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ORIGINAL PAPER

Evaluation of the urinary globotriaosylceramide (Gb3) assay by tandem mass spectrometry

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Abstract Fabry disease (FD) is an X-linked lysosomal storage disorder resulting from a deficiency of α-galactosidase A, which leads to the progressive accumulation of one biomarker, globotriaosylceramide (Gb3), prominently elevated in the urine of affected patients. Using filter paper discs saturated with urine, we evaluated the analytical performance and clinical usefulness of the urinary Gb3 assay by tandem mass spectrometry (LC-MS/MS), with respect to linearity, precision, and reproducibility. We used healthy control urine samples to validate the reference interval of urinary Gb3. This method showed a good linearity (R^2= 0.9998) in the range of 0.05-10 μg/mL. Within-run CVs were less than 5% and total CVs were within 10%. The mean recovery of Gb3 from the urine filter paper was 96.7% and the limit of quantification (S/N ≥ 5) was 0.05 μg/mL, which was sensitive enough for the diagnosis of FD. The mean concentration of Gb3 in urine samples from healthy Korean controls was 5.93 ± 3.6 μg/mmol Cr (range 0.9-16.43 μg/mmol Cr). The urinary Gb3 assay by LC-MS/MS showed good analytical performance required for the diagnosis of FD in its linearity, precision, and accuracy. Therefore, this technique could be used for a rapid and reliable first line screening, monitoring and/or diagnosis of individuals at high risk for FD.

Keywords Fabry disease, Globotriaosylceramide, Tandem mass spectrometry, Urine filter paper

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Fabry disease (FD; OMIM 301500) is an X-linked lysosomal storage disorder caused by deficiency of the lysosomal enzyme alpha-galactosidase A (α-Gal A). This abnormality leads to the accumulation of its substrates, mainly globotriaosylceramide (Gb3) throughout various cells, organs and tissues[1]. As Gb3 accumulates in the body, affected individuals suffer from chronic pain, renal failure, stroke and cardiovascular complications. Clinical manifestations of disease progression in patients with FD are commonly marked by the finding of increased Gb3 concentrations in the peripheral blood and non-neural tissues (e.g. kidney, heart, skin)[2].

Several laboratory assays have been developed to test for FD, including the α-Gal A enzyme activity in leukocytes, plasma and dried blood spots, molecular genetic analysis and Gb3 accumulation in urine and plasma. In addition, recent progress in the treatment of FD has shown that elevated Gb3 concentrations can be significantly decreased in patients receiving enzyme replacement therapy (ERT), suggesting that Gb3 could function as a surrogate biomarker[3,4]. However, the accurate measurement of Gb3 in biological samples is complicated by the inherent heterogeneity and amphiphilic nature of the Gb3 molecule[2]. C. Auray-Blais *et al*. recently reported a rapid and simple liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to analyze Gb3/creatinine ratios in urine samples collected on filter paper from patients with FD[5,6]. Gb3 concentrations in urine, derived from glycolipid accumulated in epithelial cells shed by the distal tubules of patients with PD, are increased many times more than the concentrations in blood[7]. In addition, as urine samples can be obtained non-invasively, the urinary Gb3 assay by LC-MS/MS using urine filter paper samples could be useful for population-based screening and monitoring patients being treated with

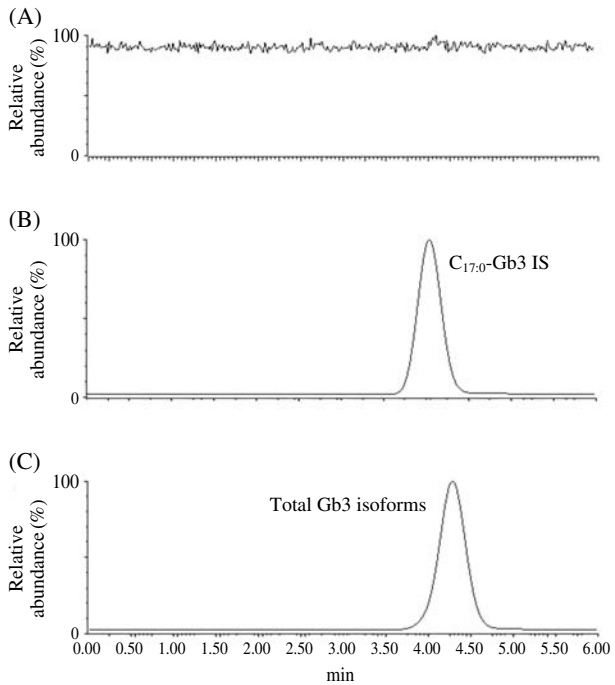


Figure 1. Total ion chromatograms in multiple reaction monitoring mode of (A) blank human urine, (B) spiked with I.S (50 μg/mL) and (C) Gb3 (10 μg/mL).

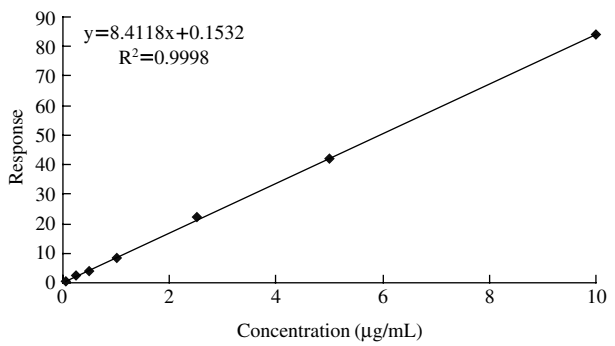


Figure 2. Calibration curve for the assay of Gb3 in urine.

ERT.

We evaluated the analytical performance and clinical usefulness of the urinary Gb3 assay by LC-MS/MS, using the filter paper method. This was the first time this method has been tried in Korea, and not only is it easy to obtain samples, using this method compared to the current methods used, it showed good analytical performance.

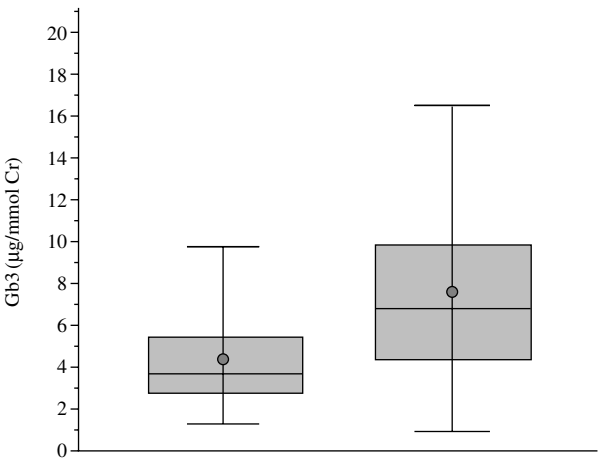
Validation of the method

Figure 1 shows the total ion chromatogram (TIC) in the multiple reaction monitoring mode (MRM) of blank normal control urine and spiked urine with internal standard (IS) and the total Gb3 isoforms. As shown in Figure 1, no significant interference in the blank urine was observed from endogenous substances at the retention time of the analytes.

The linearity of the response for Gb3 was quite acceptable at concentrations from 0.05 to 10.0 μg/mL of elutes with a correlation coefficient (r^2) of 0.9998 (Figure 2). For the intra-day results, the precision ranged from 0.7 to 3.6% and the accuracy ranged from 97.8 to 107.7% (Table 1). For the inter-day results, the precision and accuracy for the analytes met the acceptance criteria ($< \pm 15\%$) and the precision was below 10% at all concentrations tested. The limit of detection (LOD) and limit of quantification (LOQ) attained for the total Gb3 were both 0.05 μg/mL for the eluted urine

Table 1. Precision and Accuracy for the determination of Gb3 in human urine (n=5).

Concentration (μg/mL)	Precision C.V. (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
1	3.6	9.8	107.7	114.7
2	0.7	7.9	97.8	110.0
10	2.0	1.0	98.9	98.5



	Normal controls (Adult)	Normal controls (Child)
N	22	25
Mean	4.28	7.46
SD	2.12	4.11
Range	1.24-10.1	0.9-16.43
Median	3.6	6.8

Figure 3. Gb3 concentration in the urine from healthy controls. *Bars inside boxes* indicate the median values for each group, *shaded areas* indicate the 25th and 75th percentiles. *Error bars* indicate the limits of the ranges, *solid circles* indicate mean values.

filter paper samples. The mean extraction recovery of total Gb3 and IS were 96.7% and 97.9%, respectively, and the dependence on concentration was negligible.

The mean concentration of Gb3 in the urine filter paper samples from 25 control children and 22 adult controls were 7.46 ± 4.11 μg/mmol of creatinine (μg/mmol Cr) (range 0.9-16.43 μg/mmol Cr) and 4.28 ± 2.12 μg/mmol Cr (range 1.24-10.1 μg/mmol Cr), respectively (Figure 3). The 95th percentile value of Gb3 from the children and adult controls were 16.15 μg/mmol Cr and 8.66 μg/mmol Cr, respectively.

Table 2. Comparison with Gb3 concentration (μg/mmol Cr) in the urine from healthy controls.

	T. Kitagawa	*C. Auray-Blais*		This study
		Child	Adult	
N	432	32	47	47
Mean	8.99	7.9	6.1	5.93
SD	6.74	5.4	5.8	3.6
Range	1.12-64.04	1.7-NA	1.5-NA	0.9-16.43
Median	7.87	NA	NA	5.14

N: number of sample in each group; SD: standard deviation; NA: not available

Discussion

The incidence estimates for FD vary widely from 1 : 55,000 to 1 : 3,000 male births. The true incidence is likely to be higher than originally thought, due to the existence of milder variants of the disease[8]. The main complications of FD are ischemic stroke, cardiac disease, a wide variety of arrhythmias, valvular dysfunction and cardiovascular disease, as well as progressive renal failure[9]. The clinical manifestations are non-specific and are often mistaken for symptoms of other disorders, thus complicating the confirmation of the diagnosis. Misdiagnosis of FD is common, as reported in a recent study showing that it takes an average 24.6 years from the onset of symptoms to confirm the diagnosis[10]. Thus, irreversible kidney or cardiac damage frequently occurs[11]. A better long term outcome might be achieved through treatment with ERT, if the diagnosis could be made earlier[12-14]. To date, several approaches to the early diagnosis of FD have been proposed, including newborn screening[15-18]. However, newborn screening for a late onset X-linked recessive disorder, such as FD, is not generally acceptable. Therefore, it is important to develop accurate diagnostic assays for early diagnosis and monitoring of high-risk patients that have symptoms or a family history suggestive of FD. In this study, we evaluated the analytical performance and clinical usefulness of the urinary Gb3 assay with LC-MS/MS, as described by C. Auray-Blais *et al*.[6].

T. Kitagawa *et al*. evaluated a non-invasive method for measuring urinary Gb3 using LC-MS/MS in urine samples from 432 healthy controls and 68 patients with FD, in order to establish an appropriate screening method[19]. The mean concentration of Gb3 in urine samples from healthy controls was 8.99 ± 6.74 μg/mmol Cr (range 1.12-64.04 μg/mmol Cr), and a tentative cut-off level for urinary Gb3 was set at the 95th percentile value of the control group (29.2 μg/mmol Cr). C. Auray-Blais *et al*. reported that the mean concentration of Gb3 in urine samples from 32 control children and 47 adult controls were 7.9 ± 5.4 μg/mmol Cr, and 6.1 ± 5.8 μg/mmol Cr, respectively. They established 25 μg/mmol Cr (at +3SD) to be the upper limit of normal for urinary Gb3 in both adults and children[6]. In the present study, we validated the cut-off value previously described in 47 healthy control urines in order to apply the technique to Koreans. Similar results to the reference interval of normal controls of former studies were obtained (Table 2). In addition, the concentration of urinary Gb3 of a FD patient who was confirmed to have decreased α-Gal A enzyme activity (2.69 nmol/min/mg Prot, reference interval: 42.4-67.9) and had the molecular genetic test (H46R heterozygote) was 51 μg/mmol Cr, which was above the previous reference interval. Considering the concentration of urinary Gb3 of the FD patient (> 25 μg/mmol Cr) and normal controls (range 0.9-16.43 μg/mmol Cr), the concentration of 25 μg/mmol Cr, which was previously validated by C. Auray-Blais *et al*. might be the appropriate cut-off value of urinary Gb3 for FD screening in Koreans.

In this study, we used the filter paper method suggested by C. Auray-Blais *et al*. which has the following advantages[5]. First, contrary to existing Gb3 techniques, which are both time-and labor-intensive, this filter paper method eliminates the lipid extraction, glycolipid isolation, centrifugation and evaporation steps, while maintaining the sensitivity and efficiency. Second, the stability of Gb3 on filter paper was good for a 7-week period under different temperature conditions; this could reduce the total cost of these tests by minimizing the reagent consumption. Third, urine samples dried on filter paper are much easier to collect, store and ship than liquid specimens, which makes this method attractive for FD screening initiatives.

In conclusion, the urinary Gb3 assay by LC-MS/MS using urine filter paper samples was tried for the first time in Korea and showed good analytical performance, which is required for the diagnosis of FD with regard to linearity, precision, and accuracy, compared to the existing diagnostic methods used for FD. In addition, as urine samples can be obtained non-inva-

sively, the urinary Gb3 assay by LC-MS/MS using urine filter paper samples could be useful for rapid and reliable first line screening, monitoring and/or diagnosis of individuals at high risk for FD, in the clinical practice setting.

Materials & Methods

Subjects and sample processing

All samples were collected after informed consent had been obtained. Urine samples of 5-10 mL from 47 healthy volunteers as controls (25 children aged 1-12 years and 22 adults aged 27-43 years) were collected. Volunteers were excluded from the study if: they had any type of anomaly or confirmed inborn error of metabolism, malformation, other health problems.

Prior to pouring the urine onto the filter paper, mixing the sample was an important step to ensure complete suspension of all of the proteins and cellular debris. The filter paper was then dried at room temperature (at least 4-6 h). A $C_{17:0}$-Gb3 IS (1 μg/20 μL) was added to each 5-cm in diameter filter paper disc, which was punched out, folded in half and deposited into a glass vial, as described by C. Auray-Blais *et al.*[6]. The elution was performed by adding 4 mL of methanol to each vial, and shaking on a rotary shaker for 1 h. The elutes were then poured into glass tubes and centrifuged (4,000 rpm, 2 min). The supernatant (20 μL) was injected into the LC-MS/MS system.

Quantification of Gb3

Materials

A total Gb3 isoforms standard and $C_{17:0}$-Gb3 isoform IS were purchased from Matreya (Pleasant Gap, PA, USA). HPLC reagents and solvents were obtained from Honeywell Burdick & Jackson (Muskegon, MI, USA). All other chemicals were of analytical grade. The filter papers were Whatman No. 903 (Schleicher & Schuell, Keene, NH., USA).

Liquid chromatography

The LC system consisted of a Waters Alliance 2795 autosampler and pump. LC analysis of Gb3 was performed using the following step-gradient elution conditions: mobile phase A=2 mM ammonium acetate+ 0.1% formic acid in water, and mobile phase B=2 mM ammonium acetate+0.1% formic acid in methanol. The optimum flow rate was found to be 500 μL/min for minimizing ion suppression, with a column temperature at 45°C. The total analysis run time between each injection was 2.6 min.

Table 3. Selected precursor and product ion masses for the quantification of Gb3 isoforms.

Gb3 isoform	Precursor ion (m/z)	Product ion (m/z)
C16 : 0-Gb3	1046	884
C18 : 0-Gb3	1074	912
C20 : 0-Gb3	1102	940
C22 : 0-Gb3	1130	968
C24 : 1-Gb3	1156	994
C24 : 0-Gb3	1158	996
C24 : OH-Gb3	1174	1012
C17 : 0-Gb3 (I.S)	1060	898

m/z: mass to charge ration; I.S: internal standard

Mass spectrometry

Mass spectrometry of Gb3 was performed on a Quattro micro tandem quadrupole instrument (Waters Micromass, Manchester, UK) with electrospray ionization in the positive ESI mode. Two functions were established in the MS tune file: 1) one for the $C_{17:0}$-Gb3 IS; 2) one for the total Gb3 isoforms. The following parameters were used for the two MS functions: capillary voltage=4.0 kV; extractor=3.0 V; RF (radio-frequency) lens=0.0 V; cone gas flow=10 L/hr; desolvation gas flow=500 L/h; multiplier setting=650 V; cone voltage=120 V; collision energy=60 eV; dwell time =250 ms.

Quantification of Gb3

MRM was used for the measurements of $C_{17:0}$-Gb3 IS and the total Gb3 isoforms. For urinary Gb3, the TIC was taken as the sum of eight specific isoforms. The relevant MRM transitions for the Gb3 isoforms are listed in Table 3. The samples were quantified using Waters QuanLynx software, with the $C_{17:0}$-Gb3 IS added to each sample, to generate an analyte/IS response ratio for a standard curve. The ratio of Gb3 to creatinine was expressed in μg/mmol Cr. The concentration of creatinine in the urine sample was analyzed by a Cobas Integra 800 (Roche Diagnostics, Basel, Switzerland).

Validation of the method

Validation of the method was performed by spiking different concentrations of total Gb3 isoform standard solution on already dried 5-cm diameter discs of random normal urine filter paper samples. The linearity of the response was assessed by a weighted (1/x) least squares regression analysis. The calibration curve had to have a correlation coefficient of 0.99 or better. We assessed the intra-day precision (5 runs in a day) and the inter-day precision (5 different days) at different concentrations of the total Gb3 isoform standard solu-

tion (1.0, 2.0, 10.0 μg/mL). The acceptance criteria for the intra- and inter-day precision was 20% or better for the LOQ and 15% or better for the rest of the concentrations and for the accuracy it was 100 ± 20% or better for the LOQ and 100 ± 15% or better for the rest of the concentrations. The precision of the method was expressed as the relative standard deviation and the accuracy of the method was expressed in terms of bias. The LOD for the total Gb3 isoforms was determined in order to obtain a signal-to-noise ratio five times higher (S/N ≥ 5) than the signal obtained for the urine filter paper samples alone. The LOQ was determined for the total Gb3 isoforms, as the lowest quantity of Gb3 that could be measured within 20% of the target concentration. Recovery of Gb3 from the filter paper extraction procedure was determined by a comparison of the results of the spiked urine samples (1.0, 5.0 μg/mL) to the results of the eluted urine filter paper samples.

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