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

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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

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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].

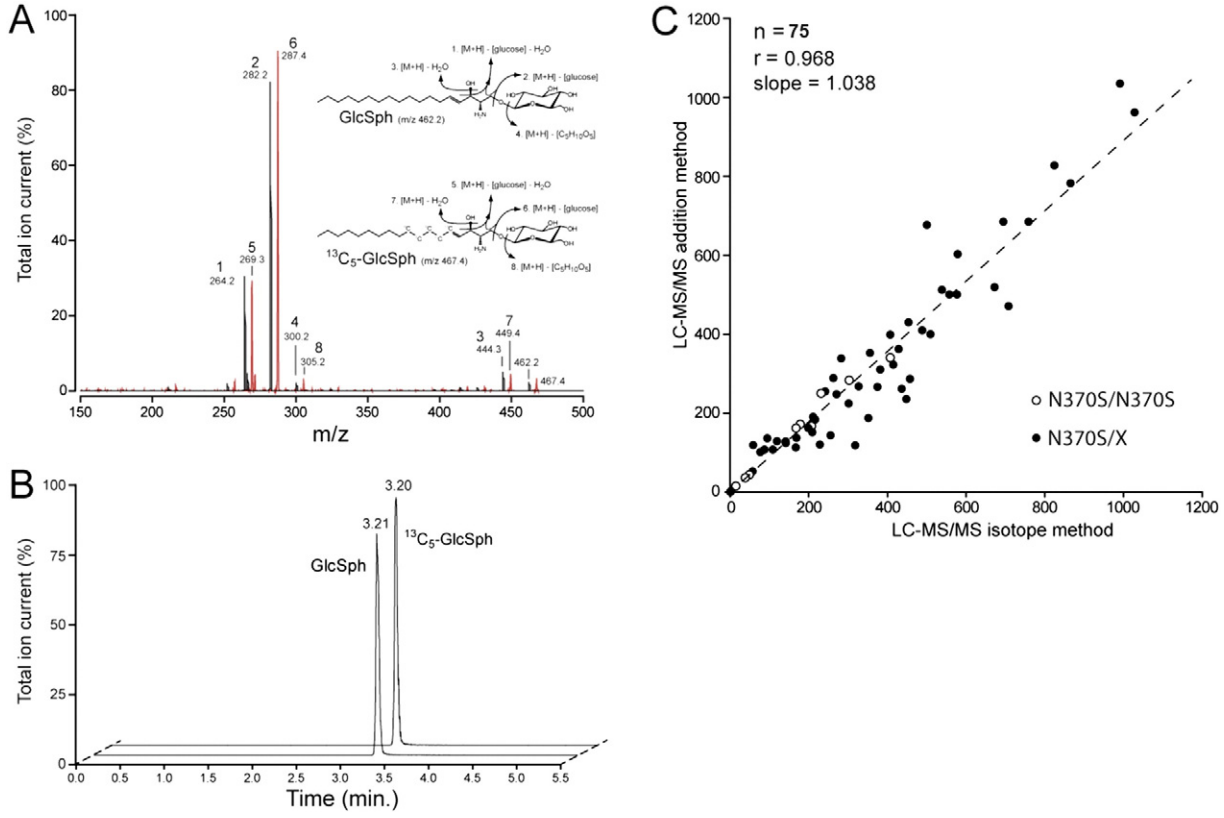

Fig. 1. Mass spectrometric detection and quantification of GlcSph. First an MS-scan was made from pure GlcSph and its isotope and their fragmentation was determined to establish the product daughter ions. (A) Fragmentation spectrum of GlcSph (*m/z* 462.3) and the internal standard [5–9] $^{13}C_5$-labelled 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.