Clinica Chimica Acta 450 (2015) 6–10

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

Rapid, single-phase extraction of glucosylsphingosine from plasma: A universal screening and monitoring tool

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ARTICLE INFO

Article history:
Received 18 May 2015
Accepted 24 July 2015
Available online 29 July 2015

Keywords:
Gaucher disease
Enzyme replacement therapy
Biomarker
Diagnosis
Glucosylsphingosine
Mass spectrometry

ABSTRACT

Background: Glucosylsphingosine (GluSph) has emerged as a biomarker for the inherited metabolic disorder, Gaucher disease (GD). We developed a simple laboratory test to measure plasma GluSph and show that elevated GluSph is diagnostic for GD as well as informing on disease burden for monitoring patients on treatment.
Methods: GluSph was measured from a single-phase total lipid extraction of 0.01 mL of plasma by liquid chromatography–electrospray ionisation–tandem mass spectrometry and concentrations extrapolated from a seven point standard curve (0.04 to 20 pmoL). A total of 1464 samples were tested and longitudinal assessment of an additional 20 GD patients.
Results: All patients with GD had elevated GluSph compared to unaffected controls and 16 other metabolic disorders. GluSph was also slightly elevated in three patients with Krabbe disease but not at concentrations to confuse a GD diagnosis. GluSph correlated with chitotriosidase in the majority of GD patients on treatment who were informative for this marker.
Conclusions: GluSph can be easily measured from 0.01 mL of plasma and is useful as a diagnostic marker for GD with the current platform suited to high-throughput screening. It outperforms other GD biomarkers for biochemical monitoring of patients receiving enzyme replacement therapy for all individuals.

1. Introduction

Gaucher disease (GD) is an autosomal recessive inborn error of glycosphingolipid metabolism arising from an absolute or functional deficiency of the lysosomal hydrolase, acid β-glucosidase, or less commonly a deficiency of its complicit activator protein, saposin C [1,2]. The consequent impairment of glycosphingolipid catabolism is characterised by the accumulation of glucosylceramide, and its cognate *lyso*-derivative, glucosylsphingosine (GluSph) leading to chronic systemic inflammation and a heterogenous multi-systemic phenotype. The major site of lysosomal storage is in the cells of the mononuclear phagocyte system (macrophages), especially those in the liver, spleen, lung and bone marrow. These cells, known as 'Gaucher cells', show characteristic lipid storage vacuoles displacing healthy cells and induce pathology largely via unknown mechanisms [3]. Clinical manifestations involve varying combinations of hepatosplenomegaly, degrees of cytopenia, skeletal complications, lung disease and central nervous system disease in the rarer variants.

Enzyme replacement therapy (ERT) for the non-neuronopathic form of GD is now well-established in the clinical environment based on a strong safety and efficacy record [4]. Predicting treatment response and the ability to tailor the dose and frequency of therapy for individual patients are supposedly addressed by the biochemical monitoring of disease regression. Several surrogate markers have demonstrable applicability to provide direct measurement of the biochemical changes that result from disease followed by correction of the blood biochemistry with treatment. Of these, the most widely used is plasma chitotriosidase; a chitinase produced by activated macrophages [5]. Although the function of chitotriosidase is not clearly understood it is thought to be produced by 'Gaucher cells' and is elevated several hundred fold in plasma from patients with GD [6]. Widely used as a biochemical marker to monitor GD patients receiving therapy, and to confirm diagnosis, chitotriosidase, a marker of inflammation, is an epiphenomenon of disease activity, therefore non-specific and reportedly elevated in many other lysosomal and non-lysosomal disorders [7–9]. Highlighting the non-specificity for GD, chitotriosidase has been used to biochemically monitor patients receiving ERT for the

Abbreviations: GD, Gaucher disease; GluSph, glucosylsphingosine; LC–ESI–MS/MS, liquid chromatography–electrospray ionisation–tandem mass spectrometry; P, Pearson correlation coefficient.

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http://dx.doi.org/10.1016/j.cca.2015.07.026
0009-8981/

related disorder, Fabry disease [10]. Moreover, up to 6% of the population, including GD patients, are deficient in chitotriosidase activity due to a 24 base pair duplication in the chitotriosidase gene [11]. This precludes the use of chitotriosidase as a biomarker in these patients and complicates interpretation in 40% of the population who are heterozygous for this null mutation. CCL18, like chitotriosidase, is non-specific but is used as a substitute biomarker for individuals genetically lacking chitotriosidase and would be the second most commonly used biomarker [12].

Other biomarkers are noteworthy, including the first report of a biomarker for GD, angiotensin converting enzyme (ACE), which like chitotriosidase and CCL18 is not specific for GD [13]. Correlating with disease activity, ACE suffers from not being useful for biochemically tracking patients receiving therapy when compared with chitotriosidase and CCL18 [14]. Given the complex network of lipid alterations that stem from glucosylceramide accumulation, plasma lipids [15] as well as HDL cholesterol [16] have also been promoted as candidate biomarkers.

More recently, the *lyso*-derivative of glucosylceramide, GluSph, has surfaced as a sensitive and specific biomarker for GD. Indeed its elevation in blood appears unique for the GD phenotype and GluSph shows evidence of correlating with other biomarkers such as chitotriosidase and CCL18, patient genotype and other disease parameters [17,18]. Here we sought to develop a rapid, semi-automated assay for GluSph that requires only 0.01 mL of plasma and demonstrate that GluSph serves not only as a biomarker to measure dynamic changes in disease burden over time, but also as a diagnostic marker for GD and may have applicability for the related metabolic disorder, Krabbe disease.

2. Materials and methods

2.1. Patient samples and materials

Plasma samples were obtained from patients referred to our Department for diagnosis and biochemical monitoring and their use in this study was approved by the Institution's Ethics Committee. All samples were de-identified with the exception of the 20 GD patients who were evaluated for longitudinal monitoring. Of these, two were untreated, 11 were receiving Velaglucerase (VPRIV® Shire, Lexington, MA), six Taliglucerase (Elelyso™ Pfizer, New York NY) and one Imiglucerase (Cerezyme® Genzyme, Cambridge, MA). All solvents were LiChrosolv gradient grade except $CHCl_3$, which was reagent grade containing 1% CH_3CH_2OH, and were purchased from Merck (Darmstadt, Germany). The internal standard, N-palmitoyl-*d3*-lactosyl ceramide (LC C16:0(*d3*)), and the glucosylsphingosine (glucopsychosine) standard were purchased from Matreya Inc. (Pleasant Gap, USA).

2.2. Chitotriosidase activity

Plasma chitotrioisdase activity was determined using the fluorescent substrate 4-methylumbelliferyl-β-D-N,N′,N″-triacetylchitotriosidase as described previously [6].

2.3. Single-phase total lipid extraction

A total single phase lipid extraction was performed from 0.01 mL of plasma with the addition of 0.2 mL of $CHCl_3$:CH_3OH (1:2) containing 10 pmol of LC C16:0(*d3*) as internal standard. Low, medium and high QC samples were prepared by the addition of GluSph to control plasma prior to extraction at concentrations of 88, 175, and 1750 pmol/mL, respectively. An eight point calibration curve was prepared at 4, 10, 20, 100, 200, 500, 1000 and 2000 pmol/mL of GluSph. Samples were mixed on a rotary mixer for 10 min, sonicated in a water bath for 30 min and then allowed to stand at room temperature for 20 min. The protein was sedimented by centrifugation at 13,000 *g* for 10 min and the supernatant removed and dried under a gentle stream of nitrogen at 40 °C. The lipid extract was reconstituted in 0.1 mL of CH_3OH containing 10 mM NH_4COOH prior to liquid chromatography/electrospray ionisation–tandem mass spectrometry (LC/ESI–MS/MS).

2.4. High pressure LC/ESI–MS/MS

Chromatographic separation was achieved using a Zorbax Eclipse C18, 2.1 mm × 50 mm column maintained at 40 °C in a 1290 Infinity Thermostatted Column Compartment, configured with an Agilent 1290 Infinity Pump and a 1290 Infinity Sampler maintained at 8 °C. A 1290 inline filter containing a 0.3 μm frit was placed in front of the column. Solvent A was 60% H_2O, 40% CH_3CN containing 10 mM CH_3COOH and solvent B was 90% $(CH_3)_2CHOH$, 10% CH_3CN containing 10 mM CH_3COOH. Initial mobile phase conditions were 90% solvent A and 10% solvent B, which was linearly ramped to 50% by 2 min and then to 100% solvent B at 8.0 min. This was held for 0.5 min followed by a return to 90% solvent A at 9 min, which was equilibrated for 1 min prior to the next injection. Injection volumes were 1 μL and the flow rate was 0.4 mL/min.

For the first minute column flow was diverted to waste and then directly into the electrospray source (ES 5500 V) of an AB SCIEX 5500 triple quadrupole tandem mass spectrometer with an ion source temperature of 400 °C. Nitrogen was used for curtain gas, 10 units; collision gas, 6 units; ion source gas 1, 30 units and ion source gas 2, 40 units. MS/MS parameters were optimised using a standard solution of 1 nmol/mL GluSph and were as follows: declustering potential of 66; entrance potential of 10, collision energy of 27, collision cell exit potential of 19 and the dwell time was 20 ms. Multiple-reaction monitoring transitions were 462.4/282.4 for GluSph and 865.5/264.4 for the LC C16:0(*d3*) internal standard. A calibration curve was constructed by plotting the concentrations of GluSph on the *x*-axis versus the chromatographic peak area ratio of GluSph to LC C16:0(*d3*) on the *y*-axis. Linear regression analysis was performed using the calibration curve data of $y = mx + b$ equation and a $1/x^2$ weighting with Analyst 1.6.2 software. Acceptable calibration criterion for linearity was the coefficient of determination $r^2 \geq 0.99$ and accuracy required each standard to be within 10% of the target concentration. The recovery of GluSph from plasma was determined by the concentration of GluSph obtained following extraction of the low, medium and high QC material as a percentage of the amount of GluSph spiked into the plasma.

3. Results and discussion

3.1. Method validation

Due to the absence of a suitable isotopic labelled internal standard for GluSph we used LC C16:0(*d3*). Although *lyso*-ceramide trihexoside was used in a previous study [18], this compound is present in significant concentrations in the plasma of patients with the related disorder, Fabry disease [19]. Indeed, *lyso*-ceramide trihexoside is used as a biomarker for Fabry disease and as such its use as an internal standard is inappropriate in the laboratory setting as it could create diagnostic confusion. Our initial choice of internal standard was the stable isotope of glucosylceramide, *N*-palmitoyl-*d3*-glucopsychosine, that we have used previously [15], however a contaminant peak was noted in the chromatography thereby also rendering this unsuitable. Fig. 1 shows that baseline separation between GluSph and LC C16:0(*d3*) was adequately achieved with retention times of 2.3 and 5.5, respectively, with no contaminants or interfering peaks. The calibration curve was linear over the biological range (4–2000 pmol/mL) as depicted in Fig. 2. Interassay CV from 15 separate runs over four months were 0.17, 0.16 and 0.16 for the low, medium and high QC material, respectively. The recovery of GluSph from plasma was >90% calculated from the concentration determined following extraction from the QC samples compared with that spiked into the plasma. The limit of detection was calculated as two times signal:noise at 2 pmol/mL and the limit of