Journal of Chromatography B, 883–884 (2012) 128–135

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of the Fabry marker lysoGb3 in human plasma by tandem mass spectrometry☆,☆☆

Ralf Krüger[a,*], Andreas Tholey[b], Thomas Jakoby[b], Rita Vogelsberger[a], René Mönnikes[a], Heidi Rossmann[a], Michael Beck[c], Karl J. Lackner[a]

[a] Institute of Clinical Chemistry and Laboratory Medicine, Medical Center of the Johannes Gutenberg University, Langenbeckstr. 1, D-55131 Mainz, Germany
[b] Institute for Experimental Medicine – Division Systematic Proteome Research, Christian-Albrechts-Universität, Niemannsweg 11, 24105 Kiel, Germany
[c] Department of Pediatrics/Villa Metabolica, Medical Center of the Johannes Gutenberg University, Langenbeckstr. 1, D-55131 Mainz, Germany

ARTICLE INFO

Article history:
Received 30 June 2011
Accepted 1 November 2011
Available online 18 November 2011

Keywords:
Morbus Fabry
Plasma
LysoGb3
Globotriaosylsphingosine
UPLC–MS/MS
Internal standard

ABSTRACT

Morbus Fabry is a hereditary metabolic disorder with low prevalence and late clinical manifestation. A defect in the α-galactosidase gene leads to lysosomal accumulation of the glycolipid globotriaosylceramide (Gb3). Gb3 may be used for monitoring of enzyme replacement therapy (ERT), but diagnostic sensitivity is limited. Recently, globotriaosylsphingosine (lysoGb3) was introduced as a promising new marker with significantly better sensitivity [23]. For Fabry diagnosis, clinical studies and possible therapy monitoring, we established a fast and reliable LC–MS/MS assay for quantification of lysoGb3 in human plasma. Protein precipitation and glycolipid extraction from EDTA plasma was performed using acetone/methanol. Samples were analyzed by UPLC–MS/MS in MRM mode. In contrast to HPLC with fluorescence detection, the LC–MS/MS method requires no derivatization, less sample preparation and less instrument analysis time (<3 min). As internal standard (ISTD), a glycine derivative of lysoGb3 was synthesized, and the product was purified by HPLC. ISTD properties such as polarity (affecting extraction and elution), ionization and fragmentation pathway were almost identical compared to the analyte. The new LC–MS/MS assay for the Fabry marker lysoGb3 shows good performance and allowed for better discrimination between Fabry patients and controls than Gb3.

1. Introduction

The lysosomal storage disease Morbus Fabry is caused by a mutated α-galactosidase gene, preventing cleavage of terminal α-galactose residues. This X-chromosomal deficiency leads to lysosomal precipitation of glycolipid intermediates, mainly Gb3 (globotriaosylceramide, ceramide trihexoside) [1–3], and subsequent accumulation in various organs and tissues [4–6]. Initially, prevalence of M. Fabry was estimated to be in the order of 1:100,000 to 1:800,000 [6,and references therein], but data from recent newborn screening initiatives suggest a remarkably higher prevalence of about 1:1000 to 1:3000 [6–8]. In this context it is important to remind that heterozygote female are often not just carriers, although hemizygote male are usually more seriously and earlier affected.

On the other hand, clinical symptoms are often diffuse and typical Fabry signs are sometimes lacking. Moreover, clinical manifestation may show up comparably late, especially in heterozygote females and in some so-called atypical or cardiac variants. In due consequence, correct diagnosis is often delayed beyond childhood. Successful pilot programs indicate that neonatal screening is feasible and allows much earlier diagnosis [9–12]. Fabry patients have a pronounced risk of myocard infarct, stroke and renal failure. Enzyme replacement therapy (ERT), which is available since 2001, can minimize clinical manifestation and delay disease progress [13,14], but earlier tissue damages are often irreversible. Immune reaction caused by recombinant protein infusion can be assessed by antibody titers. Gb3 analysis in plasma or urine is usually performed by tandem mass spectrometry and can be used to monitor therapy success [15–22], but unfortunately this parameter suffers from comparably low diagnostic sensitivity. Especially plasma Gb3 of heterozygote females or atypic variants is often in the normal range [16,17,22], whereas urinary Gb3 shows a somewhat better discrimination of heterozygotes from healthy individuals [18,19,22].

In 2008 Aerts et al. introduced a new marker for Fabry disease, namely globotriaosylsphingosine (lysoGb3) [23]. LysoGb3 is

☆ This paper is part of the special issue "LC–MS/MS in Clinical Chemistry", Edited by Michael Vogeser and Christoph Seger.
☆☆ The work was partially presented as poster at the 21st IFCC World Lab 2011 in Berlin, Germany, 15–19 May 2011.

* Corresponding author. Present address: Max Rubner-Institute, Federal Research Institute of Nutrition and Food, Haid-und-Neu-Str. 9, D-76131 Karlsruhe, Germany. Tel.: +49 0721 6625 405; fax: +49 0721 6625 404.
E-mail addresses: ralf.krueger@mri.bund.de (R. Krüger), karl.lackner@unimedizin-mainz.de (K.J. Lackner).

1570-0232/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2011.11.020

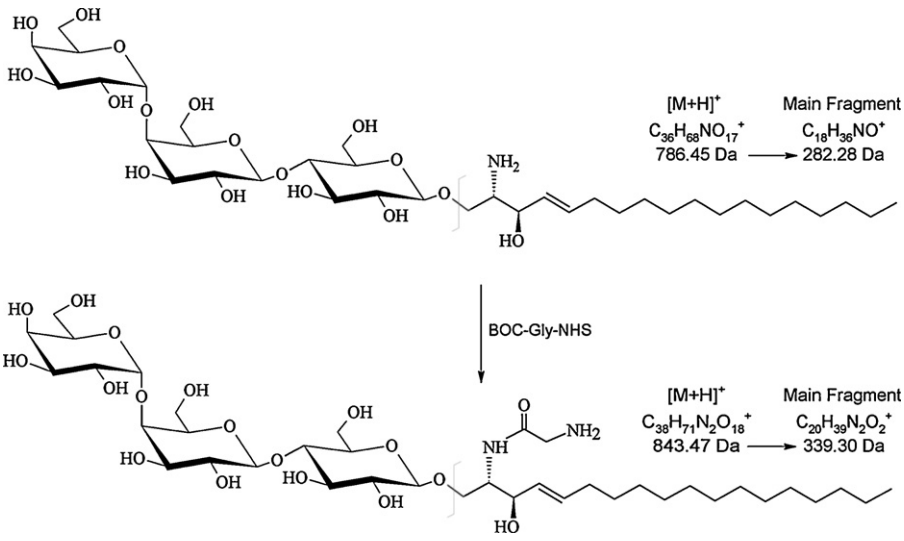

Fig. 1. Structure of lysoGb3 and synthesis of the glycine derivative (internal standard). Elemental formulas, MRM transitions and main fragmentation are also shown.

a Gb3 metabolite with free sphingosine amino group (see Fig. 1); in Gb3, this amino group is linked via an amide bond to a fatty acid. Aerts et al. could show for the first time the potential of lysoGb3 to serve as an improved Fabry marker. In addition, they discussed whether lysoGb3 may play a role in disease progression, but molecular mechanisms or clinical consequences are currently unclear. LysoGb3 enhances toxicity of the viper venom verotoxin 2, a protein which binds to the natural membrane-anchored Gb3 receptor [24], and Gb3 levels in fibroblasts were increased upon addition of lysoGb3 [23]. This indicates that lysoGb3 may inhibit the key enzyme α-galactosidase, but this could not be confirmed up to now [25]. Enhanced Gb3 synthesis may also explain the findings. Moreover, lysoGb3 seems to play a role in glomerular injury by promoting the release of secondary mediators such as TGF-β1 and CD74 [26].

A diagnostic benefit of lysoGb3 was verified by independent on-going studies from the groups of Aerts and Sakuraba, confirming that plasma lysoGb3 shows a significantly higher diagnostic sensitivity compared to plasma Gb3 [27–30], especially in case of heterozygote female [30]. Reduction of lysoGb3 levels in plasma and tissue upon ERT was clearly confirmed [28–30]. Correlation with common severity makers such as the MSSI (Mainz severity scoring index) or development of white matter lesions was present but rather weak [27,30]. Recently, Auray-Blais et al. could show that lysoGb3 is also elevated in urine of Fabry patients and that lysoGb3 normalized to urinary creatinine may also be used as monitoring parameter [25].

The latest results underline that there is a high interest to establish lysoGb3 as a clinical study parameter and as a potential parameter for monitoring disease progress and ERT in Fabry patients. In contrast, the current analytical protocols are comparably complex and allow only limited throughput. The initial method employs HPLC with fluorescence detection and requires several liquid–liquid extraction and evaporation steps, followed by subsequent derivatization with OPA (o-phtaldialdehyde) [23]. Moreover, HPLC separation of the glycolipids is comparably time-consuming (about 45 min) and no internal standard was used to control extraction efficiency. Recently, an LC–MS/MS method for quantification of lysoGb3 in urine was introduced [25]. Sample extraction and purification is performed by off-line SPE (solid phase extraction), and the analysis time is significantly improved (about 11 min).

Our goal was to develop a fast and reliable method for lysoGb3 quantification in human plasma, which should be more appropriate for high sample numbers, and to validate the method using clinical samples from Fabry patients. We applied UPLC–MS/MS in combination with a simple protein precipitation and glycolipid extraction step as sample Pretreatment to minimize sample processing. Special attention was paid to find a structurally very similar internal standard (ISTD), since possible matrix effects may be more critical without SPE purification. Since neither homologues nor isotope-coded substances are available, we synthesized and purified a glycine derivative of lysoGb3 (Gly-lysoGb3). In the following, method development, assay performance and plasma ranges for the diagnostic lysoGb3 assay are reported.

2. Experimental

2.1. Materials and chemicals

LysoGb3 (globotriaosylsphingosine) was obtained from Matreya (Pleasant Gap, PA, USA). Deionized water (18.2 MΩ) from an arium®611VF system (Sartorius, Göttingen, Deutschland) was used during ISTD synthesis, whereas HPLC grade water from Applichem (Darmstadt, Germany) was used for LC–MS/MS. HPLC grade methanol was either from Fisher Scientific (Schwerte, Germany) or from Sigma–Aldrich (Taufkirchen, Germany). Further solvents (chloroform and acetone spectroscopy grade) and all other chemicals such as formic acid and ammonium formate (both mass spectroscopy grade), trifluoroacetic acid (TFA), triethylammonium bicarbonate, and Boc-glycine N-hydroxysuccinimide ester (Boc-Gly-NHS) were obtained from Sigma–Aldrich/Fluka.

2.2. Synthesis of Gly-lysoGb3

1 mg lysoGb3 was dissolved in 1250 μL of the recommended solvent mixture A (chloroform:methanol:triethylammonium bicarbonate 0.5 M, 2:1:0.1, v:v:v) and divided into aliquots of 100 μL (80 μg). Boc-Gly-NHS was dissolved in the same solvent mixture A at a concentration of 0.1 M, and 10 μL of this reagent solution was added to each lysoGb3 aliquot, corresponding to a molar reagent excess of 10:1. The reaction mixture was incubated for 3 h in a thermomixer at 300 U/min and 26 °C. Reaction and incubation were repeated by adding another 10 μL reagent solution, thereby increasing the reagent excess to 20:1. Cleavage of the BOC protective group was performed by addition of 120 μL TFA. The quenched reaction mixture was evaporated to dryness using a vacuum centrifuge from Eppendorf (Hamburg, Germany). To remove excess

TFA, 100 μL of solvent mixture A were added and dried again in the vacuum centrifuge.

2.3. Purification of Gly-lysoGb3

Purification of Gly-lysoGb3 was performed by analytical HPLC using a LC–MS triple-quadrupole instrument (Surveyor and TSQ Quantum Discovery Max, Thermo Scientific, CA, USA). The desired product was separated from remaining educt and byproducts using a Kinetex pentafluorophenyl (PFP) phase from Phenomenex (Torrance, CA, USA). The dimensions were 150 mm × 3.0 mm with 2.6 μm core shell particles. The flow rate was 0.5 mL/min and the column temperature was 50 °C. Aliquots of the raw product from ISTD synthesis (see Section 2.2) were redissolved in 1 mL methanol and diluted 1:1 with eluent A. Eluent A was water and eluent B was methanol, and both eluents contained 0.1% formic acid. The gradient program was as follows: 1 min 5% B isocratic; at 1.01 min increase to 65% B followed by a flat gradient from 65% to 75% B until 10 min; at 10.01 min increase to 100% B until 12 min; at 12.01 back to 5% B until 18 min. The divert valve was used for fraction collection by switching to the load position from 10.0 to 10.8 min and collecting the eluting solution from the waste capillary. ESI MS/MS in MRM mode was used to monitor fraction collection. MS conditions were as follows: ESI mode positive, spray voltage 3.0 kV, sheath gas 8 AU, auxiliary gas 0 AU, capillary temperature 270 °C, capillary offset −35 V. The following transitions were monitored for MRM (dwell time was 200 ms): 786.5 > 282.3 (lysoGb3); 843.5 > 339.3 (ISTD). Collision gas pressure was 1.5 mTorr and collision energy was 35 V for both MRM traces. Purity of collected fractions was controlled by UPLC–LC–MS/MS (see Section 2.5), due to a slight memory on the PFP column. High resolution full scan spectra of the purified ISTD were acquired on an Agilent Q-TOF 6540 by direct infusion. Fraction aliquots were evaporated to dryness in a vacuum centrifuge. An adequate amount per vial was chosen (about 500 μL) so that redissolvation with 1 mL methanol resulted in an appropriate ISTD concentration (about 500 μg/L). These ISTD aliquots could be used directly for the quantitative assay.

2.4. Sample preparation EDTA-plasma

The neat lysoGb3 standard was dissolved in 1 mL of solvent mixture A as recommended by the supplier and diluted to 4 mg/L with methanol. Aliquots of 100 μL (400 μg) were dried in a vacuum centrifuge and stored at −20 °C for later use. For each analysis run, one standard aliquot was redissolved in 1 mL methanol, resulting in a working concentration of 400 μg/L. One purified ISTD aliquot was also dissolved in 1 mL methanol. For each sample, standard and control, one vial containing 1000 μL acetone:methanol (1:1, v:v) spiked with 20 μL ISTD solution was prepared. Six standards were prepared in duplicate by spiking plasma pools from healthy controls with different volumes of the diluted lysoGb3 standard (0–100 μL) resulting in plasma concentrations of 0, 40, 100, 200, 300 and 400 μg/L. 100 μL EDTA plasma was added to each vial. Pooled EDTA plasma was used in case of standards and controls. Controls were prepared at minimum two concentrations in duplicate. Samples, standards and controls were processed identically as described below. The vials were sonicated for 10 min, vortexed and centrifuged for 10 min at 4000 rpm. The supernatant was transferred into a new vial and dried in a vacuum centrifuge. For LC–MS/MS analysis, samples were redissolved in 100 μL of methanol with subsequent sonication for 10 min, thorough vortexing and centrifugation at 14,000 rpm for 10 min. The supernatant was transferred into glass microvials, and 10 μL were injected.

2.5. Quantitative LC–MS/MS assay

Quantitative UPLC–MS/MS analysis was performed on a triple quadrupole mass spectrometer Quattro Premier XE equipped with an Acquity UPLC system (Waters, Milford, MA, USA). A BEH C_{18} column (50 mm × 2.1 mm) with 1.7 μm particles was used in combination with a VanGuard precolumn with the same material (Waters, Milford, MA, USA). The flow rate was 0.6 mL/min, the column temperature was 60 °C, and the autosampler temperature was 10 °C. Elution was achieved by a linear gradient from 50% to 100% methanol in 0.8 min. 100% methanol was held until 2 min, followed by equilibration with the starting conditions. The total run time was 2.6 min. The divert valve was switched to waste until 0.7 min. Both solvents contained 0.1% formic acid and 2 mM ammonium formate. The latter is not necessary for the lysoGb3 assay, but identical solvents and columns were used also for immunosuppressant analysis. No deleterious effects of ammonium salt addition were observed. MS conditions were as follows: ESI mode positive, spray voltage 3.7 kV, desolvation gas flow 500 L/h, cone gas flow 25 L/h, desolvation temperature 200 °C, source temperature 60 °C, cone voltage 40 V. The following MRM transitions were monitored (dwell time was 100 ms): 786.5 > 282.3 (lysoGb3); 843.5 > 339.3 (ISTD). Collision gas flow was 0.35 mL/min and collision energy was 36 V for both MRM traces.

2.6. Calibration, quality controls and samples

Calibration was performed at six levels using a quadratic calibration curve through the origin with weighting $1/x^2$. Recovery was determined by spiking pools from healthy persons before and after sample extraction at two levels. Controls were prepared either from Fabry pools or by spiking pools of healthy individuals with appropriate amounts of lysoGb3. Samples from healthy volunteers ($n = 178$) were completely anonymized except birth year and sex. Samples from Fabry patients ($n = 74$) were generally taken after having obtained informed consent.

3. Results and discussion

3.1. Implementation of the quantitative LC–MS/MS assay

For analysis of patient samples in the frame of clinical studies, it is essential to have a robust method with short instrument run time and easy sample handling without complex and laborious sample preparation steps. Mass spectrometric detection was the first choice to avoid time-consuming analyte derivatization of routine samples, since lysoGb3 does not absorb UV light above 200 nm and has no natural fluorescence activity. Electrospray detection as $[M+H]^+$ (m/z 786) is straightforward due to the basic amino group. CID (collision induced dissociation) leads to a prominent fragment (m/z 282) resembling the sphingosine moiety (see Fig. 2, top panel), which gives the possibility to monitor lysoGb3 with high specificity by the MRM transition 786 > 282. In contrast to Gb3, no heterogeneous fatty acid composition has to be taken into account, and the higher polarity facilitates chromatographic separation by conventional reversed phase C_{18} columns. To enhance analytical turnover, we applied UPLC (ultra performance liquid chromatography) with sub-2-μm particles and a short 0.8 min methanol gradient, leading to a duty cycle below 3 min.

Simultaneous protein precipitation and glycolipid extraction was performed with a 1:1 mixture of acetone and methanol. This solvent is ideally suited to extract preferentially polar lipids and glycolipids from plasma. It was used for Gb3 extraction before [22], and turned out to be appropriate also in case of lysoGb3. Samples were evaporated to dryness and taken up in pure methanol for

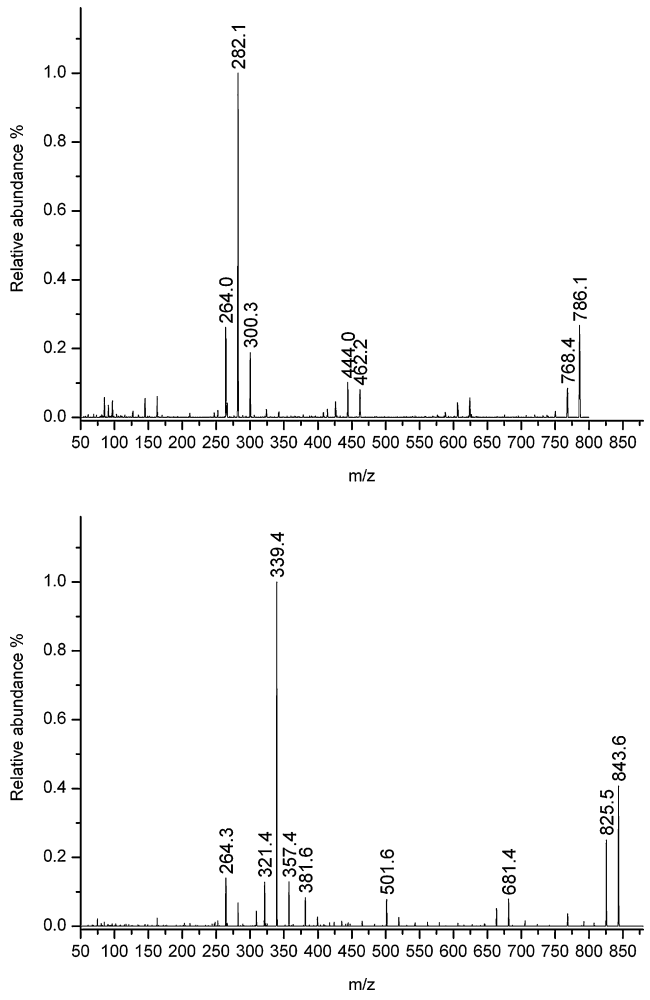

Fig. 2. Product ion spectra of lysoGb3 (top panel) and its glycine derivative/internal standard (bottom panel).

subsequent LC–MS/MS analysis without further sample preparation such as SPE. Recoveries from spiked plasma after precipitation were 90 ± 13% at 25 μg/L and 98 ± 10% at 150 μg/L, compared to a methanolic standard solution.

3.2. Choice and synthesis of the internal standard

The use of an internal standard is not only necessary to compensate for analyte losses during sample preparation, it is also inevitable during MS detection to compensate for fluctuations and matrix effects in the ESI source. Due to the comparably simple sample purification strategy, it was mandatory that the internal standard resembles the analyte lysoGb3 as much as possible. However, structurally similar molecules which are not present in humans are not easy to find. Isotope coded analogues are not available and their synthesis would be very laborious. Auray-Blais and co-workers used glucopsychosine (1-β-D-glucosylsphingadienine) from plant origin, which has one additional double bond compared to human glucopsychosine (1-β-D-glucosylsphingosine). It worked obviously satisfactory for SPE-purified urine samples, even though this molecule has a significantly lower sugar content compared to lysoGb3 (one hexose instead of three), resulting in lower polarity. However, the authors detected also a lysoGb3 analogue with one additional double-bond equivalent. In consequence, the existence of an analogue substance with only one hexose cannot be safely excluded in human individuals, for instance depending on nutrition status or plant-rich diet.

We pursued a different strategy in order to match ISTD properties such as extraction and chromatographic behaviour as close as possible, and to minimize the possibility of endogenous interferences. For this purpose, a glycine derivative of lysoGb3 was synthesized by amino acid chemistry (see Fig. 1). The free amino moiety of lysoGb3 was derivatized by a NHS-activated, N-Boc-protected glycine. The Boc-protection group was afterwards removed by treatment with TFA, leaving again a free amino group at the derivative. The free amino group is essential for polarity (extraction, chromatography) and mass spectrometric response (ionization, fragmentation). Adding a small amino acid changes polarity only marginally and restores the important amino group (compare Fig. 1), resulting in an ISTD with almost identical characteristics compared to the analyte.

Care was taken to optimize conditions such as reaction time and reagent excess. A two-step derivatization by adding the reagent twice at 10:1 molar excess, followed by a reaction time of three hours gave the best results (see Section 2.2). Generation of the desired product (m/z 843) was verified by MS.

3.3. Purification of the internal standard

Surprisingly, quantification was even possible with the crude ISTD, provided the background from remaining educt (1–6%) was subtracted individually for each sample. However, such a background is not tolerable for a quantitative diagnostic assay and limits analytical sensitivity. Moreover, side reactions were also observed, probably because sugar hydroxyl groups were not protected. Therefore, purification of the crude product was necessary. Separation was achieved by analytical HPLC, and fraction collecting using the divert valve of the mass spectrometer was monitored by MRM. We tested several columns from different suppliers, including cyano, phenyl and various C_{18} phases, and a monolithic column. Complete separation in less than 20 min was not straightforward, underlining the very similar polarity and retention characteristics of lysoGb3 and its glycine derivative. Cyano and phenyl phases gave unsatisfactory peak forms, as well as C_{18} phases designed for use with 100% aqueous solvent. Acceptable separation was achieved on C_{18} columns from Waters (HSS T3, BEH C_{18}) and Phenomenex (Kinetex C_{18} core shell). Fronting was a general phenomenon observed on most columns, despite the presence of formic acid as weak ion pairing agent. However, even addition of the stronger ion pairing agent TFA did not significantly improve peak shape. Since the product eluted always before the educt impurity, fronting led to insufficient separation and contamination of the product fraction. In contrast, the monolithic column gave good peak symmetry, but separation was insufficient and retention time difference was less than on C_{18} columns.

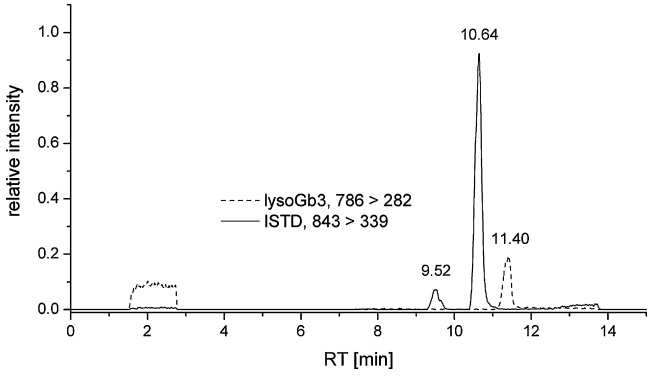

Fig. 3. Purification of internal standard: HPLC separation from reaction byproducts and impurities using a Kinetex PFP phase (150 mm × 3.0 mm, 2.6 μm).

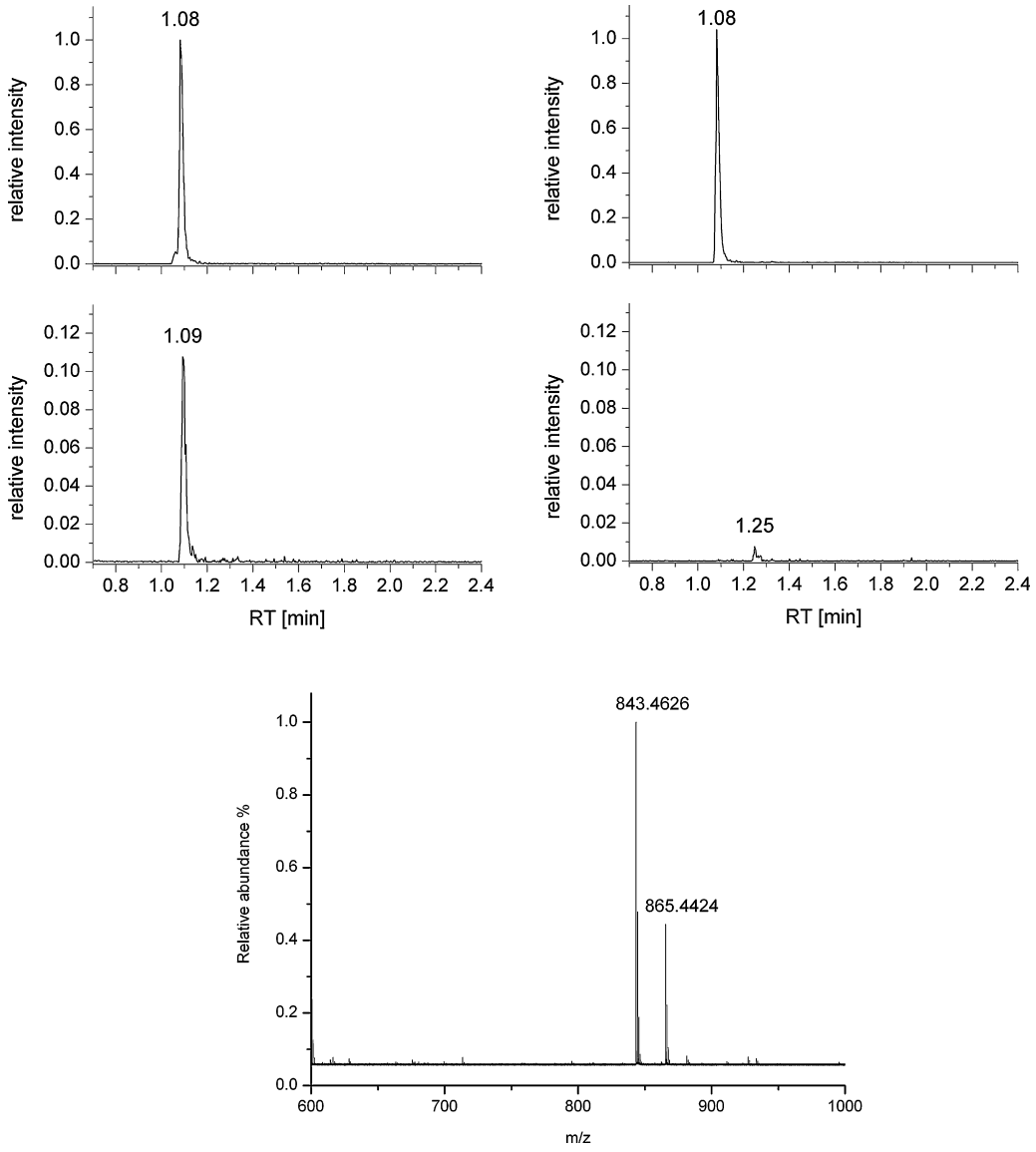

Fig. 4. (A) UPLC–MS/MS MRM traces of the internal standard Gly-lysoGb3 before (left) and after (right) purification using a short step gradient (BEH C_{18}, 50 mm × 2.1 mm, 1.7 μm). Top traces: ISTD (843 > 339); bottom traces: lysoGb3 (786 > 282). (B) TOF full scan mass spectrum of the purified ISTD (theoretical *m*/*z*: 843.4696, observed $\Delta = -8$ ppm).

Acceptable separation and best peak shape was achieved with 2.6 μm pentafluorophenyl core shell particles (Kinetex PFP, Phenomenex) using a flat 12 min methanol gradient (see Fig. 3). Tests were performed on 2 mm columns, but finally a 3 mm column with 15 cm length was chosen for purification of higher amounts in order to enhance peak capacity. Baseline separation of the product (RT 10.6 min) from side products (RT 9.5 min, probably OH-derivatives) and educt (RT 11.4 min) was achieved, and the fraction from 10.0 to 10.8 min was collected.

Purity of the collected fraction was checked by UPLC–MS/MS on the BEH C_{18} column, because a slight memory effect was observed on the PFP column. Fig. 4A shows MRM traces of the crude product (left) and the purified ISTD fraction (right). In case of the raw product, peaks appear in both traces, indicating that remaining educt is present. The additional impurity corresponding to the peak at RT 9.5 in Fig. 3 (probably from reaction of sugar hydroxyl groups) is also visible in front of the main product peak. In contrast, only the desired peak in the ISTD trace was detected after injection of the purified glycine derivative. Purity was further verified by TOF-MS of the collected fraction (Fig. 4B), showing the expected peaks corresponding to the desired product with a mass deviation of −8 ppm using external mass calibration. The product amount after synthesis and purification was roughly estimated to about 40% from the peak intensities of the final purified ISTD solution.

Compared to the educt lysoGb3, its glycine derivative showed an almost identical fragmentation pattern, except of the expected shift by 57 Da, the mass of the glycine residue (see Fig. 2). Consequently, detection was possible using the MRM transition 843 > 339. Moreover, the retention time in the routine assay was almost identical, and the same electrospray and CID parameters could be used (cone voltage, collision energy). Recovery from spiked plasma samples using the standard procedure for lysoGb3 was 79 ± 17% at 25 μg/L and 91 ± 11% at 150 μg/L, only slightly less than for the analyte itself (90% and 98%). Recovery was slightly concentration dependent, but the quotient of the two recoveries for lysoGb3 and for the Gly-lysoGb3 ISTD was stable over the concentration range (1.14 at 25 μg/L and 1.08 at 150 μg/L), providing a constant, concentration-independent response. These results underline that the glycine derivative is ideally suited as ISTD for the quantitative lysoGb3 LC–MS/MS assay.

Table 1
Linearity results for lysoGb3 quantification (dilution series).

Dilution series	Low	Medium	High
Mean value ± SD, μg/L	26.1 ± 1.9	106.8 ± 10.0	406.6 ± 31.2
CV %	7.4	9.4	7.7
Recovery ± SD, %	100.3 ± 4.9	100.5 ± 5.1	97.4 ± 7.5
Recovery max. %	107.4	109.9	105.0
Recovery min. %	93.3	95.1	85.6
Linear range, μg/L	2.3–29.1	6.6–120	31.4–438
Linear regression	$y = 28.303x - 0.8193$	$y = 104.36x + 0.3434$	$y = 448.75x - 13.18$
Regression coefficient, R^2	0.9918	0.9930	0.9988

3.4. Assay validation

Validation of the diagnostic assay was performed essentially according to CLSI criteria using pooled plasma from Fabry patients and spiked plasma pools from healthy controls. Linearity was tested by dilution series of spiked pools (see Table 1) analyzed in three separate runs. Recoveries over the dilution range were between 85.6% and 109.3%. The lower limit of quantification (LLOQ) was 2.3 μg/L, giving a linear analytical working range of more than two orders of magnitude (2.3–448 μg/L). Analytical sensitivity was estimated by sample dilution, since a very low lysoGb3 background was observed even in healthy controls and no real blank samples were available. The limit of detection (LOD) was calculated from all subsequent sample dilutions which did not lead to further signal reduction, therefore representing the assay background (mean 0.20 ± 0.16 μg/L). Signals of less diluted samples (next dilution step) were significantly higher ($p < 0.005$; 0.65 ± 0.35 μg/L). By this way, the LOD (mean plus 3 SD) was calculated to 0.7 μg/L (mean plus 10 SD: 1.8 μg/L).

Imprecision was assessed at various concentration levels (see Table 2) and was generally between 6% and 16%. In addition, we analyzed also a plasma pool with very low lysoGb3 content. Its concentration was below the linear range, and consequently imprecision was above 40%. However, this pool could be used as a negative control, since it was reliably below the cut-off of 2.3 μg/L. For all other controls within the linear range, imprecision was satisfactory, but concentration-dependant, so that low pools gave slightly higher CVs than high pools. No significant difference between Fabry pools and spiked pools was found. Total imprecision as well as estimates of between-day, between-run and within-run imprecision were calculated according to CLSI protocols [31]. Total CV was between 10% and 16% for all controls in the quantitative concentration range. The bias of the spiked pools was between 3 and 12%, giving an estimate for the inaccuracy of the assay.

Methanolic stock solutions of the standard and ISTD were stable for at least two months at −20 °C. Lyophylized aliquots of STD and ISTD were also stored at −20 °C and were used up to six months after freezing without any sign of reduced signal intensity in the assay. Moreover, we did not observe rising intensities in the analyte MRM trace with storage time of ISTD aliquots, indicating that the Gly-lysoGb3 is stable at −20 °C. Consequently, ISTD synthesis and purification is not necessary every time the assay is used, but can instead be performed batch-wise in advance. Samples and controls were also frozen at −20 °C and showed no significant decrease after six months storage. Up to three freeze–thaw cycles did not have a noticeable influence on quantitative results.

3.5. Diagnostic performance

In order to verify the assay with clinical samples and to establish normal and pathological lysoGb3 ranges for our assay, 178 healthy control samples and 74 samples from Fabry patients were analyzed. Fig. 5 shows UPLC–MS/MS chromatograms of a Fabry pool (upper panel) with a lysoGb3 concentration of 62.9 μg/L, and a healthy control (lower panel) with a lysoGb3 concentration below the LLOQ of 2.3 μg/L. In agreement with earlier reports [23,27–30], lysoGb3 values of the majority of Fabry patients were above the analytical cut-off, whereas control levels were all below the LLOQ.

LysoGb3 levels of the examined Fabry and control collectives are summarized in the following (compare Fig. 6). All determined ranges are given as 5–95th percentile. The median of the lysoGb3 level in healthy controls was 0.48 μg/L (mean 0.55), with a range of 0.12–1.12 μg/L and a maximum of 1.85 μg/L. Since this range is clearly below the LLOQ, it represents essentially the method background. In contrast, the level in Fabry patients was much higher with a median of 11.95 μg/L (mean 28.99, range 1.34–108.46, maximum 185.70). In fact, only 13 out of 148 samples (8.8%) gave no detectable lysoGb3 signal at all above the lower linearity limit (2.3 μg/L). This is in sharp contrast to Gb3, where levels in the normal range are found in many Fabry patients, especially female patients and patients currently under ERT. It has to be noted that the majority of the investigated Fabry patients in the present study received regular ERT. However, even this collective has significantly increased lysoGb3 values. Moreover, no significant difference was observed between patients with known ERT status and the rest of the collective without ERT information. This result allows no conclusion about ERT success, since the non-ERT collective might be less severely affected. Any effect of ERT can only be examined by

Table 2
Imprecision and inaccuracy results for lysoGb3 quantification. Low and high controls refer to spiked pools from healthy volunteers, and pool control refers to a pool from Fabry patients. Within-run CV was determined by batch analysis of individually prepared samples ($n = 10$). Estimate of between-day, between-run, within-run and total CV were calculated according to CLSI guidelines [31]. Samples were prepared in duplicate and measured in two separate runs with at least 2 h in between ($n = 20$).

Control	Mean (μg/L)	Bias (%)	CV % all results	CV % within-run (batch)	CV % estimate of between-day	CV % estimate of between-run	CV % estimate of within-run	CV % total (CLSI)
Low A	17.6	−12.0	13.4	10.0	–	–	–	–
High A	95.3	−4.7	8.7	8.9	–	–	–	–
Low B	42.7	+6.7	14.7	–	9.0	0[a]	12.6	15.5
High B	195.0	−2.5	10.5	–	5.7	0[a]	9.8	11.3
Pool	63.9	–	15.6	–	11.9	0[a]	10.7	16.0

[a] Negative values are set to zero according to CLSI guideline.

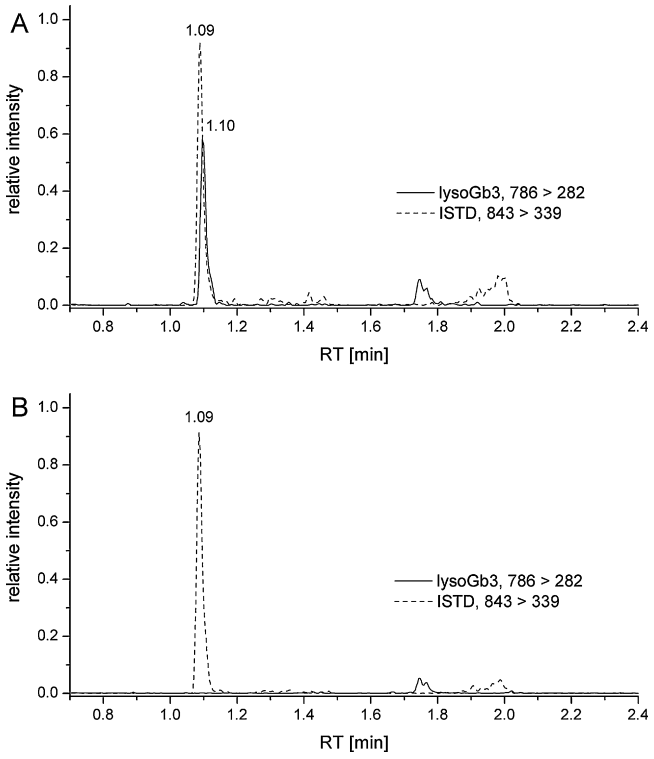

Fig. 5. Analysis of EDTA plasma from a Fabry patient pool (A) with a lysoGb3 mean value of 62.9 μg/L and a healthy control (B) with a lysoGb3 mean value below 2.3 μg/L (UPLC–MS/MS, BEH C_{18}, 50 mm × 2.1 mm, 1.7 μm).

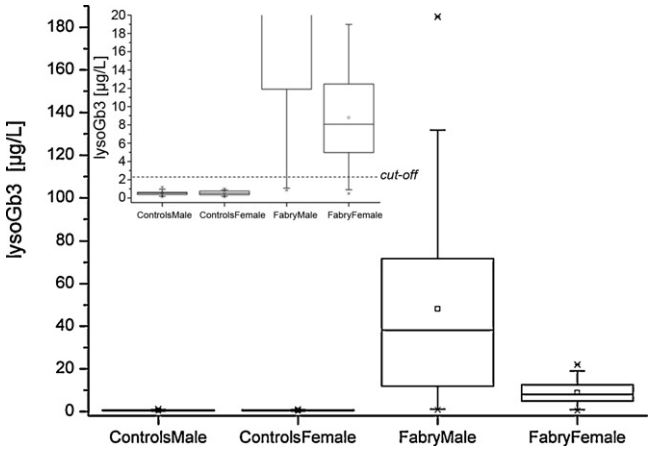

Fig. 6. Comparison of plasma lysoGb3 concentrations in Fabry patients and healthy controls. The square represents the mean average and the line within the box represents the median. The box represents the 25–75th percentile, and the whiskers represent the 5–95th percentile (see text for range numbers). The crosses represent the 1–99th percentile, and the dash gives the maximum and minimum values, respectively.

testing individual persons before and after start of ERT. Such a comparison was not possible in the present patient collective and was also not the topic of this study.

No significant difference was observed between male and female controls (male: median 0.49 μg/L mean 0.54, range 0.08–1.08; female: median 0.48 μg/L, mean 0.55, range 0.06–1.26). In contrast, lysoGb3 concentrations in plasma of male Fabry patients were significantly higher than in plasma of female Fabry patients, as expected. The median in male Fabry was 38.25 μg/L (mean 48.13, range 3.05–129.25), and the median in female Fabry was 8.80 μg/L (mean 8.78, range 1.21–18.27).

Using the lower limit of the linear range as cut-off (2.3 μg/L), diagnostic specificity of the lysoGb3 assay was 100%, since all samples from healthy controls were clearly below this cut-off. Diagnostic sensitivity was 95% for male Fabry patients, and 88% for female Fabry patients, even in the present collective with a majority of ERT patients. In contrast, sensitivity of Gb3 in the same collective was remarkably lower (55% for male Fabry, and only 33% for female Fabry), using the established reference range of <4.0 mg/L [22].

3.6. Preliminary correlation with other Fabry features

Correlation with other Fabry markers or genotype was not the focus of the present investigation. However, some preliminary data were obtained already during method setup. 74 Fabry patients have been analyzed for plasma lysoGb3 and Gb3 levels, but correlation between Gb3 and lysoGb3 levels was only weak ($R = 0.817$). LysoGb3 was high in all patients with low enzyme activity, with values above 14 μg/L at <20% residual activity and above 22 μg/L at <10% residual activity. Although such a result may be expected, one has to keep in mind that the analogous correlation between Gb3 levels and enzyme activity is rather poor, which is one reason that Gb3 is not well suited as primary diagnosis marker.

All patients with rather low lysoGb3 levels had missense mutations, similar to Gb3. This trend was more pronounced for male Fabry ($p = 0.005$) with a median of 22.7 μg/L (mean 27.6), in contrast to 73.8 μg/L (mean 76.0) for nonsense mutations and 49.8 μg/L (mean 71.9) for other mutations (including insertions, deletions and splice site mutations). Significance was lower ($p = 0.15$) in case of female Fabry patients (median 7.3 μg/L vs. 10.6 μg/L and 9.6 μg/L; mean 7.6 vs. 10.8 and 10.3). High levels were found especially in case that the mutation leads to a truncated protein or if mutations on exons 4, 5 or 7 were present ($p = 0.01$). This makes sense at least for exons 4 and 5, since these include coding regions for active sites of the enzyme.

The highest lysoGb3 plasma values were found for two mutations which lead to truncated protein versions and which are known to have severe impact on enzyme function [3]. The classical Fabry mutation W340X causes a stop at position 340 on exon 7, and a male patient presented with 135 μg/mL plasma lysoGb3. The highest level of 185 μg/L was found in a male patient with the mutation G183S on exon 4, which affects the splice site between exons 3 and 4, most probably leading to skipping of exon 3 [32].

We found only 7 out of 74 patients with lysoGb3 levels in the normal range. The genetic background was unknown in three cases. Three patients had the mutation A143T, which is known as late-onset variant or cardiac variant, respectively [33]. All three patients (one male, two female) were between 35 and 40 years old, and plasma Gb3 was also in the normal range. Another patient with normal lysoGb3 was a 44-year-old female with the missense mutation G325S in exon 6. In contrast, three male family members between 13 and 20 years of age had moderately elevated lysoGb3 plasma levels between 6.1 and 24.5 μg/L, but normal Gb3 (2.9–4.0 mg/L). This observation highlights the improved diagnostic sensitivity of the marker lysoGb3 compared to Gb3.

4. Conclusions

For quantitative analysis of the Fabry marker lysoGb3, an LC–MS/MS method was implemented and successfully validated. The method is short and requires only minimal sample preparation. The ISTD Gly-lysoGb3 is easy to synthesize and to purify with the optimized conditions. We found that Gly-lysoGb3 is ideally suited for LC–MS/MS due to the very similar properties compared to the analyte lysoGb3, including recovery, chromatographic retention, and fragmentation. By this way, matrix effects are minimized.

The presented method proved to be easy, robust and fast, and is therefore suited for therapy monitoring of Fabry patients.

The method enables analysis of larger Fabry collectives, and further examination of the mutation background or enzyme activity might substantiate the correlation with lysoGb3 plasma levels. Future investigations should also evaluate correlation of plasma lysoGb3 with ERT success, clinical symptoms (e.g. the MSSI score), and age. Especially children have usually low Gb3 values, and monitoring via Gb3 is clearly not ideal. LysoGb3 monitoring may offer an alternative, but this may require age-specific reference ranges for children. Furthermore, a comparison of plasma and urine lysoGb3 would be of high interest, especially in view of the observed discrepancies between plasma and urine Gb3. This could be different for lysoGb3.

Acknowledgements

Financial support from Fabry Outcome Survey (FOS) and Shire is gratefully acknowledged. AT and TJ were supported by the DFG-Cluster of Excellence “Inflammation at Interfaces”.

References

[1] R.O. Brady, New Engl. J. Med. 276 (1967) 163.
[2] C.M. Eng, R.J. Desnick, Hum. Mutat. 3 (1994) 103.
[3] S.C. Garman, D.N. Garboczi, J. Mol. Biol. 337 (2004) 319.
[4] H. Fabry, J. Inherit. Metab. Dis. 24 (2001) 3.
[5] J.T.R. Clarke, Ann. Intern. Med. 146 (2007) 425.
[6] D.P. Germain, Orphanet J. Rare Dis. 5 (2010) 30.
[7] M. Spada, S. Pagliardini, M. Yasuda, T. Tukel, G. Thiagarajan, H. Sakuraba, A. Ponzone, R.J. Desnick, Am. J. Hum. Genet. 79 (2006) 31.
[8] W.L. Hwu, Y.H. Chien, N.C. Lee, S.C. Chiang, R. Dobrovolny, A.C. Huang, H.Y. Yeh, M.C. Chao, S.J. Lin, T. Kitagawa, R.J. Desnick, L.W. Hsu, Hum. Mutat. 30 (2009) 1397.
[9] Y. Li, C.R. Scott, N.A. Chamoles, A. Ghavami, B.M. Pinto, F. Turecek, M.H. Gelb, Clin. Chem. 50 (2004) 1785.
[10] M.H. Gelb, F. Turecek, C.R. Scott, N.A. Chamoles, J. Inherit. Metab. Dis. 29 (2006) 397.
[11] P.J. Meikle, D.J. Grasby, C.J. Dean, D.L. Lang, M. Bockmann, A.M. Whittle, M.J. Fietz, H. Simonsen, M. Fuller, D.A. Brooks, J.J. Hopwood, Mol. Genet. Metab. 88 (2006) 307.
[12] D. Marsden, H. Levy, Clin. Chem. 56 (2010) 1071.
[13] A. Mehta, M. Beck, F. Eysken, C. Feliciani, I. Kantola, U. Ramaswami, A. Rolfs, A. Rivera, S. Waldek, D.P. Germain, Q. J. Med. 103 (2010) 641.
[14] M. Beck, Hum. Genet. 121 (2007) 1.
[15] K. Mills, A. Johnson, B. Winchester, FEBS Lett. 515 (2002) 171.
[16] F. Boscaro, P. Pieraccini, G. la Marca, G. Bartolucci, L. Luceri, F. Luceri, G. Moneti, Rapid Commun. Mass Spectrom. 16 (2002) 1507.
[17] C. Nelson, C.C.C. Sung, S. Araghi, D. Wilkens, K. Zhang, J.J. Thomas, S.M. Richards, Clin. Chem. 51 (2005) 237.
[18] M. Fuller, P.C. Sharp, T. Rozaklis, P.D. Whitfield, D. Blacklock, J.J. Hopwood, P.J. Meikle, Clin. Chem. 51 (2005) 688.
[19] K. Mills, P. Morris, P. Lee, A. Vellodi, S. Waldek, E. Young, B. Winchester, J. Inherit. Metab. Dis. 28 (2005) 35.
[20] P.D. Whitfield, J. Calvin, S. Hogg, E. O'Driscoll, D. Halsall, K. Burling, G. Maguire, N. Wright, T.M. Cox, P.J. Meikle, P.B. Deegan, J. Inherit. Metab. Dis. 28 (2005) 21.
[21] G. Fauler, G.N. Rechberger, D. Devrnja, W. Erwa, B. Plecko, P. Kotanko, F. Breunig, E. Paschke, Rapid Commun. Mass Spectrom. 19 (2005) 1499.
[22] R. Krüger, K. Bruns, S. Grünhage, H. Rossmann, J. Reinke, M. Beck, K.J. Lackner, Clin. Chem. Lab. Med. 48 (2010) 189.
[23] J.M. Aerts, J.E. Groener, S. Kuiper, W.E. Donker-Koopman, A. Strijland, R. Ottenhoff, C. van Roomen, M. Mirzaian, F.A. Wijburg, G.E. Linthorst, A.C. Vedder, S.M. Rombach, J. Cox-Brinkman, P. Somerharju, R.G. Boot, C.E. Hollak, R.O. Brady, B.J. Poorthuis, PNAS 105 (2008) 2812.
[24] M. Takenaga, R. Igarashi, M. Higaki, T. Nakayama, K. Yuki, Y. Mizushima, J. Infect. Chemother. 6 (2000) 21.
[25] C. Auray-Blais, A. Ntwari, J.T.R. Clarke, D.G. Warnock, J.P. Oliveira, S.P. Young, D.S. Millington, D.G. Bichet, S. Sirrs, M.L. West, R. Casey, W.-L. Hwu, J.M. Keutzer, X.K. Zhang, R. Gagnon, Clin. Chim. Acta 411 (2010) 1906.
[26] M.D. Sanchez-Niño, A.B. Sanz, S. Carrasco, M.A. Saleem, P.W. Mathieson, J.M. Valdivielso, M. Ruiz-Ortega, J. Egido, A. Ortiz, Nephrol. Dial. Transplant. 26 (2011) 1797.
[27] S.M. Rombach, N. Dekker, M.G. Bouwman, G.E. Linthorst, A.H. Zwinderman, F.A. Wijburg, S. Kuiper, M.A. vd Bergh Weerman, J.E.M. Groener, B.J. Poorthuis, C.E.M. Hollak, J.M.F.G. Aerts, Biochim. Biophys. Acta 1802 (2010) 741.
[28] T. Togawa, T. Kodama, T. Suzuki, K. Sugawara, T. Tsukimura, T. Ohashi, N. Ishige, K. Suzuki, T. Kitagawa, H. Sakuraba, Mol. Genet. Metab. 100 (2010) 257.
[29] T. Togawa, I. Kawashima, T. Kodama, T. Tsukimura, T. Suzuki, T. Fukushige, T. Kanekura, H. Sakuraba, Biochem. Biophys. Res. Commun. 399 (2010) 716.
[30] M.J. van Breemen, S.M. Rombach, N. Dekker, B.J. Poorthuis, G.E. Linthorst, A.H. Zwinderman, F. Breunig, C. Wanner, J.M. Aerts, C.E. Hollak, Biochim. Biophys. Acta 1812 (2011) 70.
[31] Clinical and Laboratory Standards Institute, User Verification of Performance for Precision and Trueness; Approved Guideline – Second Edition. CLSI Document EP15-A2, CLSI, Wayne, PA, USA, 2005.
[32] L.-W. Lai, O. Whitehair, M.-J. Wu, M. O'Meara, Y.-H.H. Lien, Clin. Genet. 63 (2003) 476.
[33] C.S. Nance, C.J. Klein, M. Banikazemi, S.H. Dikman, R.G. Phelps, J.C. McArthur, M. Rodriguez, R.J. Desnick, Arch. Neurol. 63 (2006) 453.