Chapter 4

Mass spectrometric quantification of glucosylsphingosine in plasma and urine of type 1 Gaucher patients using an isotope standard

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Mass spectrometric quantification of glucosylsphingosine in plasma and urine of type 1 Gaucher patients using an isotope standard

Mina Mirzaian [a], Patrick Wisse [b], Maria J. Ferraz [a], Henrik Gold [b], Wilma E. Donker-Koopman [a], Marri Verhoek [a], Herman S. Overkleeft [b], Rolf G. Boot [a], Gertjan Kramer [a], Nick Dekker [a], Johannes M.F.G. Aerts [a,b,*]

[a] Department of Medical Biochemistry, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[b] Department of Biochemistry, Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands

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ABSTRACT

Deficiency of glucocerebrosidase (GBA) leads to Gaucher disease (GD), an inherited disorder characterised by storage of glucosylceramide (GlcCer) in lysosomes of tissue macrophages. Recently, we reported marked increases of deacylated GlcCer, named glucosylsphingosine (GlcSph), in plasma of GD patients. To improve quantification, [5–9] $^{13}C_5$-GlcSph was synthesised for use as internal standard with quantitative LC-ESI-MS/MS. The method was validated using plasma of 55 GD patients and 20 controls. Intra-assay variation was 1.8% and inter-assay variation was 4.9% for GlcSph (m/z 462.3). Plasma GlcSph levels with the old and new methods closely correlate ($r = 0.968$, slope $= 1.038$). Next, we analysed GlcSph in 24 h urine samples of 30 GD patients prior to therapy. GlcSph was detected in the patient samples (median 1.20 nM, range 0.11–8.92 nM), but was below the limit of quantification in normal urine. Enzyme replacement therapy led to a decrease of urinary GlcSph of GD patients, coinciding with reductions in plasma GlcSph and markers of Gaucher cells (chitotriosidase and CCL18). In analogy to globotriaosylsphingsone in urine of Fabry disease patients, additional isoforms of GlcSph differing in structure of the sphingosine moiety were identified in GD urine samples.
In conclusion, GlcSph can be sensitively detected by LC-ESI-MS/MS with an internal isotope standard. Abnormalities in urinary GlcSph are a hallmark of Gaucher disease allowing biochemical confirmation of diagnosis.

1. Introduction

Gaucher disease (GD) is caused by deficiency of the enzyme glucocerebrosidase (GBA) resulting in lysosomal accumulation of its lipid substrate glucosylceramide (GlcCer) in tissue macrophages. These lipid-laden phagocytes, named Gaucher cells, prominently accumulate in liver, spleen, and bone marrow of GD patients. Their presence is thought to cause the hepatosplenomegaly, pancytopenia and bone complications expressed by GD patients [1]. The majority of GD patients develop a non-neuropathic (type 1) course of disease. More rarely, in severely affected patients the clinical manifestation involves the central nervous system at infantile or juvenile age (type 2 and type 3 GD, respectively). Two therapeutic interventions are available for treatment of visceral manifestations of GD. Firstly, enzyme replacement therapy (ERT), based on chronic intravenous administration of recombinant GBA, results in prominent improvement of visceral symptoms [2–4]. With substrate reduction therapy (SRT), oral administration of the iminosugar *N*-butyl-deoxynojirimycin aims to reduce GlcCer synthesis and thus reduce its accumulation [5]. SRT with Miglustat is used for mildly to moderately affected patients in whom ERT is not a therapeutic option [6]. Very recently the improved inhibitor Eliglustat has also been registered by the FDA as drug for treatment of type 1 GD [7,8]. The availability of costly therapies for type 1 GD has stimulated the search for biomarkers that can assist in diagnosis and individualised patient management. Several protein biomarkers for type 1 GD have been identified in the blood of patients. At least two of these, chitotriosidase and CCL18, are known to be produced by Gaucher cells and directly secreted into the blood [9,10]. Plasma chitotriosidase and CCL18 levels reflect disease progression and are presently used to monitor disease progression and response to therapeutic intervention [11]. Of note, the primary storage lipid GlcCer is only modestly increased in blood of symptomatic type 1 GD patients [12]. In contrast, glucosylsphingosine (GlcSph), the deacylated form of GlcCer, has been found to be markedly increased in plasma of type 1 GD patients [13]. This finding was confirmed in a more recent study by Rolfs et al. [14]. Our initial investigation showed an average 200 fold elevation in plasma GlcSph levels in symptomatic type 1 GD patients examined prior to ERT. In response to therapy, plasma GlcSph was found to decrease, mimicking corrections of chitotriosidase and CCL18 [13]. Inhibition of GBA activity in cultured macrophages with a highly specific irreversible inhibitor led within one day to a sharp increase in GlcSph, again supporting the idea that visceral Gaucher cells are a major source of plasma GlcSph [13]. Recent

* Corresponding author at: Department of Biochemistry, Leiden Institute of Chemistry, Leiden University, Einsteinweg. 55, 2300 RA Leiden, The Netherlands.
E-mail address: j.m.f.g.aerts@lic.leidenuniv.nl (J.M.F.G. Aerts).

Table 1
Measured transitions and possible structures of glucosylsphingosine isoforms.

Transition	Possible structure	ΔMass	RT (min)
462.3 > 282.3	GlcSph	0	3.37
460.3 > 280.3	GlcSph di-ene	−2	3.28
476.3 > 296.3	GlcSph [+OH, C=C] or GlcSph [+CH_2]	+14	2.84
476.3 > 278.3			2.83
478.3 > 298.3	GlcSph [+OH]	+16	2.90
478.3 > 280.3			2.89

investigations with an induced Gaucher mouse model also detected elevations in plasma GlcSph in symptomatic animals [15–17].

Given the interest in GlcSph as potential biomarker for GD, we improved its mass spectrometric detection. For this purpose we synthesised [5–9] $^{13}C_5$-GlcSph for use as internal standard with quantitative LC-ESI-MS/MS. We here report on the improved quantification of GlcSph manifestation and therapy of type 1 GD. In addition, we here describe the prominent occurrence in GD urine, but not blood, of additional elevated *m/z* transitions reflecting isoforms of GlcSph differing in the sphingosine moiety from regular GlcSph. This finding resembles observations earlier made for heterogeneity in lysoGb3 in urine of Fabry disease patients [18].

2. Materials and methods

2.1. Materials and standards

LC-MS grade methanol, water, formic acid, and HPLC grade chloroform were purchased from Biosolve (Valkenswaard, The Netherlands).and LC-MS grade butanol from Merck Millipore, Billerica, USA. Ammonium formate and GlcSph (D-glucosyl-β1-1′-D-*erythro*-sphingosine) were obtained from Sigma-Aldrich and Avanti Polar Lipids (Alabaster, USA), respectively.

2.2. Patients, plasma and urine samples

EDTA plasma (28 males and 27 females) and 24 hour urine samples were collected prior to therapy from Dutch patients (17 males, 13 females) suffering from type 1 GD, known by referral to the Academic Medical Center. Diagnosis of GD in patients was confirmed by genotyping and demonstration of deficient glucocerebrosidase activity in leukocytes or fibroblasts [19]. Plasma and urine samples were stored frozen at −20 °C until further use. Most patients were compound heterozygotes for the N370S and one other mutation in the GBA gene, with the exception of 5 patients homozygous for N370S. Plasma samples of 10 normal males and 10 females and urine samples from 21 male and 12 female healthy subjects were used as controls. Approval had been obtained from the institutional ethics committee and informed consent according to the Declaration of Helsinki.

2.3. Determination of plasma glucosylceramide

Glucosylceramide levels in plasma of GD patients and controls were analysed as previously described [20].

2.4. Synthesis of [5–9] $^{13}C_5$-labelled glucosylsphingosine

Isotope labelled GlcSph was synthesised as described earlier for [5–9] $^{13}C_5$-labelled globotriaosylsphingosine [21].

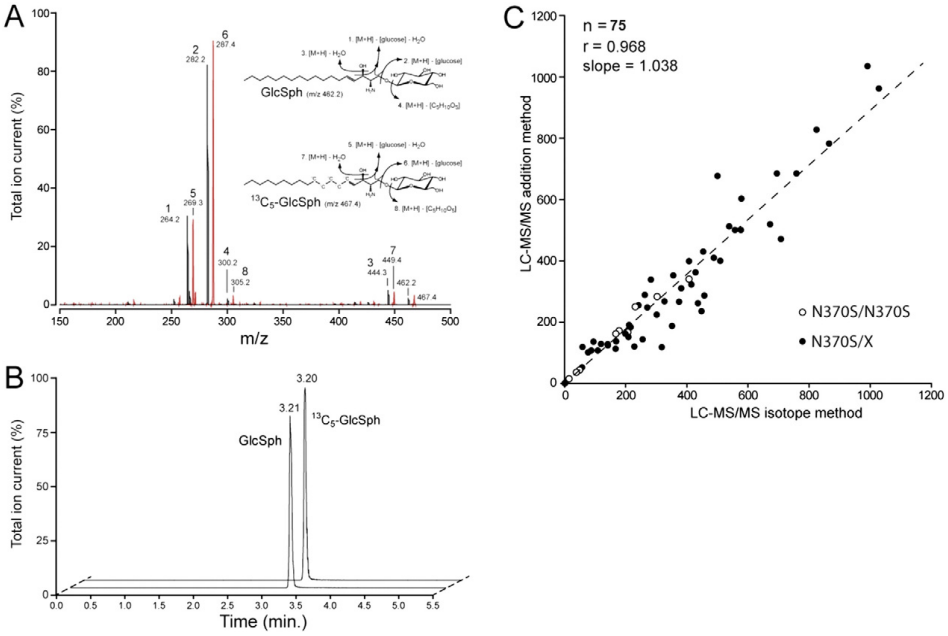

Fig. 1. Mass spectrometric detection and quantification of GlcSph. First an MS-scan was made from pure GlcSph and its isotope and their fragmentation was determined to establish the product daughter ions. (A) Fragmentation spectrum of GlcSph (*m/z* 462.3) and the internal standard [5–9] $^{13}C_5$-labelled GlcSph (*m/z* 467.3). Predominant peaks represent product ions corresponding to unlabelled and labelled sphingosine, numbers 2 and 6, respectively. (B) Elution pattern of GlcSph (*m/z* 462.3 > 282.3) and [5–9] $^{13}C_5$-labelled GlcSph (*m/z* 467.3 > 287.3) from UPLC. (C) Correlation of GlcSph quantification using the addition of GlcSph (addition method) or [5–9] $^{13}C_5$ -labelled GlcSph (isotope method) for quantification. 20 controls and 55 patients with type 1 GD were included.

2.5. Isolation of glucosylsphingosine from plasma and urine

GlcSph from plasma and urine was extracted using the method of Bligh and Dyer [22] with an overall recovery of 89% and 85%, respectively. Briefly, to 10 μL of plasma, 500 μL $CHCl_3$/MeOH (2:3, vol:vol) was added and samples were centrifuged for 3 min at 16,000 ×*g* to remove protein precipitate. Subsequently, 100 μL $CHCl_3$ and 260 μL MQ-H_2O were added for phase separation. After vigorous mixing and centrifugation for 3 min at 16,000 ×*g*, the lower organic phase was collected, and upper phase was re-extracted with 300 μL $CHCl_3$. Pooled lower phases were desiccated in a heat block, set at (37 °C) using a mild N_2 flow. Dried samples were re-dissolved in 100 μL MeOH, of which 10 μL was analysed by LC-ESI-MS/MS. For isolation of GlcSph from urine, 800 μL $CHCl_3$/MeOH (1:1, vol:vol) was added to 360 μL urine. After vigorous mixing, phases were separated by centrifugation as described above, and the lower phase was collected. Subsequently, the upper phase was re-extracted with 400 μL $CHCl_3$, and lower phases were pooled. After drying, the samples were re-dissolved in 100 μL MeOH, of which 10 μL was analysed.

An improved extraction was developed to recover optimally all GlcSph isoforms from urine. To urine (400 μL), 100 μL of internal standard [5–9]-$^{13}C_5$-labelled GlcSph, 0.1 pmol/μL was added and stirred briefly. Next, 900 μL methanol and 500 μL $CHCl_3$ were added. The samples were left at room temperature for 30 min, stirred occasionally and centrifuged for 10 min at 16,000 ×*g* in an Eppendorf centrifuge to spin down protein. The supernatant was transferred to a glass tube of 5 mL and next 500 μL aqua formate buffer 100 mM, pH 3.2 and 500 μL $CHCl_3$ were added while stirring. The tubes were centrifuged for 5 min at 3220 ×*g* to separate the phases. The upper methanol/water phase was collected (extract A). The lower phase was extracted by subsequent addition of 1000 μL methanol and 900 μL aqua formate buffer 100 mM, pH 3.2 and stirring for 1 min. The samples were centrifuged for 5 min at 3220 ×*g*, the upper phase removed, pooled with extract A and dried at 35 °C under a nitrogen stream. The residue was dissolved in 900 μL H_2O saturated with butanol, and then 900 μL butanol saturated with H_2O was added. The sample was stirred and centrifuged for 5 min in an Eppendorf centrifuge (16,000 ×*g*). The upper phase was transferred to a 1 mL tube with screw cap and the sample was dried under a gentle stream of nitrogen at 35 °C. The residue was dissolved in 150 μL methanol, stirred for 1 min, and sonicated for 30 s in a bath sonifier. The samples were centrifuged for 10 min at 16,000 ×*g* in an Eppendorf centrifuge to precipitate any insoluble material. The recovery of GlcSph analogues with this method was >90%.

2.6. Further optimization of LC-ESI-MS/MS analysis

Analytes were separated on an Acquity BEH C18 reversed phase column (column dimensions 2.1 × 50 mm, particle size 1.7 μm, Waters, Milford, USA), using the following eluents: Eluent A: H_2O: formic acid 99.5/0.5 (%) *v*/*v* containing 1 mM ammonium formate and Eluent B: methanol: formic acid 99.5/0.5 (%) *v*/*v* containing 1 mM ammonium formate. A mobile-phase gradient was used during a 6.50 min run: 0.00 min, 0% B; 2.50 min 100% B; 6.00 min 100% B; 6.05 min 0% B; and 6.50 min 0% B. The flow rate was 0.25 mL/min, and the eluent was diverted to waste between 0.00 and 2.00 min to keep the electrospray and the cone free of contaminants; data were collected between 2.00 and 6.05 min and after 6.05 min the eluent was again diverted to waste.

2.7. LC-ESI-MS/MS quantification of glucosylsphingosine

For quantification of plasma glucosylsphingosine, three methods were compared.

The first method (addition method) required duplicate measurements of samples that were spiked or not. In a sample, 10 pmol of C18-GlcSph (D-glucosyl-β1-1′-D-*erythro*-sphingosine) was added as spike for quantification. GlcSph concentrations were calculated by comparing signal intensities of endogenous GlcSph levels with the signal intensity of the spiked plasma.

The second method (isotope method) implied the addition of 12.5 pmol [5–9] $^{13}C_5$-labelled glucosylsphingosine as internal standard per sample in a duplicate measurement. The ratio of the peak areas of plasma glucosylsphingosine and [5–9] $^{13}C_5$-labelled internal standard was plotted against a ratio calibration curve of spiked GlcSph (0–2 nM) and 12.5 pmol of internal standard isolated from control plasma.

The third method involved the optimised extraction and LC-MS/MS analysis described above in combination with using the isotope-labelled internal standard. With all three methods ionisation suppression effects of the matrix, as well as variations in ionisation efficiency between standards and analytes of interest are excluded.

Optimization of ion source parameters, ionisation conditions, and chromatographic separation of GlcSph were performed as described previously [13]. Optimised ion source parameters are shown in Supplemental Table 1. Data were analysed using MassLynx software (version 4.1, Waters, Manchester, UK). Limit of quantification (0.3 nM) was defined as a signal to noise ratio (S/N) higher than 5. The intra-assay and inter-assay variations for plasma were determined as described previously [13]. Intra-assay variation for urine was determined by analyzing a pooled urine sample spiked with 3 different concentrations of GlcSph (0.3, 1.3, and 4.5 nM) in one batch, whereas inter-assay variation was determined by analyzing individually prepared samples in different runs over different days.

Table 2
Plasma glucosylsphingosine assay comparison: spike addition versus isotope addition.

	Addition method	Isotope method
LOQ[a]	0.3 nM	0.3 nM
Intra assay variation	3% ($n = 6$)	1.8% ($n = 6$)
Inter assay variation	6.8% ($n = 6$)	4.9% ($n = 6$)

Assay variation was determined at a 1 μM plasma GlcSph concentration. Data are presented as coefficient of variation (CV).
[a] LOQ is defined by a peak/noise ratio of 5.

2.8. Detection of GlcSph isoforms

Using the third method, various isoforms of GlcSph were detected in (see Table 1 for *m*/*z* transitions). Optimised ion source parameters are shown in Supplemental Table 2.

2.9. Stability of GlcSph isoforms

Urine samples were analysed prior and after 16 months storage at −20 °C. No significant differences in absolute concentrations of GlcSph in relation to storage were observed, indicating stability. The concentrations of various GlcSph analogues ranged always between 66 and 123%.

2.10. Determination of Gaucher cell plasma markers

Chitotriosidase and CCL18 were determined as described previously [23].

Table 3
Assay precision for glucosylsphingosine determination in urine.

	Intra assay variation	Inter assay variation
0.3 pmol/ml urine	10.2% ($n = 10$)	9.4% ($n = 10$)
1.3 pmol/ml urine	5.8% ($n = 10$)	7.0% ($n = 10$)
4.5 pmol/ml urine	3.0% ($n = 10$)	3.9% ($n = 10$)

Table 4
Stability of glucosylsphingosine in urine.

Spiked concentration (pmol/ml)	Measured concentration (pmol/ml)			
	$T = 0$	$T = 5$ days (−20 °C)	$T = 5$ days (4 °C)	$T = 5$ days (RT)
0.5	0.66	0.78	0.59	0.51
1.5	1.46	1.54	1.37	1.91
5	4.94	5.40	5.21	5.59

2.11. Statistical analyses

Results are presented as median and range. Mann–Whitney *U* testing was used for the following comparisons: Glucosylsphingosine levels of control subjects vs. GD patients. All correlations were tested by the rank correlation test (Spearman coefficient, ρ). Results were considered to be statistically significant when two-tailed *P*-values were <0.05.

3. Results

3.1. [5–9] $^{13}C_5$-labelled GlcSph detection by LC-MS/MS

To set up an improved LC-tandem mass spectrometric assay to quantify GlcSph in plasma and urine, we evaluated the use of [5–9] $^{13}C_5$-labelled GlcSph as internal standard. First the detection conditions were optimised by direct infusion of unlabelled and isotope labelled glucosylsphingosine. Two abundant peaks with *m/z* of 462.3 and 467.3 were detected, corresponding to the single protonated form $(M + H)^+$ of unlabelled and isotope labelled GlcSph, respectively (Fig. 1A). Fragmentation of either unlabelled or isotope labelled GlcSph by collision induced fragmentation yielded two main stable product ions (number 1 and 2 for unlabelled and 5 and 6 for isotope labelled glucosylsphingosine), corresponding to a loss of glucose with (number 1 and 5) or without a H_2O molecule (number 2 and 6) (Fig. 1A). In addition, minor fragments of GlcSph with loss of an H_2O molecule only (number 3 and 7), or sphingosine only (number 4 and 8) were detected (Fig. 1A). Electrospray and collision settings were optimised for fragmentation and detection of precursor–product with *m/z* 462.3 > 282.3 and 467.3 > 287.3 (see Supplemental Table 1). Since isotope labelled

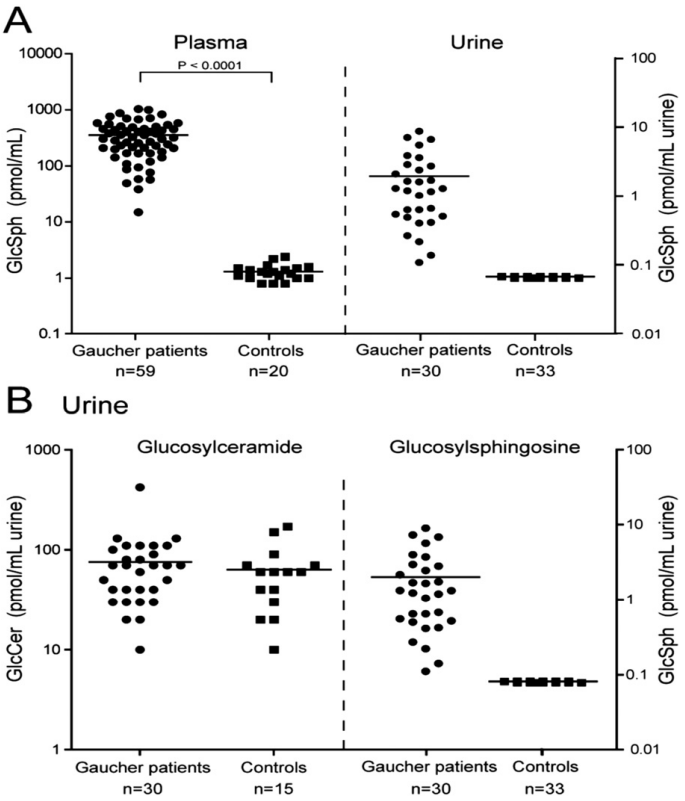

Fig. 2. Glucosylceramide and GlcSph levels. (A) GlcSph in plasma and urine of controls and type 1 GD. (B) GlcCer and GlcSph in urine of controls and type 1 GD patients.

and unlabelled GlcSph have identical chemical properties, similar electrospray and collision settings were used. As expected, the elution times were similar for unlabelled and isotope labelled GlcSph (3.21 and 3.20 min, respectively) (Fig. 1B).

3.2. Assay validation

Assay linearity, limit of quantification, and intra- and inter-assay variation were determined as described previously [13]. Briefly, serial dilutions of unlabelled and isotope labelled GlcSph were spiked to control plasma and subsequently extracted. Calibration curves were linear from the limit of quantification (LOQ) 0.3 nM to at least 2 μM, covering all concentrations found in plasma from GD patients (data not shown). The limit of quantification was identical for both methods (Table 2). Intra-assay and inter-assay variations using the isotope labelled standard were 1.8% and 4.9%, respectively, compared to 3% and 6.8% for the addition method, illustrating the benefit of using an isotope labelled standard in the assay. The reduced variability of the stable isotope based method is probably caused by an improved correction for variations in extraction efficiency, injection volume variability, matrix effects and mass spectrometer performance.

Subsequently the assays (addition method and isotope method) were compared by determination of plasma GlcSph levels in a cohort of patients with type 1 GD. As expected there is agreement of plasma concentrations between both methods (correlation plot slope of 1.038) (Fig. 1C). Plasma concentrations measured with both methods correlated with a rho value of 0.968, validating the use of the isotope method for assessing GlcSph concentrations in patient samples.

3.3. GlcSph in urine of type 1 GD patients

To test whether the assay was also applicable for measuring GlcSph in urine of GD patients, we validated the assay by supplementing 24 hour control urine with serial dilutions of unlabelled and isotope labelled glucosylsphingosine. The calibration curves of isolated GlcSph were linear from LOQ until at least 25 nM, covering all concentrations found in urine of GD patients. The limit of detection was 0.1 nM. To test assay precision and stability of GlcSph in urine, we supplemented urine with 0.3, 1.3, and 4.5 nM GlcSph, in the range of concentrations encountered in urine of GD patients. Intra-assay and inter-assay variations were good even at very low GlcSph concentrations (Table 3). Stability of GlcSph in urine was tested by incubation of spiked urine samples for 5 days at 4 °C, room temperature, or storage at −20 °C. GlcSph was found to be stable at all conditions (Table 4).

3.4. Glucosylsphingosine in plasma and urine samples of type 1 GD patients and control subjects

As reported earlier [13], GlcSph was found to be markedly increased in plasma of untreated symptomatic Gaucher patients (Gaucher, median 230.7 nM, range 15.6–1035.2 nM; Controls, median 1.3 nM, range 0.8–2.7 nM), (Fig. 2A). GlcSph was clearly detectable in urine samples of Gaucher patients (Gaucher, median 1.20 nM, range 0.11–8.92 nM), whereas in control urine only traces of GlcSph below the limit of quantification were detected (median 0.01 nM; range 0.001–0.071 nM) (Fig. 2A). The use of creatinine to normalise the amounts of GlcSph in urine neither improved nor worsened the discrimination of Gaucher patients from control subjects. Glucosylceramide was not found to be increased in urine of GD patients ($n = 30$) compared to control samples ($n = 15$) (Gaucher, median 70 nM, range 10–420 nM; Controls, median 60 nM; range 10–170 nM) (Fig. 2B).

3.5. Correlation of urinary GlcSph with plasma glycosphingolipids and established protein biomarkers

Previously, we showed that plasma GlcSph correlated significantly with plasma glucosylceramide, the primary storage lipid in GD [13]. Urinary GlcSph did however not correlate with plasma glucosylceramide (rho = 0.1069; $P = 0.6109$), nor with GlcSph present in plasma (rho = 0.2467; $P = 0.197$). Also no correlation could be found with protein biomarkers that reflect total storage cell burden (plasma CCL18 rho = 0.2352; $P = 0.3324$; urine CCL18 rho = 0.1483; $P = 0.374$, plasma chitotriosidase rho = 0.16; $P = 0.4227$). A good correlation was found between urine GlcSph and liver volume (rho = 0.7292; $P = 0.0001$).

3.6. Effect of enzyme therapy on urinary glucosylsphingosine levels

In order to study the effect of ERT on GlcSph in urine of type 1 GD patients, we collected 24 hour urine samples from two patients prior to and during 100 months of ERT. Both patients responded clinically well to ERT. Treatment resulted in a pronounced reduction in chitotriosidase and CCL18 and plasma GlcSph levels in both patients (Fig. 3). Similar to plasma GlcSph, urine GlcSph showed a sharp initial reduction followed by a more gradual decrease in both patients. In patient B an increase of plasma GlcSph occurred after approximately 90 months of therapy (Fig. 3B). Concomitantly, urinary GlcSph levels increased indicating a strong relation between plasma and urinary GlcSph.

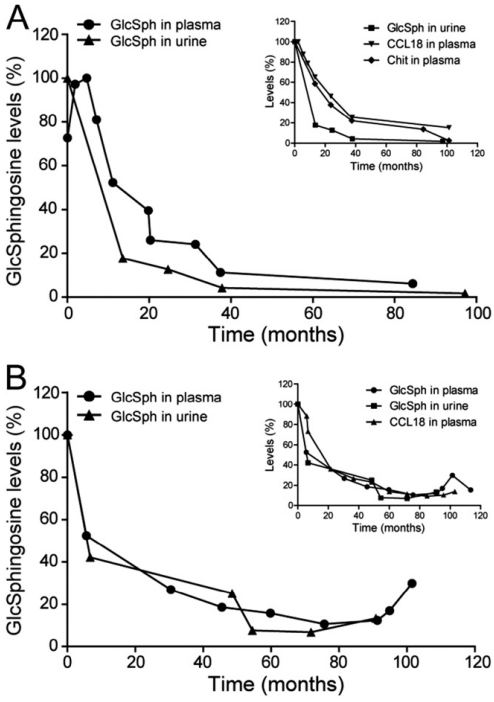

Fig. 3. GlcSph response to enzyme therapy in plasma and urine of two type 1 GD patients. (A) Urinary and plasma GlcSph in patient A. Insets compare urinary GlcSph response with of plasma levels and Gaucher biomarkers chitotriosidase (chit) and CCL18. (B) Idem patient B, deficient in chitotriosidase.

3.7. Presence of GlcSph isoforms in plasma and urine

We firstly monitored in plasma samples of GD patients and normal subjects various precursor–product transitions shown in Table 1. As can be seen in Fig. 4A, GlcSph in plasma of type 1 GD patients is largely the regular sphingoid base (*m/z* 462.3 > 282.3).

We next studied GlcSph in urine of type 1 GD patients. During the course of our investigation Auray-Blais et al. reported the occurrence of multiple isoforms of globotriaosylsphingosine (lysoGb3) in urine of Fabry disease patients, postulated to differ in the chemical structure of the sphingosine moiety [18,24–26]. One of these isoforms of lysoGb3 is the di-ene structure as we had also observed ourselves independently in plasma samples [27].

Investigation of urine of GD patients revealed that the regular GlcSph (*m/z* 462.3 > 282.3) is a minor component as compared to the situation in plasma (Fig. 4B). Relatively more abundant in urine is the signal from GlcSph di-ene (*m/z* 460.3 > 280.3 as compared to that for regular GlcSph. Elevated in GD urine samples are some additional structures: *m/z* 476.3 > 296.3, 476.3 > 296.3, 478.3 > 280.3 and 478.3 > 298.3, and to lesser extent *m/z* 434.3 > 254.3, 450.3 > 252.3, and 496.3 > 284.3. The same increments in parent masses of 14 and 16 Da for the elevated additional structures (*m/z* 476.3 and 478.8), compared with regular GlcSph (*m/z* 462.3), are similar to the increased masses earlier reported for abundant urinary lysoGb3 isoforms by Auray-Blais et al. [18]. This suggested to us that possibly similar heterogeneity occurs in the

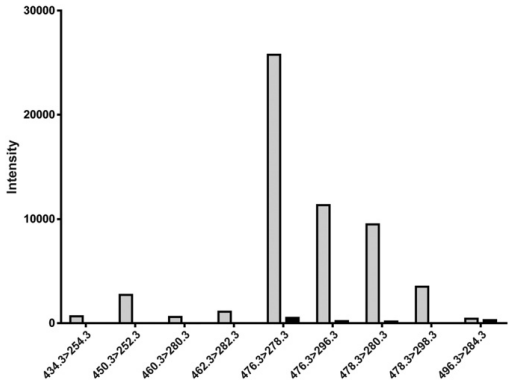

Fig. 5. Degradation of isoforms by recombinant glucocerebrosidase. Urine was incubated with excess of recombinant glucocerebrosidase resulting in almost complete (98.6%) conversion of normal glucosylsphingosine to sphingosine. All the proposed glucosylsphingosine analogues were likewise degraded by treatment with recombinant glucocerebrosidase. Shown is residual isoform after treatment with and or without enzyme. Left bar: untreated, right bar: treated. Of note, Only 496.3 > 284.3 was poorly digested suggesting that this (very minor) component is not related to glucosylsphingosine.

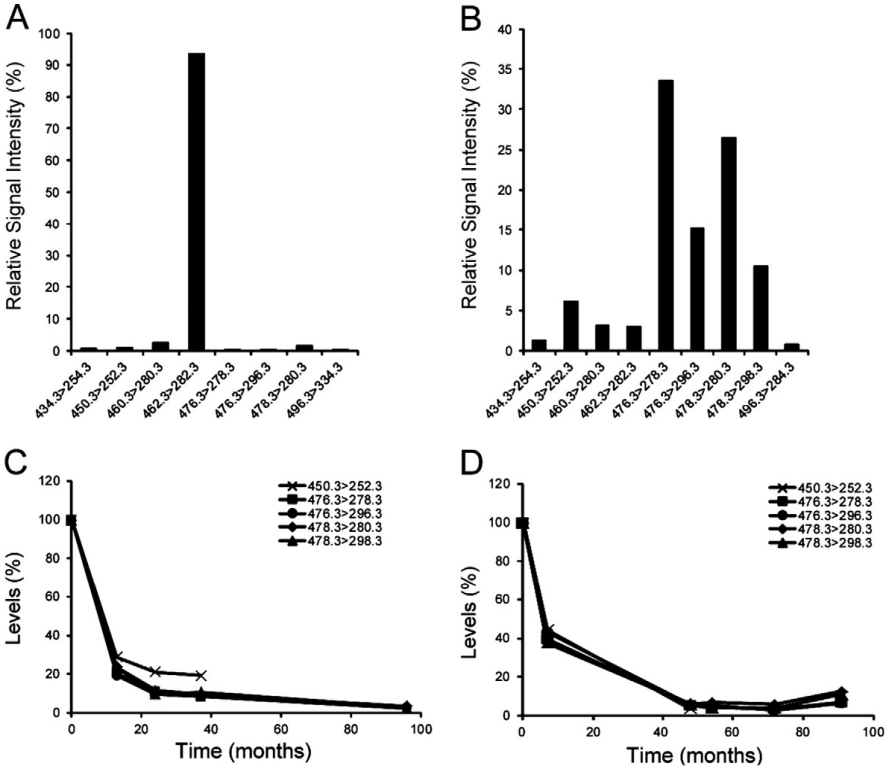

Fig. 4. GlcSph isoforms in urine of GD patients and their response to enzyme replacement therapy. (A) Relative signal intensities of the transitions for GlcSph and isoforms measured in plasma of three type 1 GD patients (mean). (B) Relative signal intensities GlcSph isoforms in urine of four type 1 GD patients (mean). (C, D) Response to enzyme therapy of the five most abundant GlcSph isoforms in urine of two type 1 GD patients.

sphingosine moiety of urinary GlcSph of GD patients as observed for urinary lysoGb3 of Fabry disease patients. We therefore next investigated the effect of therapy of type 1 GD patients on the additional structures in urine. Fig. 4C and D shows that the abundant structures all reduce comparably with treatment. This similar response suggested a relationship between the additional structures and regular GlcSph.

Next, we determined experimentally whether the observed additional structures in Gaucher urine are indeed GlcSph isoforms. For this purpose, urinary lipids were digested with recombinant glucocerebrosidase (Cerezyme, Genzyme). Like the finding for regular GlcSph (m/z 462.3 > 282.3) and GlcSph di-ene (m/z 460.3 > 280.3), also more than 98% of the additional structures with m/z 476.3 > 296.3, 478.3 > 298.3, 434.3 > 254.3, and 450.3 > 252.3 were digested by glucocerebrosidase (Fig. 5). This finding demonstrates the presence of a beta-glucosyl moiety in all these structures. Only the structure with transition m/z 496.3 > 284.3 was poorly digested by glucocerebrosidase suggesting that this (very minor) component is likely not related to GlcSph. As mentioned, Auray-Blais et al. have earlier proposed structures for the urinary lysoGb3 isoforms. Based on her proposal, possible hydroxylated and/or methylated and unsaturated structures for the noted isoforms of GlcSph are listed in Table 1. Evidently, purification of the additional structures from large quantities of GD urine followed by NMR analysis will be required to establish unequivocally their identity.

4. Discussion

Kanfer et al. were the first to identify GlcSph in spleen of GD patients [27]. Next, accumulation of this sphingoid base in brain of GD patients was found to correlate with central nervous system (CNS) involvement [28,29]. Following the availability of more sensitive detection methods, we again looked into the presence of GlcSph in plasma of type 1 GD patients. We and Rolfs et al. demonstrated a several hundred fold increase in GlcSph levels in plasma of GD patients [13,14]. It was firstly proposed by us that plasma GlcSph levels may assist clinicians in decision making on initiation and optimizing individual dosing of therapy [13]. We now developed a robust mass spectrometric detection method using an isotope-labelled standard for more sensitive quantification of GlcSph. The isotope labelled internal standard allows compensation for losses during extraction, ionisation efficiency, and mass spectrometric performance. Since the isotope-labelled standard is chemically identical to the analyte of interest, the elution pattern from UPLC as well as the ionisation efficiency in the mass spectrometer is identical to the analyte. The present method is superior to the one used previously, where a known amount of GlcSph was added to a clinical sample and quantification was performed by subtracting signal intensities [13]. It allows for higher throughput and delivers more reliable data.

A second aim of our investigation was to establish whether GlcSph is also elevated in urine of type 1 GD patients. Indeed, we could demonstrate for the first time elevated levels of GlcSph in urine of symptomatic type 1 GD patients. Of interest in this connection is the comparison with the situation in Fabry disease, a lysosomal storage disorder where the glycosphingolipid globotriaosylceramide and its derivative globotriaosylsphingosine (lysoGb3) accumulate in plasma [30]. In Fabry disease, lysoGb3 is also elevated in urine of patients as well as that of Fabry mice [31]. However, investigations by Auray-Blais et al. revealed that the regular lysoGb3 is only a minor component in urine of Fabry disease patients [18,24–26]. Not the regular lysoGb3, but other analogues of lysoGb3 with different sphingosine structure (hydroxylated, branch methylated, and multi-unsaturated isoforms as proposed by Auray-Blais et al.) are the most abundant signals [18]. In the present study we made a very similar observation for GlcSph in urine of GD patients: not the regular sphingoid base transition (m/z 462.3 > 282.3), but other m/z transitions with parent masses of +14 and +16 Da are the more abundant signals in urine of type 1 GD patients (see Fig. 4). Importantly, in plasma of type 1 GD patients regular GlcSph is the prominent form (>90%) and monitoring of this structure is to be preferred for assessment of type 1 GD patients.

Although all investigated GD patients showed elevated levels of urinary GlcSph, its apparent chemical heterogeneity makes it less attractive for monitoring GD patients. At present it is unclear what the precise structures of the GlcSph isoforms are, how they originate and which enzymes are responsible for their formation. It cannot be excluded that the pattern of GlcSph isoforms is interindividually different or even influenced by external factors. Clearly more insight in these possibilities will have to be obtained by further investigations on this topic. The noted structural heterogeneity in the urinary GlcSph and the possible physiological relevance of this is intriguing. A similar, extensive structural heterogeneity in the sphingoid base of sphingolipids is also prominent in the epidermis. For example, in human skin sphingolipids with a 6-hydroxylated sphingosine are abundant and elevated in atopic dermatitis. Neither the biosynthesis nor the (patho)physiological roles of these unique lipids have been established; see ref. [32] for a review.

In conclusion, a robust sensitive LC-MS/MS assay to measure GlcSph in (unconcentrated) urine samples was developed exploiting the advantages of an isotope labelled standard. A marked elevation of GlcSph in urine of untreated type 1 GD patients was demonstrated, but also the occurrence of a considerable number of apparent GlcSph isoforms, particularly those with +14 and +16 Da parent masses. Given the complexity of urinary GlcSph, the use of plasma GlcSph measurement with the new method is recommended to monitor therapy efficacy in type 1 GD patients and the biochemical confirmation of diagnosis of Gaucher disease.

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Supplemental data

Supplemental Table 1. Instrument parameters Waters TQD: Regular GlcSph

Parameter	Setting
Cone voltage	30 V
Capillary voltage	3.50 kV
Extractor voltage	1 V
Source temperature	150°C
Desolvation temperature	450°C
Cone gasflow	50 L/hr
Desolvation gasflow	500 L/hr
Collision gasflow	0.15 mL/min
Collision energy	20 V
Ion mode	ES+
Dwell time	0.1 s
Transitions (GlcSph)	m/z 462.3 > m/z 282.3
Transitions [5,6,7,8,9] $^{13}C_5$ GlcSph)	m/z 467.3 > m/z 287.3

Supplemental Table 2. Instrument parameters Waters TQD: GlcSph Isoforms

Parameter	Setting
Cone voltage	30 V
Capillary voltage	3.50 kV
Extractor voltage	2 V
Source temperature	140°C
Desolvation temperature	450°C
Cone gasflow	50 L/hr
Desolvation gasflow	950 L/hr
Collision gasflow	0.12 mL/min
Collision energy	20 V
Ion mode	ES+
Dwell time	0.05 s