity by reverse-phase HPLC.

In addition to the synthetic material, 1-mg batches of nonlabeled lysoGb3 derived from natural sources were obtained from Sigma (product G9534, lot numbers 071K2130 and 078K1124). LC-MS–grade methanol, water and formic acid and HPLC-grade $CHCl_3$ were purchased from Biosolve. Ammonium formate

(LC-MS grade) was purchased from Sigma-Aldrich Chemie GmbH.

EXTRACTION OF LysoGb3 FROM PLASMA

LysoGb3 was extracted from plasma by a modification of the method of Bligh and Dyer *(13)*. Briefly, 50 μL plasma was pipetted in an Eppendorf tube, 25 μL internal standard ([5,6,7,8,9] $^{13}C_5$-labeled lysoGb3, 0.1 nmol/mL in methanol) was added, and the tube was stirred briefly. Subsequently, 25 μL H_2O, 300 μL methanol, and 150 μL chloroform were added with 1 min of stirring after each addition. The samples were left for 30 min at room temperature. After centrifugation for 10 min at 15 700*g* in a 5415 D Eppendorf centrifuge to spin down precipitated protein, the supernatants were transferred to clean Eppendorf tubes and 150 μL chloroform and 225 μL H_2O were added. After stirring, the tubes were centrifuged for 2 min at 15 700*g* to separate the 2 phases. From the upper phase, 400 μL was transferred to a 2-mL clean tube (extract A). The lower phase was extracted by subsequent addition of 300 μL methanol and 300 μL H_2O and stirring for 1 min. The samples were centrifuged for 2 min at15 700*g* and the upper phase was removed, pooled with extract A, and taken to dryness at 35 °C under a nitrogen stream. The residue was dissolved in 100 μL methanol, stirred for 1 min, and sonicated for 30 s in a bath sonifier. The samples were centrifuged for 10 min at 15 700*g* in the Eppendorf centrifuge to precipitate any insoluble material. The recovery of lysoGb3 with this method was >98%. The isotope-labeled standard is presently not available for distribution because of its scarcity.

EXTRACTION OF LysoGb3 FROM URINE

LysoGb3 was extracted from urine by a modification of the method of Bligh and Dyer *(13)*. LysoGb3 was nearly quantitatively recovered in the upper phase. The upper phase containing the lysoGb3 was taken to dryness, dissolved in n-butanol, and extracted with water *(11)*. LysoGb3 was nearly quantitatively recovered in the butanol phase. The overall recovery of lysoGb3 with this method was >80%.

Briefly, to 400 μL of urine in an Eppendorf tube, 40 μL of internal standard ([5,6,7,8,9] $^{13}C_5$-labeled lysoGb3, 10 pmol/mL) was added and stirred briefly. Then, 960 μL methanol and 500 μL $CHCl_3$ were added with brief stirring. The samples were left at ambient temperature for 30 min, stirred occasionally, and centrifuged for 10 min at 15 700*g* in a 5415 D Eppendorf centrifuge to spin down precipitated protein. The supernatant was transferred to a glass tube, and 500 μL H_2O (Milli-Q) and 500 μL $CHCl_3$ were added with stirring. The tubes were centrifuged for 5 min at 2500*g* in a 5810 R Eppendorf centrifuge to separate the phases. The upper methanol/water phase was transferred to a 2-mL Eppendorf tube. No attempt was made to completely recover the upper phase, because care was taken not to disturb the interphase, which may contain protein in urines from Fabry patients with proteinuria. The sample was dried under a gentle stream of nitrogen at 35 °C, and the residue was dissolved in 800 μL butanol saturated with water. Then 800 μL H_2O saturated with butanol was added and the sample was stirred briefly and centrifuged for 5 min in the Eppendorf centrifuge at 15 700*g*. The upper (butanol) phase was transferred to a 1.5-mL polypropylene tube and the sample was dried under a gentle stream of nitrogen at 35 °C. No attempt was made to completely recover the upper phase, because care was taken not to disturb the interphase. Theoretically the combined extraction efficiency was >90%. Because the upper phases were not completely removed the actual recovery was lower than the theoretical recovery, namely >80%. The presence of the isotope-labeled internal standard corrected for all losses during sample cleanup. The residue was dissolved in 100 μL methanol. The samples were sonicated for 30 s in a bath sonifier and centrifuged for 5 min in the Eppendorf centrifuge at 15 700*g*.

INSTRUMENTATION

A Waters Acquity^{TM} TQD instrument was used in all experiments. The instrument consisted of a UPLC system combined with a tandem quadrupole mass spectrometer as mass analyzer. Data were analyzed with Masslynx Software (Waters).

OPTIMIZATION OF UPLC CONDITIONS

Analytes were separated on an Acquity BEH C18 reversed-phase column (2.1 × 50 mm, particle size 1.7 μm) (Waters) by using the following eluents: eluent A was 37% methanol, 0.5% formic acid in UPLC grade water containing 1 mmol/L ammonium formate; eluent B was 0.5% formic acid in methanol containing 1 mmol/L ammonium formate. A mobile-phase gradient was used during a 5.50-min run: 0.00 min, 0% B; 2.50 min, 100% B; 4.00 min, 100% B; 5.00 min, 0% B; 5.50 min, 0% B. The flow rate was 0.25 mL/min. The eluent was diverted to waste between 0.00 and 2.30 min to keep the source free of contaminants; data were collected between 2.30 and 4.05 min, and after 4.05 min the eluent was again diverted to waste.

OPTIMAL CONDITIONS FOR LysoGb3 DETECTION BY MS/MS

Standard solutions of lysoGb3, [5,6,7,8,9] $^{13}C_5$-lysoGb3,and a mixture of both compounds were prepared with concentrations of 1 nmol/mL in 5 mmol/L ammonium formate/0.5% formic acid in methanol. The compounds were introduced in the mass spectrometer by direct infusion and the optimal tuning

Table 1. MS/MS instrument parameters.

Capillary voltage	3.50 kV
Cone voltage	55 V
Source temperature	140 °C
Desolvation temperature	450 °C
Cone gas	50 L/h
Desolvation gas	950 L/h
Collision gas	0.12 mL/min
Collision voltage	40 V
Type	Multiple reaction monitoring
Ion mode	ES^+ (electrospray positive)
Dwell time	0.100 s
Interchannel delay	0.005 s
Interscan delay	0.005 s
Transitions (lyso-Gb3)	*m/z* 786.4 to *m/z* 282.3
Transitions [5,6,7,8,9] $^{13}C_5$ lyso-Gb3)	*m/z* 791.4 to *m/z* 287.3
Transitions(lyso-ene-Gb3)	*m/z* 784.4 to *m/z* 280.3
Fit weight	None
Smooth method	Mean
Smooth iterations	2
Smooth width	2
Point of origin	Force

conditions for both compounds in ES^+ (electrospray positive) mode were determined, i.e., the signal response was optimized as a function of capillary and cone voltage and of flow and temperature of desolvation gas (nitrogen). Optimal conditions of collision-induced dissociation for the main transitions *m/z* 786.4 > 282.3 $[M+H]^+$ for lysoGb3 and 791.4 > 287.3 $[M+H]^+$ for $^{13}C_5$-lysoGb3 were established as a function of collision energy and collision gas (argon) flow. The optimal conditions for the detection of lysoGb3 by UPLC-ESI-MS/MS are shown in Table 1.

DETERMINATION OF LysoGb3 BY HPLC

The measurement of lysoGb3 using derivatization with orthophthaldialdehyde, HPLC separation with fluorescence detection was performed as previously described *(11)*.

CHARACTERIZATION OF COMMERCIALLY AVAILABLE STANDARD

Calibration curves from 0–1–2–5 pmol containing 0.5 pmol of [5,6,7,8,9] $^{13}C_5$-lysoGb3 per injection were constructed for commercially available lysoGb3 from 2 different lot numbers obtained from Sigma G9534. The absolute amounts injected were plotted against the internal standard ratio. Linear responses were obtained: $y = 2.26x$, $R^2 = 1.000$ for lot number 071K2130 and $y = 1.00x$, $R^2 = 0.999$ for lot number 078K1124. The latter standard had been used in previous studies on lysoGb3 from our laboratory *(5, 10, 11)*. The calibration curve of the unlabeled standard synthesized in our laboratory, ($y = 2.52x$, $R^2 = 0.999$), was highly comparable with the unlabeled Sigma standard (lot number 071K2130). However, a remarkable difference was found with the slope of the calibration curve of the commercial standard with lot number 078K1124. These results were confirmed by HPLC measurements (data not shown). This finding highlights the need for certified lipid standards.

Results and Discussion

MASS SPECTROMETRIC ANALYSIS OF UNLABELED AND [5,6,7,8,9] $^{13}C_5$-LABELED LysoGb3

Mass spectra were recorded from the newly synthesized unlabeled and [5,6,7,8,9] $^{13}C_5$-labeled lysoGb3 injected as a mixture of 1 nmol/mL each. Fig. 1A shows the mass spectra with the expected *m/z* values of 786.4 for the protonated form of unlabeled lysoGb3 and *m/z* 791.4 for [5,6,7,8,9] $^{13}C_5$-lysoGb3. In addition to the protonated forms, the Na^+-adducts (*m/z* 808.4 and 813.3, respectively) were detected. This mixture was used to optimize the conditions for detection by MS/MS (Table 1). Fig. 1B shows the fragmentation spectrum of lysoGb3 under optimal conditions of collision-induced dissociation for the main transition *m/z* 786.4 > 282.3 $[M+H]^+$. Under these conditions the main positively charged product of collision-induced dissociation was a fragment of *m/z* 282.3$[M+H]^+$ due to the loss of trisaccharide and H_2O from the parent molecule (Fig. 1B, insert). The optimal conditions for collision-induced dissociation of [5,6,7,8,9] $^{13}C_5$-lysoGb3 (main transition 791.4 > 287.3 $[M+H]^+$) were identical. This newly synthesized isotope-labeled internal standard had a chemical structure identical to lysoGb3 and was thus ideally suited to compensate for losses during sample preparation and most importantly for matrix effects during MS detection.

MATRIX EFFECT: THE BENEFIT OF USING AN ISOTOPE-LABELED INTERNAL STANDARD

During the initial stages of method development we compared the absolute signal responses of a calibration curve of 0–0.01–0.25–0.05–0.1–0.25–0.5 and 1.0 pmol of pure standard lysoGb3 and a calibration curve of the same amount of standard lysoGb3 spiked in plasma and extracted by the modified method of Bligh and Dyer (see above) *(14)*. All samples contained 0.25 pmol of [5,6,7,8,9] $^{13}C_5$-lysoGb3. The calibration line (area lysoGb3 vs pmol injected) of the pure standard was $y = 8532x$, $R^2 = 0.993$. For

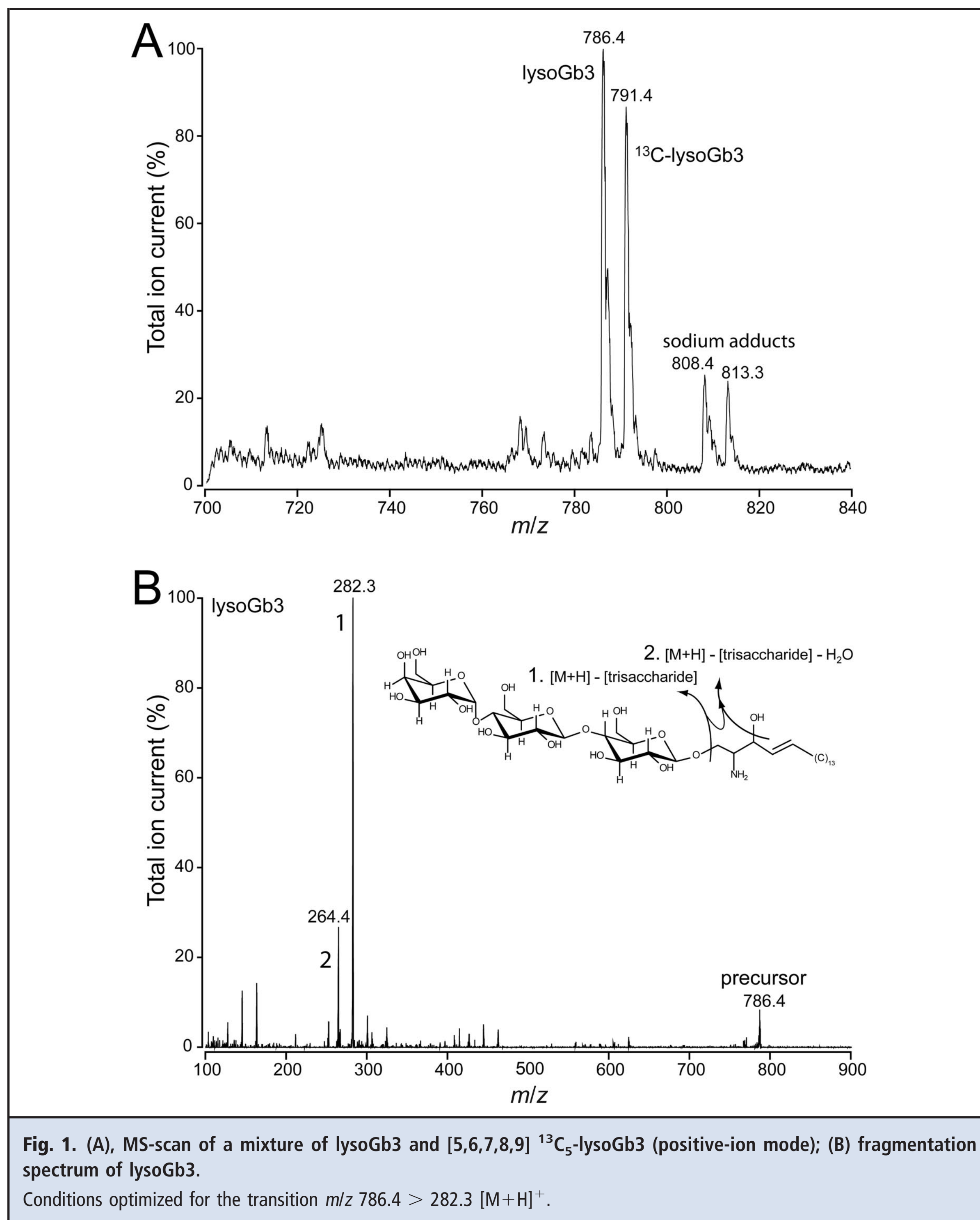

Fig. 1. **(A), MS-scan of a mixture of lysoGb3 and [5,6,7,8,9] $^{13}C_5$-lysoGb3 (positive-ion mode); (B) fragmentation spectrum of lysoGb3.**

Conditions optimized for the transition *m/z* 786.4 > 282.3 $[M+H]^+$.

spiked plasma extracted by the Bligh and Dyer method the calibration line was $y = 15790x$, $R^2 = 0.993$. The slope of the latter calibration line was 1.85 times that of the pure standard, indicating a signal enhancement effect of the extraction procedure. When the absolute amounts injected were plotted against the ratio of the area of lysoGb3/area of [5,6,7,8,9] $^{13}C_5$-labeled internal standard, the following calibration lines were obtained: $y = 2.941x$ for the pure standard and $y = 2.830x$ for supple-

mented plasma. The ratio of the similar slopes was 1.039, demonstrating the value of using the labeled internal standard for correction of the matrix effect.

CARRYOVER AND REPRODUCIBILITY OF RETENTION TIMES

Carryover between runs was tested by injecting methanol after a run with the highest concentration of pure standard lysoGb3 (500 pmol/mL) and plasma supplemented with lysoGb3 (500 pmol/mL). This corresponded to 2.5 pmol of lysoGb3 per injection. No signal of lysoGb3 could be detected, demonstrating that the carryover was essentially zero. The between-run variability in the retention time of lysoGb3 was 3.15 (0.02) min, CV = 0.5%. As expected, identical retention times were found for lysoGb3 and [5,6,7,8,9] $^{13}C_5$-labeled lysoGb3.

QUANTIFICATION OF LysoGb3 IN PLASMA

A calibration curve of lysoGb3 in normal control plasma was constructed. Normal control plasma was spiked with lysoGb3 a concentrations from 0–2–5–10–20–50–100–200 pmol/mL, internal standard was added, and the samples were extracted as described in the Materials and Methods. The ratio of the area of lysoGb3/area of [5,6,7,8,9] $^{13}C_5$-labeled internal standard was plotted against the concentration of lysoGb3. A linear response was obtained over the entire concentration range ($y = 0.025x$, $R^2 = 0.998$). To estimate the imprecision of the new method, control plasma was supplemented with lysoGb3 at a high concentration comparable to concentrations found in hemizygote Fabry patients as well as a low concentration comparable to concentrations in heterozygotes. The results of 10 consecutive duplicate determinations were calculated by EP Evaluator® and yielded the following data: for the high concentration, intraassay, 71.7 (2.5) pmol/mL, CV = 3.5%; interassay, 71.7 (4.6) pmol/mL, CV = 6.3%; for the low concentration, intraassay, 17.5 (1.2) pmol/mL, CV = 7%; and interassay 17.5 (0.2) pmol/mL, CV = 1.3%. We compared the data obtained with the current UPLC-ESI-MS/MS method with those obtained with the HPLC method using the new lysoGb3 standards. Good linearity and a good correlation were found between the results obtained by both methods ($y = 1.13x$, $R^2 = 0.974$).

Next, the lysoGb3 concentrations in plasma samples of 10 classically affected Fabry hemizygotes, 10 classically affected Fabry heterozygotes, and 20 healthy individuals (10 males and 10 females) were determined by mass spectrometry (Fig. 2). Although the concentration of lysoGb3 in plasma samples of male Fabry hemizygotes was 100 times higher than in those of healthy individuals, the wide linear dynamic range and the great sensitivity allowed accurate quantification in both groups (Fig. 2). No difference in lysoGb3 concentration was found between male and female control samples. LysoGb3 was found to be completely stable in plasma stored at −20 °C as judged from more than 20 consecutive lysoGb3 measurements of a reference plasma sample during a period of at least 2 years (data not shown). The limit of detection with the mass spectrometric method (signal-to-noise ratio of 3) was 0.05 pmol/mL. The signal-to-noise ratio in the case of control plasma samples was always >10 (Fig. 3), allowing accurate quantification of plasma lysoGb3 even in normal individuals. These results compare favorably with those of a recently published MS/MS method in which the limit of quantification was not sufficiently low to allow quantification of lysoGb3 in healthy individuals *(9)*. These authors used a glycine derivative of lysoGb3, Gly-lysoGb3, as an internal standard. The synthesis of this compound is less complicated than the synthesis of [5,6,7,8,9] $^{13}C_5$-labeled internal standard described here. However, the structure of this compound is closely related, but not identical to lysoGb3, which may affect both the extraction efficiencies as well as the mass spectrometric behavior in different matrices. It should be noted that the measured absolute amounts of lysoGb3 in plasma of hemizygous and heterozygous Fabry patients were lower than previously determined by HPLC measurements *(5, 10, 11)*. This difference may be attributed to an impurity of commercial lysoGb3 standard used in these studies (see Materials and Methods).

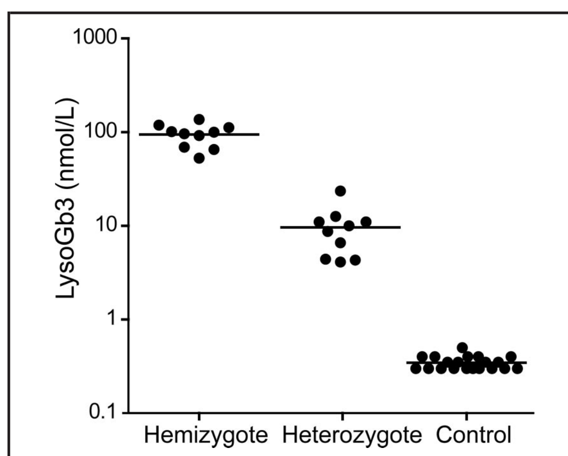

Fig. 2. LysoGb3 concentrations in plasma of 10 classically affected Fabry hemizygotes, 10 classically affected Fabry heterozygotes, and 20 controls (semi-logarithmic plot).

Hemizygote: 94.4 (25.8) pmol/mL, range 52.7–136.8 pmol/mL. Heterozygote: 9.6 (5.8) pmol/mL, range 4.1–23.5 pmol/mL. Control: 0.4 (0.1) pmol/mL, range 0.3–0.5 pmol/mL. MRM, multiple reaction monitoring.

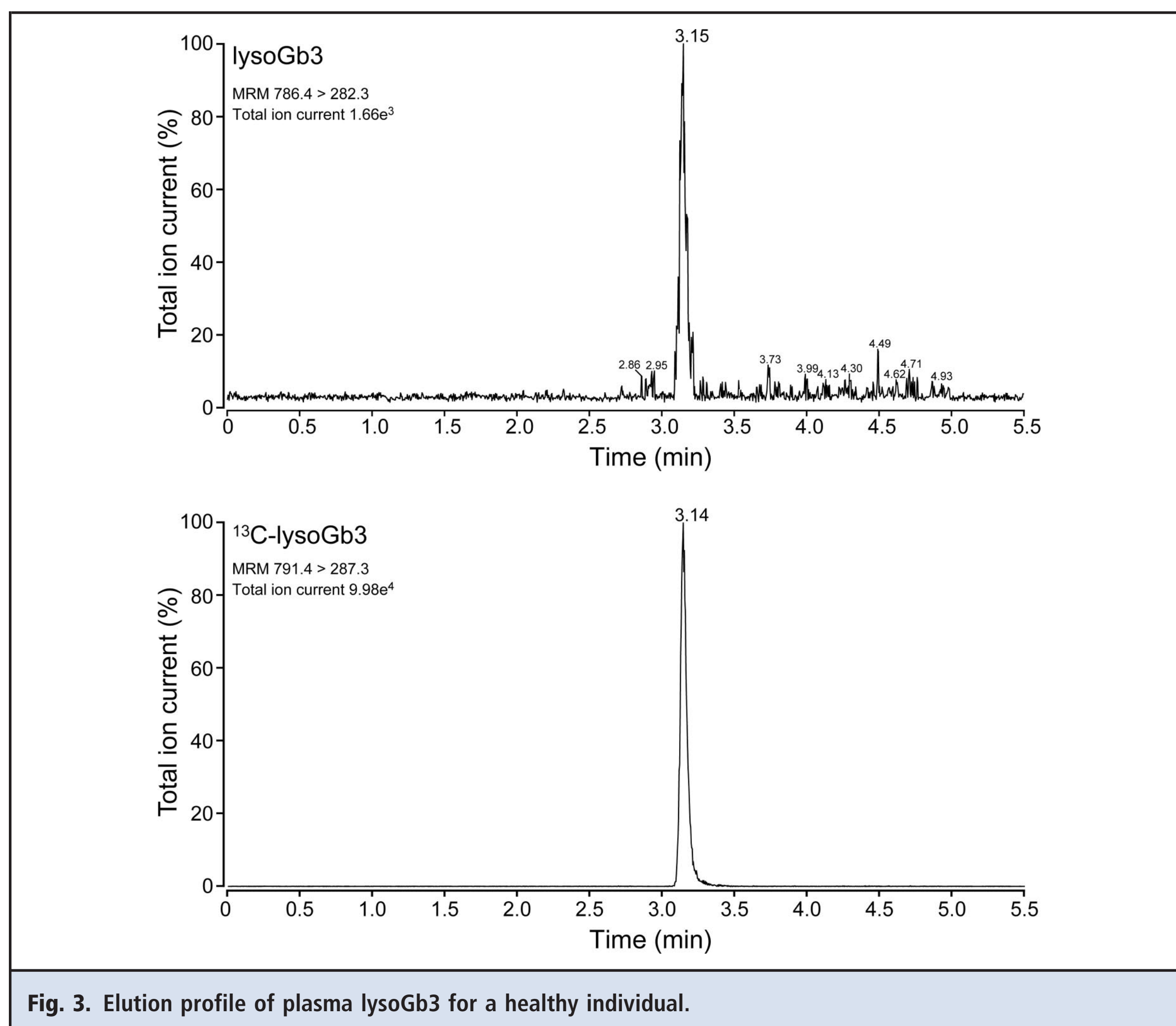

Fig. 3. Elution profile of plasma lysoGb3 for a healthy individual.

PRESENCE OF A Lyso-ene-Gb3 IN PLASMA AND URINE FROM FABRY PATIENTS

We have previously shown that, in addition to lysoGb3, a related compound with higher polarity accumulates in patients with Fabry disease *(5)*. This compound was tentatively identified by Auray-Blais and coworkers as a lyso-ene-Gb3 *(8)*. Prompted by this finding, we monitored the transition 784.4 > 280.3 in addition to the transitions 786.4 > 282.3 (lysoGb3) and 791.4 > 287.3 ([5,6,7,8,9] $^{13}C_5$-labeled lysoGb3). A peak with a retention time slightly shorter than the retention times of lysoGb3 (Fig. 4A) and that of labeled lysoGb3 (Fig. 4B) was found in plasma (Fig. 4C) and urine of male Fabry patients (urine not shown). These data suggest that compound X is indeed a lysoGb3 with a sphingosine base containing 1 additional double bond, consistent with the findings of Auray-Blais and colleagues *(8)*. Although we did not establish the position of the double bond, the most likely position is that of a 4E, 14Z-sphingadiene (4E, 14Z-18:2-sphingosine) *(15)*. The fragmentation spectrum of the peak of the chromatogram fits with lyso-ene-Gb3 (see online Supplemental Fig. 2). The fragmentation spectrum of lyso-ene-Gb3 is highly comparable to that of lysoGb3, allowing the quantification of lyso-ene-Gb3 with ([5,6,7,8,9] $^{13}C_5$-labeled lysoGb3 as the internal standard.

QUANTIFICATION OF LysoGb3 AND Lyso-ene-Gb3 IN PLASMA AND URINE FROM FABRY PATIENTS

The concentrations of lysoGb3 and lyso-ene-Gb3 were determined in plasma and urine of 9 male patients with classical Fabry disease from whom a plasma sample and a 24-h urine sample were collected at the same time before treatment. The results for lysoGb3 in plasma are shown in Fig. 4D and are comparable to those in Fig. 1. Lyso-ene-Gb3 was found to be increased in all examined plasma samples, but the concentrations were con-

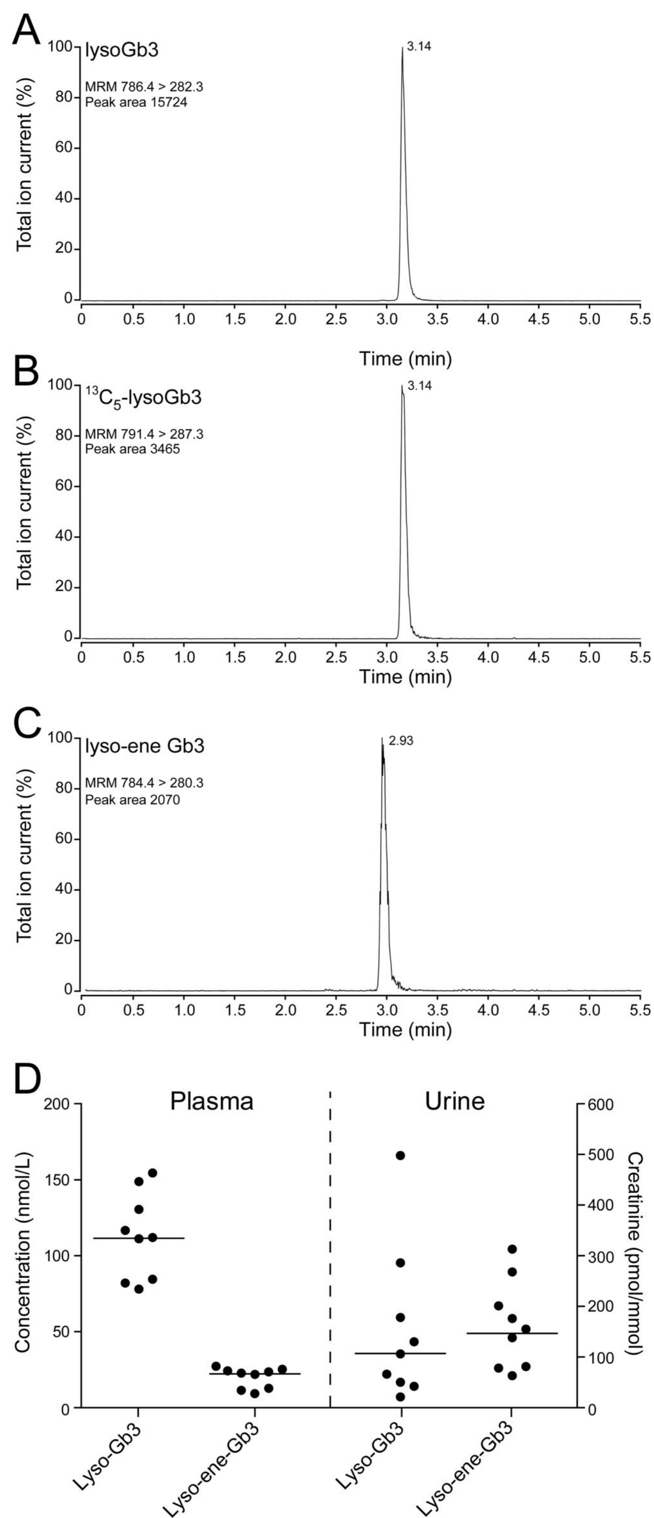

Fig. 4. Detection of lyso-ene-Gb3 in plasma.

(A), Elution profile of lysoGb3; (B), elution profile of [5,6,7,8,9] $^{13}C_5$-lysoGb3; (C), elution profile of lyso-ene-Gb3; (D), quantification of lyso-ene-Gb3 in plasma and urine from Fabry patients: comparison with lysoGb3.

sistently lower than those of lysoGb3 (10%–25% of total lysoGb3) (Fig. 4D).

For the determination of lysoGb3 and lyso-ene-Gb3 in urine, 400 μL of urine was extracted as described in Materials and Methods. The second sample clean-up step by butanol extraction was necessary to obtain sufficiently clean analyte preparations. A calibration curve of lysoGb3 in control urine was constructed. Control urine from a healthy individual was supplemented with lysoGb3 in concentrations ranging from 0–25–50–100–250–500–1000–2500–5000 fmol/mL, internal standard (1000 fmol/mL) was added, and the samples were extracted. No lysoGb3 could be detected in normal urine. The ratio of the area of lysoGb3/area of [5,6,7,8,9] $^{13}C_5$-labeled internal standard was plotted against the concentration of the lysoGb3 standard. A linear response was obtained over the entire concentration range ($y = 0.00122x$, $R^2 = 0.998$). The limit of detection, defined as a signal-to-noise ratio of 3, was 15 fmol/mL; the limit of quantification, defined as signal-to-noise ratio of 10, was 50 fmol/mL. Thus, the detection capability of the method for the determination of lysoGb3 in urine was comparable to that for plasma (see above). The intraassay CV for this assay was 11.2% for a urine sample with a low concentration of lysoGb3 [97.2 (10.9) fmol/mL, n = 10] and 5.3% for a urine sample with a high concentration of lysoGb3 [1106 (58) fmol/mL, n = 10]. The interassay CV was 7.7% for the urine sample with a low concentration [95.0 (7.3) fmol/mL, n = 10] and 9.5% for the sample with a high concentration of lysoGb3 [1186 (112) fmol/mL, n = 10]. The reproducibility of the retention times was comparable to that in plasma [3.14 (0.01) min for lysoGb3, n = 10]. Carryover was determined by processing a urine sample with a highest concentration of lysoGb3 of the calibration curve (5000 fmol/mL). No carryover was observed.

The concentrations of lysoGb3 and lyso-ene-Gb3 expressed per millimole creatinine are shown in Fig. 4D. Creatinine in urine was measured by the Jaffe reaction on the Cobas P800 (Roche Diagnostics) *(16)*. Both lysoGb3 and lyso-ene-Gb3 were detectable in all urine samples from classical Fabry patients, but not in samples from healthy individuals. In urine, the concentrations of lyso-ene-Gb3 were comparable to or even higher than those of lysoGb3 (30%–80% of total lysoGb3, Fig. 4D). In contrast, the concentrations of lyso-ene-Gb3 in plasma were consistently lower than those of lysoGb3. The concentrations of lysoGb3 in plasma were in the nanomolar range, and the concentrations in urine were in the picomolar range, in agreement with earlier findings *(8)*. This means that lysoGb3 and lyso-ene-Gb3 are likely reabsorbed by the kidney and barely excreted in the urine. The total urinary excretion per 24 h in these 9 patients varied from 0.3 to 3.8 nmol/24 h. for lysoGb3 and from 0.8 to 3.0 nmol/24 h. for lyso-ene-Gb3. The use of 24-h urine collections allowed us to determine the daily excretion of lysoGb3 and lyso-ene-Gb3. In principle, random urine samples could also be used for the quantification of lysoGb3 and lyso-ene-Gb3 in urine. In our view, plasma is the preferred material for diagnostic purposes and follow-up of treatment of patients with Fabry disease given that the concentrations of lysoGb3 and lyso-ene-Gb3 in plasma are 2–3 orders of magnitude higher than in urine. In addition, the plasma compartment is likely to be more representative of the tissue content of lysoGb3 than urine.

Conclusions

Our UPLC-ESI-MS/MS method takes advantage of an isotope-labeled internal standard with a chemical structure identical to the analyte. Variations in recovery during the extraction procedures are corrected for and matrix effects are minimized. The assay is robust, sensitive, accurate, and reproducible. All classically affected hemizygote Fabry patients and all heterozygous Fabry females with relatives with classic disease manifestation examined so far showed abnormally high plasma concentrations of lysoGb3. Thus, next to mutation analysis, the method is a very useful diagnostic tool to identify female carriers of classic Fabry disease. Because the method allows accurate quantification of lysoGb3 in plasma of healthy individuals, it should be particularly useful in the analysis of lysoGb3 concentrations in plasma of patients with an atypical clinical presentation of Fabry disease, who might present with lysoGb3 concentrations within reference intervals or only slightly increased *(9)*. The concentrations of lysoGb3 and lyso-ene-Gb3 in the urine of male hemizygotes with the classic presentation of Fabry disease are 40–480-fold lower than in plasma, and for urine a more elaborate sample clean-up is required. For diagnostic purposes, we recommend the determination of lysoGb3 in plasma.

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