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An easy and sensitive method for determination of globotriaosylceramide (Gb3) from urinary sediment: Utility for Fabry disease diagnosis and treatment monitoring

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ABSTRACT

Background: Fabry disease is an X-linked disorder that results from the deficiency of the lysosomal enzyme α-galactosidase A. The defect leads to the accumulation of globotriaosylceramide (Gb3). The detection of Gb3 accumulated in different tissues may help in the diagnosis and enzyme replacement therapy monitoring. For this reason, we developed a simple method available to clinical laboratories to measure this analyte.
Methods: Gb3 excretion was determined by the incubation of urine sediment glycolipids from Fabry patients with agalsidase α and subsequent determination of galactose produced.
Results: The amount of urinary Gb3 in Fabry hemizygotes was significantly higher ($p = 0.00001$) than the amount in normal controls. Patients undergoing enzyme replacement therapy with agalsidase α showed a significantly lower content of Gb3 in urine sediment. This method showed a good recovery and comparability with a previously validated method.
Conclusions: We developed an easy method for quantification of Gb3 in urine samples from Fabry patients, by the use of the specific recombinant enzyme for this glycolipid, that does not require complex infrastructure. Urinary Gb3 as measured by this enzymatic method could be useful for the diagnosis and monitoring of treatment in Fabry patients.

1. Introduction

Fabry disease (McKusick 301500) is an X-linked disorder of glycosphingolipid catabolism that results from the deficiency of the lysosomal enzyme α-galactosidase A (α-D-galactoside galactohydrolase, EC 3.2.1.22; α-Gal A) [1,2]. This defect leads to the accumulation of its substrates, mainly globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1Ceramide; Gb3) throughout various cells, organs and tissues [3]. Clinical manifestations in males with Fabry disease include acroparesthesia, angiokeratomas, hypohidrosis, and corneal opacities of early onset, and the development of renal, cardiac and cerebral complications, leading to early death [4]. In contrast, heterozygous females are either asymptomatic or have mild to severe manifestations [5].

The diagnosis of Fabry hemizygotes disease is based on the demonstration of reduced levels of α-Gal A activity in different samples [6]. However, this assay is inconclusive in heterozygous females, which shows ranges of activity between low to normal levels. Genetic analysis may be the best diagnostic procedure. More than 350 different mutations were detected in α-Gal A gene from patients with Fabry disease [7]. Recently, enzyme replacement therapy with human recombinant α-Gal A was introduced. This therapy significantly reverses the main clinical manifestations of Fabry disease [8].

The detection of Gb3 accumulated in different tissues may help in the diagnosis and enzyme replacement therapy monitoring. Gb3 from urine sediment comes from desquamated tubular cells and may reflect renal storage. Moreover, it was suggested as a possible surrogate biomarker of disease progression and therapy evaluation. Several chromatographic and spectrometric methods of Gb3 quantification have been developed [9,10] that require expensive equipment, generally restricted to research laboratories. For this reason, it would be practical to have a simple method available to clinical laboratories to measure this analyte.

2. Materials and methods

2.1. Patients

Thirty four hemizygotes and 41 heterozygotes Fabry (Table 1) patients were recruited by AADELFA. AADELFA is a medical association whose objective is the study and dissemination of knowledge of lysosomal storage diseases in Argentina. The protocol was approved by the scientific committee of AADELFA according to provisions of the Declaration of Helsinki in 1995. The nature and purpose of the study and its possible risks were explained to all volunteers. All the patients gave their informed consent previous to the participation in the study.

The diagnosis of male patients with Fabry disease was established by clinical examination, reduced enzymatic activity and genetic test. The females were obligate carriers as demonstrated by pedigree analysis, and diagnosis was confirmed by genetic test. Twenty heterozygotes and 26 hemizygotes were undergoing enzyme replacement

Abbreviations: Gb3, globotriaosylceramide; α-Gal A, α-galactosidase A; ERT, enzyme replacement therapy.

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Table 1
Demographic data of the groups of patients and control individuals.

	Male controls	Female controls	Hemizygous patients	Hemizygous + ERT	Heterozygous patients	Heterozygous + ERT
N	22	20	8	26	21	20
Age, y (Mean ± SD)	23.89 ± 11.14	42.23 ± 17.34	20.75 ± 12.80	28.12 ± 10.82	38.62 ± 18.45	44.10 ± 17.31
Range	15–55	16–55	10–51	10–52	17–70	15–72

therapy (ERT) with agalsidase α (Replagal, Shire HGT, Lexington, KY). None of the patients suffered from hypersensitivity reactions, nor anti agalsidase antibodies have been analyzed. Forty-two healthy individuals matched for age and sex served as controls. Demographic data of the groups of patients and control individuals is shown in Table 1.

2.2. Isolation of urinary sediment glycolipids

Twenty-four-hour urine samples were collected and provided by the patients. Moreover, sixteen patients provided 2 samples, the first at diagnosis and the second after 6 to 24 months of the initiation of ERT. Two hundred ml aliquots of the 24 h urine were centrifugated at 10,000 ×*g* for 15 min at 4 °C. The sediments were stored at −20 °C until use. Glycolipids were extracted according to the method of Vance and Sweeley [11]. Briefly, 15 ml of Chloroform: Methanol 2:1, v/v was added to the urine sediment, and incubated overnight at room temperature. Four milliliters of water was added to the tube and centrifugated to separate phases. Upper aqueous phase was removed, and the lower phase was brought to dryness. One milliliters of methanol and 0.1 ml 1.0 mol/l NaOH was added to the dried residue, and incubated at 37 °C overnight. After the addition of 2 ml of Chloroform and 0.5 ml water and separation of the phases, the upper phase was removed. The lower phase, corresponding to the glycolipid extract, was brought to dryness and used for Gb3 determination.

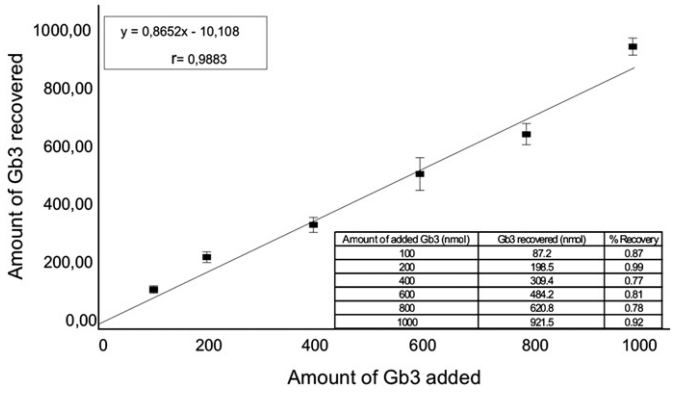

Fig. 1. Recovery curve of Gb3 obtained by the addition of known amounts of Gb3 to aliquots of urinary sediment from normal controls.

2.3. Gb3 determination

Glycolipid extract was dispersed in 100 μl of 0.15 mol/l acetate buffer pH = 4.5; and sodium taurodeoxycholate (Sigma, St Louis, MO) was added to emulsify the glycolipids at a final concentration of 0.046 mol/l. Gb3 present in the urinary glycolipids was hydrolyzed to galactose and lactosylceramide by the addition of 10 μl of 1 mg/ml agalsidasa α (Shire HGT) and incubation at 37 °C overnight. Agalsidase alfa is available in a 1 mg/ml solution, and was stored aliquoted at −20 °C until use without significant loss of enzymatic activity. To quantify the galactose produced by the enzymatic hydrolysis, 3 mm-filter paper discs were impregnated with the mixture reaction solution. After drying the paper discs at room temperature for 4 h, galactose was determined by a modified enzymatic fluorometric method using galactose dehydrogenase, diaphorase and resazurine [12]. The results were expressed as Gb3 nmol per 24 h urine.

2.4. Validation of method

The linearity of the response and the recovery were assessed by adding varying amounts (100–1000 nmol/24 h urine) of standard Gb3 (Matreya, Pleasant Gap, PA) to urinary sediment from normal controls. The detection and quantification limits were determined as the mean value + 3 SD and the mean + 5 SD respectively, from 10 normal control urines [13]. The within- and between-run precision was assessed by measuring 3 samples 3 times in the same run and in 3 different runs, respectively.

2.5. Comparability with a previously validated method

For comparison of Gb3 results from this enzymatic method with a previously validated method, urinary Gb3 from twenty two of the samples were also analyzed using a HPLC method, performed at the Shire HGT's central laboratory.

2.6. Statistical analysis

Mean values of samples from patients and normal controls and their standard deviation (Fig. 3) were calculated and statistical means comparison was performed using paired Student's *t*-test at the level of $p<0.05$.

3. Results

The linearity of the response for Gb3 was acceptable at concentrations between 100 to 1000 nmol/24 h with a correlation coefficient of

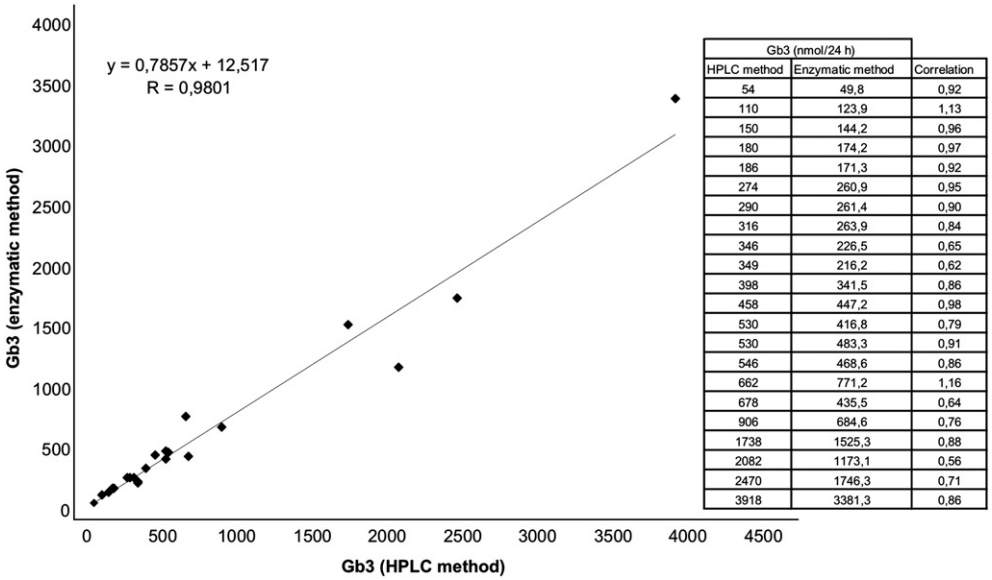

Fig. 2. Comparison of the results of urinary Gb3 from 22 samples as assayed by this method and a previously validated HPLC method. Correlation is calculated as the ratio between enzymatic method and HPLC.