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Tandem mass spectrometry of globotriaosylceramide: quantification in plasma and urine for diagnosis and therapy monitoring in Fabry disease

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SUMMARY

Objective: An assay for measuring globotriaosylceramide (Gb_3Cer) levels in plasma and urine of patients with Fabry disease (FD) has been established.
Settings: Institute of Inherited Metabolic Disorders, General Faculty Hospital and 1st Faculty of Medicine of Charles University, Prague.
Material and Methods: Gb_3Cer concentrations were measured in 31 FD hemizygotes with classic form of the disease, 4 FD hemizygotes with cardiac variant, 33 FD female carriers and 6 carriers of cardiac variant. The control group consisted of 40 healthy men and 22 healthy women. Urine or plasma are diluted with acetone : methanol : water (45 : 45 : 10) and centrifuged to remove protein. The detection of relevant analytes is accomplished with flow injection analysis-electrospray ionization by triple quadrupole mass spectrometer operating in the multiple-reaction monitoring mode. The calibration standard C17:0-Gb_3Cer is used.
Results: The assay is linear up to 20 mg/l urine and 50 mg/l plasma. The limits of detection and quantitation of Gb_3Cer are 0.04 mg/l and 0.13 mg/l, respectively. The repeatability and reproducibility are under 5%. Established reference ranges in urine of males are up to 119 mg Gb_3Cer/mol creatinine, in plasma 4–11 mg Gb_3Cer/l. The ranges for men with FD are 440–1586 mg Gb_3Cer/mol creatinine in urine and in plasma 13–50 mg Gb_3Cer/l. Reference range in urine of females is up to 179 mg Gb_3Cer/mol creatinine, in FD carriers the range is 31–667 mg Gb_3Cer/mol creatinine. Fourteen Fabry patients have been tested both before treatment and in the course of the enzyme replacement therapy. Decreasing Gb_3Cer concentration was found in all investigated patients in plasma whereas in urine only in eight of them.
Conclusion: Compared to the formerly used and relatively insensitive chromatographic techniques, no solvent extraction is needed and only a single dilution step is required to prepare sample for MS/MS analysis. Because of its specificity, sensitivity, and high-throughput, the assay provides useful diagnostic mean in classic form of Fabry disease and a model of detection strategy for other lysosomal disorders.
Key words: Fabry disease, globotriaosylceramide, tandem mass spectrometry, enzyme replacement therapy.

SOUHRN

Chrastina P., Martincová O., Berná L., Ledvinová J.: Tandemová hmotnostní spektrometrie globotriaosylceramid – kvantifikace v plasmě a moči pro diagnózu a monitorování léčby u Fabryho choroby
Cíl studie: Zavedení metody pro stanovení globotriaosylceramidu (Gb_3Cer) v plasmě a moči u pacientů s Fabryho chorobou (FD).
Název a sídlo pracoviště: Ústav dědičných metabolických poruch, Všeobecná fakultní nemocnice a 1. lékařská fakulta Univerzity Karlovy, Praha.
Materiál a metody: Koncentrace Gb_3Cer byla stanovena u 31 hemizygotů s klasickou formou FD, 4 hemizygotů s kardiální variantou, u 33 žen přenašeček a 6 přenašeček kardiální varianty. Kontrolní skupinu tvořilo 40 zdravých mužů a 22 zdravých žen. Moč nebo plasma byly naředěny směsí aceton : metanol : voda (45 : 45 : 10) a centrifugací odstraněny bílkoviny. Pro stanovení byla použita metoda přímého nástřiku do tandemového hmotnostního spektrometru s ionizací elektrosprejem (FIA-ESI-MS/MS). Jako vnitřní standard byl použit C17:0-Gb_3Cer.
Výsledky: Lineární rozsah metody je do 20 mg/l pro moč a do 50 mg/l pro plasmu. Limity detekce a kvantifikace Gb_3Cer jsou 0,04 mg/l a 0,13 mg/l. Opakovatelnost a reprodukovatelnost jsou nižší než 5%. Referenční rozmezí pro moč u mužů je 0–119 mg Gb_3Cer/mol kreatininu, pro plasmu 4–11 mg Gb_3Cer/l. Rozmezí hodnot u mužů s FD jsou 440–1586 mg Gb_3Cer/mol kreatininu v moči a 13–50 mg Gb_3Cer/l v plasmě. Referenční rozmezí pro moč u žen je 0–179 mg Gb_3Cer/mol kreatininu, rozmezí u přenašeček je 31–667 mg Gb_3Cer/mol kreatininu. 14 pacientů s FD bylo vyšetřeno před léčbou a během enzymové substituční terapie (enzyme replacement therapy). Snížená koncentrace Gb_3Cer vlivem léčby byla nalezena u všech vyšetřovaných pacientů v plasmě, zatímco v moči pouze u osmi.
Závěr: V porovnání s dříve používanými metodami a relativně intenzivními chromatografickými metodami není pro přípravu vzorku pro MS/MS analýzu nutná extrakce, vzorek se pouze naředí. Díky své specificitě, senzitivitě a vysoké propustnosti je metoda použitelná pro diagnostiku klasické formy FD a je modelem pro diagnostiku dalších poruch.
Klíčová slova: Fabryho choroba, globotriaosylceramid, tandemová hmotnostní spektrometrie, enzymová substituční terapie.

Fabry disease (FD) is an X-linked recessive disorder (OMIM 301 500) [1]. The defect of lysosomal α-galactosidase A (α-GalA, EC 3.2.1.22) leads to the lysosomal accumulation of glycolipid structures with terminal α-galactose moiety in the carbohydrate chain, primarily globotriaosylceramide (Gb_3Cer, Gal(α1→4)Gal(β1→4)Glc(β1→1)Cer). Patients suffer from pain and acroparesthesia in the limbs, angiokeratomas, hypohidrosis, corneal and lenticular opacities, renal and cardiac disease. The disease manifests in all hemizygous men, while the

majority of female heterozygotes display symptoms to a variable extent, depending on the degree of X-chromosome inactivation. Recently, treatment of Fabry disease by supplementation of an absent enzyme with recombinant α-galactosidases A has been established (enzyme replacement therapy, ERT) [2].

The proof of storage of non-degraded glycolipid substrate is of high diagnostic importance especially in easily obtainable sources such as plasma and urine of patients. In Fabry males and also in some females, massive excretion of Gb_3Cer in urine and its significant increase in blood plasma are typical features, which can be exploited for diagnosis [1]. Nowadays, tandem mass spectrometry (MS/MS) has become an efficient and sensitive technique also for quantification of lipids [3]. Therefore, adaptation of assay of Boscaro et al. [4] for measuring Gb_3Cer in plasma and urine has been developed and is used to test individuals from our cohort of Fabry patients. Also, some patients were tested both before treatment and in the course of ERT.

Material and method

Patients

Gb_3Cer concentrations were measured in 31 FD hemizygotes with classic form of the disease, 4 FD hemizygotes with cardiac variant, 33 FD female carriers and 6 carriers of cardiac variant [5]. Diagnosis of all patients was confirmed by enzymatic analysis of α-galactosidase activity in leukocytes and by mutation analysis [5]. The control group consisted of 40 healthy men (age 37 ± 4.6 year) and 22 healthy women (age 30 ± 5.6 year). Urine and plasma samples were originally taken for diagnostic purposes and then stored in our Institute.

Part of the study was focused on analysis of Gb_3Cer levels in plasma and urine of 14 hemizygotes (age 43 ± 2.4 year) and three FD carriers (age 59 ± 1.6 year) from nine families who receive enzyme replacement therapy (ERT). These patients were administered standard dose of recombinant α-galactosidase A every two weeks from 5 to 40 months (mean 21 ± 2.8 month). We received one sample of plasma and urine from each patient. Written informed consent of patients and local ethical committee approval were obtained. In all urine samples creatinine was determined on automatic analyzer Hittachi 902 (Jaffe, Pliva-Lachema Diagnostika).

Preparation of samples and MS/MS analysis

All samples were investigated on API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, USA) with TurboIonSpray interface in combination with a PE 200 Autosampler and a PE series 200 microgradient system was used. Twenty microlitres of plasma or urine were added to 980 µl of a mixture acetone : methanol : H_2O (45 : 45 : 10) containing 100 ng of an internal standard (C17:0-Gb_3Cer, a gift from Genzyme, USA). The sample was ultrasonicated for 10 min and centrifuged to remove protein (13 000 rpm, 5min). Twenty microlitres of supernatant were analysed by flow injection analysis-electrospray ionization by tandem mass spectrometer (FIA-ESI-MS/MS) at a solvent flow rate of 10µl/min, resulting in a run-time of 10 min for each sample. Instrument operated in positive mode using multiple/reaction monitoring (MRM) system. This approach enables quantification of all major Gb_3Cer isoforms by distinguishing them by fatty acid bound in the lipid (ceramide) moiety. The monitored ion transitions for individual Gb_3Cer isoforms, corresponding to the loss of a galactosyl residue, were m/z 1046.6→884.6, 1074.7→912.7, 1102.7→940.7, 1128.5→966.5, 1130.5→968.5, 1154.2→992.2, 1156.2→994.2, 1158.2→996.2, 1170. 5→1008.5, 1172. 5→1010.5 and 1174.5→1012.5. The scan time for each isoform was 1000 ms. The needle, orifice and collision energy voltages were 5.5 kV, 200 V and 80 eV, respectively. The source and desolvation gas temperatures were held constant at 150 °C, with flow rates of 12 and 5 liters of nitrogen per minute, respectively. In collision gas cell nitrogen at gas intensity 4 units was used. Quantification was achieved by relating the signal of Gb_3Cer to the signal of internal standard and calculating concentration from external calibration. Composition of Gb_3Cer isoforms was calculated dividing the total concentration of Gb_3Cer by the isoform signal ratio from measured MRM spectra.

Results

In biological material Gb_3Cer and all other sphingolipids exist in multiple molecular forms due to heterogeneity of lipid (ceramide) moiety. Therefore, to quantify the total Gb_3Cer, the multiple-reaction monitoring mode was used for determination of all the isoforms detected. In urine and plasma a total of 11 Gb_3Cer isoforms were detected and identified [6]. Analytical characteristics of FIA-ESI-MS/MS method are shown in Table 1.

Table 1. Analytical characteristics of the method for Gb_3Cer measurement by FIA-ESI-MS/MS

Characteristics	Plasma	Urine
Linearity interval	0-50 mg Gb_3Cer/l	0-20 mg Gb_3Cer/l
Limit of detection	0.04 mg Gb_3Cer/l	
Limit of quantification	0.13 mg Gb_3Cer/l	
Repeatability	2.6 %	3.0 %
Reproducibility	4.0 %	4.0 %
Recovery	97.9 %	94.3 %

Established reference ranges in urine and in plasma are presented in Table 2. We found significantly increased concentrations of Gb_3Cer both in plasma and urine of all 31 classic FD hemizygotes compared to healthy males ($p < 0.001$, Table 2). In contrast, four patients with cardiac variant and a very low residual α-galactosidase A activity in plasma had urinary Gb_3Cer excretion in the normal range but elevated Gb_3Cer va-

lues in plasma ($p < 0.001$, Table 2). Concerning the urines of confirmed classic female carriers, the range of Gb_3Cer overlapped the control range.

Table 2. Concentration of Gb_3Cer in plasma and urine of FD patients (males and females) and of controls (mean ± SEM, range)

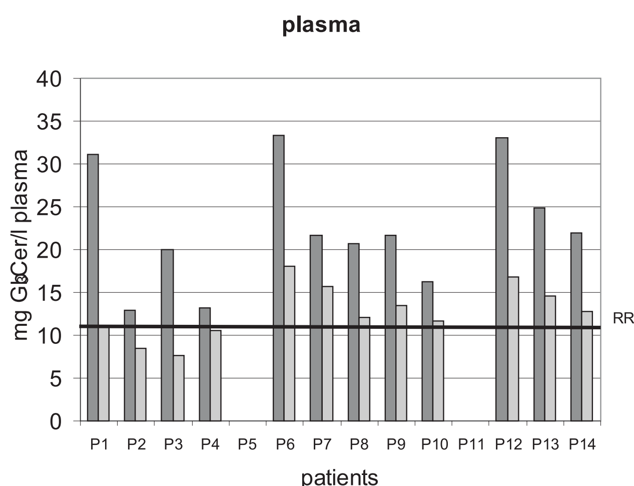
Group	Plasma $Gb_3Cer^{a)}$	Urinary excretion of $Gb_3Cer^{b)}$
Classic FD patients	26.0 ± 2.1** (n=25) 13-50	870.3 ± 71.1** (n=31) 440-1586
FD cardiac variant	12.5 ± 2.2** (n=3) 9-17	59.7 ± 13.9 (n=4) 33.7-97.5
Classic FD carriers	not analyzed	113.3 ± 19.5* (n=33) 31-667
FD cardiac carriers	not analyzed	52.8 ± 6.6 (n=6) 32-69.5
Male controls	7.7 ± 0.3 (n=28) 4-11	60.8 ± 4.6 (n=40) 0-119
Female controls	not analyzed	72.3 ± 11.3 (n=22) 0-179

a) mg/l, b) mg/mol creatinine

*Statistically significant differences between patients and controls: *p < 0.05, **p < 0.001.*

In 14 patients the levels of plasma and urine Gb_3Cer before and in the course of ERT were compared (Fig. 1). In plasma decreasing tendency of Gb_3Cer level was clearly evident, however, only in 5 patients the Gb_3Cer fell to normal range. In urine the decrease of Gb_3Cer excretion was observed in eight patients, whereas in five patients the level of Gb_3Cer remained unchanged or even increased, probably due to the state of progression of the renal affection at commencement of treatment (Fig. 1). Only in patients P2, P3, P5 and P10 the concentration of Gb_3Cer fell to normal range. In a group of Fabry carriers the urine of three female patients was tested before and in the course of ERT. Gb_3Cer in pre-treatment urine of two carriers was in normal range. In one carrier (C3) with very high excretion before treatment (668 mg/mol creatinine) the concentration of Gb_3Cer fell dramatically to the normal range (163 mg/mol creatinine).

Distribution of Gb_3Cer isoforms was analyzed in healthy male controls and in patients with classic FD before and in the course of ERT.



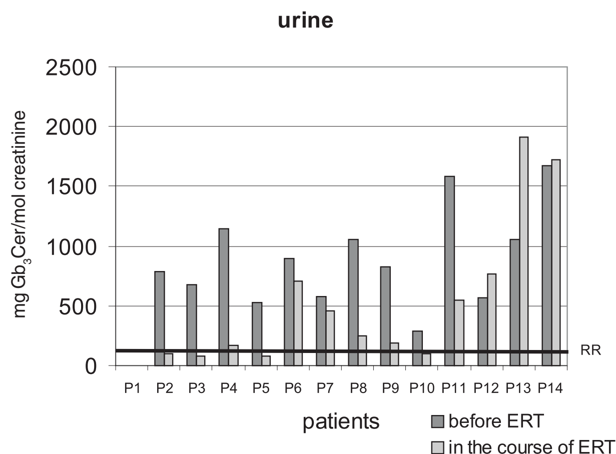


Fig. 1. Gb_3Cer in plasma and urine of FD patients before and in the course of ERT

Note: P5, P11 – plasma samples were not available

P1 – the only urine sample on ERT was collected after renal transplantation, therefore it was not possible to compare the samples before and in the course of ERT

RR – reference range

In normal urine the major Gb_3Cer isoforms were C18:0 (58%) and C16:0 (18%); C22 (C22:0, C22:1), C24 (C24:0, C24:1, C24:2, C24-OH) family isoforms were present in minor quantities (less than 5%). The similar profile was found in normal plasma with predominating C16:0 (57%) and C18:0 (16%) acids. In urine from patients C24:0, C24:1 and C22:0 isoforms dominated (77% in comparison to 24% in normal urine). In plasma of patients Gb_3Cer with high proportion of C16:0 (55%) and C24:1 (13%) fatty acids were found.

When we compared the distribution of fatty acids in the lipid (ceramide) moiety of Gb_3Cer during ERT, urine samples of patients with decrease of Gb_3Cer revealed marked shift towards C18:0/C16:0 fraction (60% in comparison to 23% before ERT). We assume that ERT initiated positive tendency to reach the normal state in isoform pattern. In plasma we observed these changes too, but they were not so evident (data not shown).

Discussion

Since 1969 the measurement of Gb_3Cer in urinary sediment and/or the whole urine of patients with suspected Fabry disease have become an important laboratory indicator leading to the final diagnosis [7–10]. All the methods were multiprocedural requiring the collection of 24 hr urine or urinary sediment, an extraction and purification of a lipid extract and a glycolipid analysis based mostly on chromatographic methods with different way of selective determination of the critical compound. These procedures were, in spite of their diagnostic reliability, laborious, time-consuming, with low throughput and therefore not suitable for the effective and exact measurement of large series of samples.

In 2002 a novel approach to Gb_3Cer determination in plasma and in whole urine by MS/MS providing high efficiency and sensitivity was reported [4, 11, 12].

In our study we adapted a MS/MS procedure of Boscaro et al. [4] using the whole urine without a lipid extraction prior to MS/MS. We optimized the method for a new API 2000 triple quadrupole tandem mass spectrometer, which allowed us to quantify also the minor Gb_3Cer isoforms and to evaluate in total 11 isoforms of this lipid. For quantification of a Gb_3Cer internal standard C17:0-Gb_3Cer was used which is not present in human plasma and urine and which makes the method more precise [6, 11]. Our data clearly show reliability of this approach in the cohort of Fabry male patients with classic phenotype in comparison to a control group using both plasma and urine for Gb_3Cer determination.

In urine the data were related to creatinine measured by conventional method, which was proved as the most practical parameter for the expression of Gb_3Cer concentration. Urinary Gb_3Cer originates from shed cells of distal renal tubules and therefore may reflect renal storage ("chemical biopsy") [13]. Surprisingly, Gb_3Cer data related to sphingomyelin [9], another cell membrane lipid measured by the same MS/MS assay (data not shown), gave practically the same results, albeit there is no direct relationship between urine creatinine and sphingomyelin. This also confirms the findings of others [6, 14] and supports the general acceptance of Gb_3Cer/creatinine relation in patients with normal creatinine concentration in urine (range 3–12 mmol/l).

The levels of Gb_3Cer in urine and plasma samples of classic Fabry hemizygotes were clearly above the reference range of controls, which was more prominent in urine (14.5-fold in urine, 3.4-fold in plasma, $p < 0.001$). In the cardiac variant, no elevated urinary Gb_3Cer excretion was found in contrast to increased plasma values. However, this elevation in plasma was not so dramatic as in the classic type of Fabry disease (1.6-fold increase in the cardiac variant) because the defect in cardiac variant is restricted only to the heart [15].

In female carriers the mean level of Gb_3Cer in urine was only 1.6 times greater than the mean level of the control group.

However, it must be pointed out that calculation of the mean value in heterozygous group is not very meaningful and gives only very rough orientation because it does not reflect individual biological diversity of each carrier due to the inactivation of X-chromosome [5]. In spite of that, the elevation of urine Gb_3Cer excretions in this group was found statistically significant when compared to female controls ($p < 0.05$). As expected, only normal Gb_3Cer values were found in urines of six heterozygotes with cardiac variant.

Plasma from FD females was not archived for Gb_3Cer analysis because of its very low informative value in this group of patients as demonstrated by previous study [16]. We can conclude that the measurement of urinary Gb_3Cer in females may give useful information in some cases but in general, its diagnostic value in heterozygotes is lower – similarly as determination of α-galactosidase activity. Therefore diagnosis of female carriers must be always confirmed by mutation analysis [5].

We compared Gb_3Cer levels in samples before and in the course of ERT. In plasma the levels of Gb_3Cer lowered in all our patients, while in urine we observed three groups of patients – in some patients the levels of Gb_3Cer lowered significantly ($p < 0.01$), in three remained almost the same and in two patients the amounts of Gb_3Cer were higher than before ERT. We did not find any relationship between the ERT responses, the age of the patients and the length of ERT.

These results show how variable response in terms of decreasing Gb_3Cer concentration can be expected in different patients in spite of their clinical improvement. Similar observation was reported for other groups of patients [16, 17]. This discrepancy is most visible in patients P13 and P14 after more then 3 years of receiving enzyme supplementation. In these patients ERT had a positive effect on lowering plasma Gb_3Cer whereas urine excretion of Gb_3Cer increased. In contrast to Mills et al. [17], we did not observe any elevation or stagnation of Gb_3Cer level in plasma of patients without response in urine. Such findings could suggest the existence of transportation problems of recombinant enzyme to some target tissues, e.g. the kidney. Similar suspicion arose from the examination of autopsy and biopsy tissues of treated patients ([18], M. Elleder, personal communication). These problems open new developments in treatment of Fabry disease, e.g. modification of infused enzyme to allow better targeting and cellular and tissue uptake. Another important factor determining the response could be the state of disease progression and irreversible cell damage at the beginning of the treatment. This observation points to the necessity of early treatment.

It is evident from our results and from results of other investigators [14, 16, 17] that Gb_3Cer can serve as a marker of disease severity and response to treatment in most male and some female patients with classic Fabry phenotype, i. e. in those with elevated Gb_3Cer prior to ERT. However, this marker is unhelpful in cardiac variants and in numerous female carriers who initially have near-normal Gb_3Cer concentration in plasma and urine.

In this study, we have shown different distribution of Gb_3Cer isoforms in plasma and urine before and in the course of ERT in Fabry patients. ERT initiated positive tendency to reach the normal state in the isoform pattern. More distinct changes were observed in urine. The evaluation of isoform profile during ERT should be another useful biomarker of treatment efficiency in patients with positive Gb_3Cer response. The attempt to use only one Gb_3Cer isoform (C24:1-Gb_3Cer) for discrimination between Fabry patients and unaffected controls has been reported [19].

According to our results evaluation of the whole pattern of 11 Gb_3Cer isoforms gives better and more sensitive reflection of ERT effectiveness and should help in recognizing the actual biological response.

Conclusion

FIA-ESI-MS/MS method based on evaluation of 11 main Gb_3Cer isoforms has been established for esti-

mation of plasma and urine Gb_3Cer in a cohort of Czech Fabry patients with classic phenotype. In female carriers the method has limited value due to the overlapping the control range. The method can serve to monitor the effect of the treatment in some patients with elevated Gb_3Cer prior to ERT. In the patients the pattern of Gb_3Cer isoforms showed marked shift towards fatty acids with longer chain, i. e. C24 family. ERT initiated positive tendency to reach the normal state in the isoform pattern.

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