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LC–MS/MS Studies on Sample Quality Control of Fabry Disease*

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Abstract Fabry disease(FD) is a rare X-linked recessive hereditary lysosomal storage disease. One of the most commonly used biomarkers for detecting FD is Lyso-GB3, a globotriaosylsphingosine, which is usually analyzed by liquid chromatography-tandem mass spectrometry(LC-MS/MS), because of its high specificity and sensitivity. Due to the complexity of LC-MS/MS technology, efficient and reliable diagnosis of FD by LC-MS/MS was unavailable in most hospitals in China. Therefore, patients' samples need to be prepared at local hospitals and then sent to central laboratories for LC-MS/MS analysis. As a result, samples undergo a lengthy shipment and storage process, potentially compromising sample quality and thus precision of diagnosis. We first established and validated a novel LC-MS/MS-based method and to guarantee FD sample quality, we studied the effects of pre-analytical variables on the analysis of Lyso-GB3. These pre-analytical variables include storage temperature and time of plasma, freeze-thaw cycles of plasma, degree of hemolysis of plasma, storage temperature and time of whole blood, and centrifugation temperature. Finally, we analyzed 86 FD samples and 100 normal human samples and made correlation studies on the relationship between Lyso-GB3 level and FD classification. Results showed Lyso-GB3 level started decreasing post 4 days when plasma was stored at 20°C. In addition, hemolysis significantly influenced the FD sample quality and severe hemolysis caused the Lyso-GB3 level decreased by 57.8%. Other pre-analytical variables have little impact on FD sample quality. Under the conditions of the standard operational procedures before analysis, the sensitivity and specificity for screening FD is 100%, which ensures the accuracy of the method. Furthermore, we used plasma Lyso-GB3 level to distinguish classical FD patients from non-classical FD patients. When we set the cut-off value as 55.15 μg/L, the sensitivity and the specificity were 71% and 100%, respectively. The area under the receiver operating characteristic curve was 0.83. Our findings can help standardize the operational procedures in medical labs to guarantee high FD sample quality and provide a reference for identification of Chinese FD classification.

Key words Fabry disease, Lyso-GB3, LC-MS/MS, pre-analytical variables, quality control, disease classification
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Fabry disease(FD) is a rare X-linked recessive hereditary lysosomal storage disease[1], which is caused by a mutation in the gene encoding α-galactosidase A[2-3], resulting in either partial or complete loss enzymatic activity. Therefore, α-galactosidase A's substrate-glycosphingolipids are gradually accumulated in various organs of the human body, including heart, kidney and pancreas, eventually causing life-threatening lesions[4]. These glycosphingolipids include globotriaosylceramide (GB3) and globotriaosylsphingosine(Lyso-GB3). According to the patients' clinical manifestations, Fabry disease is classified into two types. One is the classical type, in which the α-Gal A activity is almost completely lost, resulting in damage to multiple

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system organs. The other is the non-classical type, which is characterized by a partial decrease in α-Gal A activity, resulting in damage to hearts or kidneys[5]. Due to the high rate of missed diagnosis or misdiagnosis, the precise morbidity rate of Fabry disease remains unclear. According to Germain[6], the morbidity rate is estimated to be between 1/446 000 to 1/117 000. No precise data are available on the general morbidity rate of the Fabry disease patients in China, while it is reported that the morbidity rate of the Fabry disease in patients with renal failure is 0.12%[7].

At present, the methods for clinical diagnosis of Fabry disease mainly include detection of α -galactosidase A (α -Gal A) enzyme activity[8], analysis of gene mutation[3], as well as analysis of the two disease markers GB3[9-11] and Lyso-GB3[4,11-12]. The α-Gal A enzyme activity detection has a higher diagnosis rate for male patients compared with female ones, because the α-Gal A enzyme activity of some female patients is within the normal range[13-14]. The analysis of gene mutation has a somewhat high rate of false-negatives[15-16], because of the incomplete gene mutation database[17-18]. GB3 is a neutral glycolipid formed by the covalent bond between trihexosides and N-sphingosine. To detect Fabry disease, plasma levels of GB3 are tested. However, the plasma GB3 concentrations of female patients are not only lower than those of males, but often within the normal physiological range[19]. A more reliable method to detect GB3 as an indicator of FD is to measure urinary total GB3 including GB3's isoforms, which can be used to diagnose both male and female patients with Fabry disease[20-21]. However the fact that GB3 has many isoforms poses a challenge to the separation and detection.

Recently, Lyso-GB3 was introduced as a novel biomarker for Fabry disease[22]. Lyso-GB3 is a deacetylated ceramide trihexoside which is a cationic and amphiphilic compound having a polar sugar group and is easily soluble in water. The concentration of Lyso-GB3 in plasma surpasses that of GB3 by several orders of magnitude and does not have as many isoforms as GB3. Lyso-GB3 concentrations may be moderately increased in women with normal concentrations of GB3[19]. Therefore, compared with detection of α-Gal A activity, gene mutation and GB3, analysis of plasma Lyso-GB3 is more accurate for diagnosing Fabry disease[14,16-17], especially for female patients with Fabry disease[13,19].

Lyso-GB3 detection by LC-MS/MS is a standard practice in developed countries, Gold *et al*.[23] and Boutin *et al*.[24] have established LC-MS/MS methods to detect Lyso-GB3 in plasma and urine. However, only a few laboratories are able to analyze Lyso-GB3 in China. Our laboratory has established and validated a LC-MS/MS method for detecting Lyso-GB3. Due to China's large population, a large number of undiagnosed Fabry patients exist[25], and these patients are distributed in many places throughout the country where most local hospitals lack LC-MS/MS facilities. Therefore, preparing samples at local hospitals and sending them to a well-equipped laboratory for analysis represents a labor- and material-saving way to overcome this challenge. However, FD patients' samples may undergo a lengthy shipment and storage process, potentially compromising samples' quality. Therefore, it is necessary to evaluate the pre-analytical variables that affect the patient samples to ensure the authenticity and reliability of Lyso-GB3 detection[26].

We not only studied pre-analytical factors such as storage temperature and time of plasma samples, freeze-thaw cycles of plasma samples that were evaluated by the other group[24], but also the degree of hemolysis, the storage temperature and time of whole blood samples, and the centrifugation temperature. After we determined the conditions for guaranteeing the sample quality, we followed that to perform sample collection. In collaboration with China Rare Diseases Association, we collected and analyzed 86 FD plasma samples over a two-year period, and selected 100 healthy human samples for comparison to verify the sensitivity and specificity of the method. In addition, we are the first group who determined the correlation between Lyso-GB3 level and Chinese FD classification.

1 Materials and methods

1.1 Chemicals and instruments

LC-MS grade methanol(MeOH) and acetonitrile (ACN) were purchased from Thermo Fisher (USA). LC-MS grade water was purchased from Sigma-Aldrich(USA). LC-MS grade formic acid(FA) and phosphoric acid(H_3PO_4) were purchased from Sigma-Aldrich(USA). HPLC grade ammonium hydroxide (NH_4OH) was purchased from Jindu Biotechnology

(China).

Lyso-GB3 and its standard(glucosylsphingosine, GSG) were purchased from Matreya LLC(Pleasant Gap, PA, USA). Their structure diagrams are shown in Figure 1. GSG was selected as Lyso-GB3's internal standard because it has a similar hydrophobic sphingosine moiety and a similar polar sugar group as Lyso-GB3. Therefore, it is expected that these two molecules will display similar binding characteristics on solid phase extraction columns and chromatographic columns, and have similar ionization process in mass spectrometers.

Lyso-GB3 was dissolved in chloroform：methanol： water (2： 1： 0.1) and the final concentration of the calibrator stock solution was 50 mg/L. GSG was dissolved in chloroform：methanol (4： 1) and diluted to a final working concentration of 500 μg/L.

For analysis of the degree of hemolysis, a BC-5310 automated blood cell analyzer(Mindray, China) was used. For LC-MS/MS analysis, a LC-20ADXR high performance liquid chromatograph(Shimadzu, Japan) and a Qtrap 5500 tandem mass spectrometer (AB sciex, USA) were used.

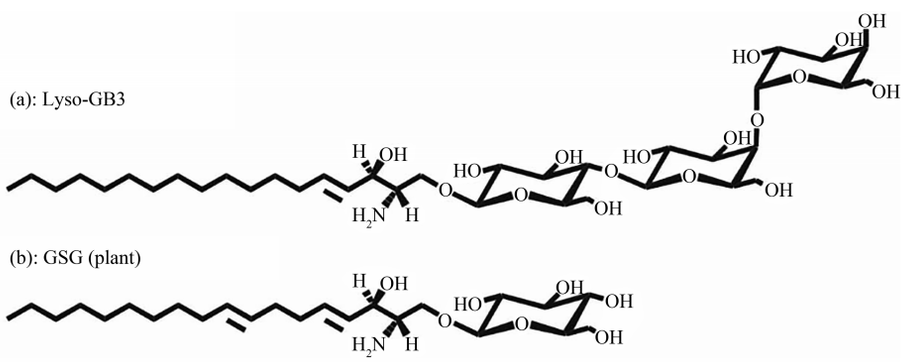

Fig. 1 The structure diagrams of Lyso−GB3 (a) and GSG (b)

1.2 Preparation of assay verification

The Lyso-GB3 stock solution was diluted with a mixture of solvents (50% ACN, 50% H_2O, 0.1% FA) to prepare 14 different concentrations of the calibration standards: 0.061, 0.122, 0.244, 0.488, 0.97, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 μg/L, which were used to evaluate the linearity of the response(3 aliquots were prepared for each concentration). The lower limit of detection(LOD) was defined as the signal-to-noise ratio of the chromatographic peak greater than 3(*S*/*N*>3) , and the lower limit of quantification (LOQ: *S*/*N*>10) met a stated acceptable imprecision (*CV*<20%) and trueness (bias<15%).

Low(1 μg/L), medium(30 μg/L) and high (300 μg/L) QCs of Lyso-GB3 were obtained by spiking pooled plasma with appropriate amounts of Lyso-GB3 standard solutions. To evaluate intraday imprecision and accuracy, 5 aliquots were prepared for each concentration, with a total of 15 aliquots. To evaluate interday imprecision and accuracy, the same QCs were continuously prepared in 3 batches and analyzed. At a minimum, the imprecision of each concentration should not exceed 15% *CV* and the bias of each concentration should not exceed ±15%.

To evaluate the carryover effects, 10 replicates of a low concentration(2 μg/L) sample were injected into the LC-MS/MS. Subsequently, 10 replicates each of high-concentration(300 μg/L) and low-concentration (2 μg/L) samples were alternately tested. A *t*-test was used to compare the means of the two sets of low-concentration injections.

1.3 Preparation of samples for the study on pre−analytical variables

Fresh plasma and whole blood were selected for this study. The mixed plasma was obtained from 12 Fabry volunteers and divided into a total of 108 aliquots(100 μl per aliquot) for studying the effects of storage temperature and time and freeze-thaw cycles on the stability of plasma Lyso-GB3.

The mixed EDTA anti-coagulated whole blood was obtained from 6 Fabry volunteers with the same blood type and divided into a total of 63 aliquots (500 μl per aliquot) for studying the effects of

hemolysis, the storage temperature and time of whole blood samples, and centrifugation temperature. Volunteers were only included in this study after signing the informed consent form.

1.3.1 Storage temperature and time of plasma samples

93 aliquots of plasma were selected in this study. The first 3 aliquots were marked as baseline (these aliquots were also marked as baseline for studying the effect of freeze-thaw cycles) and immediately analyzed by LC-MS/MS. A total of 90 aliquots were stored at −20, 4 and 20℃ for 12 h, 1 d, 2 d, 4 d, 8 d, 12 d, 16 d, 20 d, 24 d and 28 d, respectively. 3 aliquots were prepared for each condition.

1.3.2 Freeze-thaw cycles of plasma samples

15 aliquots of plasma were selected in this study. The number of freeze-thaw cycles was set at 1, 2, 3, 4, and 5 respectively with the storage temperature of −80℃ , and three aliquots were prepared for each condition. The first freeze-thaw interval was 24 h, with the follow-up intervals greater than 12 h.

1.3.3 Degree of hemolysis of plasma samples

18 aliquots of whole blood were selected in this study. The first 3 aliquots that were centrifuged at 4℃ immediately were marked as baseline (these aliquots were also marked as baselines for studying the effects of storage temperature and time of whole blood samples and centrifugation temperature). Next 15 aliquots were stored at a −20℃ re-thawed after 4, 8, 12, 16 and 20 min respectively to achieve different degrees of hemolysis. The degree of hemolysis was marked as level 1 (4 min), level 2(8 min), level 3 (12 min), level 4(16 min), level 5(20 min), and three aliquots were prepared for each level. Aliquots samples were centrifuged and the supernatants analyzed using a BC-5310 automated blood cell analyzer to measure the hemoglobin (Hb) concentration.

1.3.4 Storage temperature and time of whole blood samples

36 aliquots of whole blood were used and stored at 4℃ and 20℃ for 12, 24, 36, 48, 60 and 72 h respectively before centrifuging, and three aliquots were prepared for each condition.

1.3.5 Centrifugation temperature

9 aliquots of whole blood were centrifuged at 20, 30 and 40℃ respectively, and at 3 000 r/min for 10 min. For each condition, three biological replicates were prepared.

1.4 86 FD samples and 100 healthy samples for clinical validation

After determining the conditions for guaranteeing sample quality, we collaborated with Chinese Fabry Disease Association to collect 86 samples from FD patients and 100 samples from healthy people. The plasma Lyso-GB3 levels of the 86 samples were measured by LC-MS / MS. The sensitivity and specificity of the method were calculated. At the same time, we also studied the correlation between FD classification and Lyso-GB3 concentration.

1.5 Sample pretreatment

A solid phase extraction plate(Waters Oasis MCX μEluton plate 30 μm) was pre-treated with 600 μl of MeOH and 600 μl H_3PO_4(2% in water), respectively. Aliquots(100 μl) of the test samples (plasma, standard solution, QCs) were thoroughly mixed with 250 μl of H_3PO_4(2% in water) and 250 μl of GSG(5 μg / L in MeOH) in centrifuge tubes, followed by centrifugation. The resulting supernatant was loaded into each well of the solid phase extraction plate. After loading the samples, the wells were washed first with 600 μl of 2% FA in water and then washed with 600 μl of 2% FA in methanol. Lyso-GB3 and GSG were then eluted into a 96-well plate with 300 μl of 2% ammonia in methanol. After the eluates were dried sufficiently by nitrogen, the residues were reconstituted in 400 μl of ACN 50%/FA 0.1%/water, and the resulting solutions were shaken for 15 min before LC-MS/MS analysis.

1.6 Mass spectrometry and liquid chromatography conditions

The MS conditions were set as follows: ESI mode was positive; ion spray voltage was 5 500 V; curtain gas was 40 Psi; cad gas was 12 Psi; gas 1 was 30 Psi; gas 2 was 60 Psi; the desolvation temperature was 650℃ . The following multiple-reaction monitoring(MRM) transitions were monitored, with a dwell time of 40 ms: 786.5>282.4(Lyso-GB3) and 460.4>280.4(IS). Declustering potential was set to 120 V and 80 V in the MRM traces of Lyso-GB3 and GSG, respectively. Collision energy was set to 53 V and 30 V in the MRM traces of Lyso-GB3 and GSG, respectively.

The mobile phase A consisted of 5% ACN, 95% water and 0.2% FA, and the mobile phase B consisted

of ACN and 0.2% FA. The flow rate was set at 0.3 ml/min. The two mobile phases formed the following gradient: 0−4.5 min(20%−100%, B), 4.5−7.0 min(100%, B), 7−9 min(100%−20%, B). The analytes were separated on an X Select HSS T3 column (2.5 μm, 2.1 × 100 mm) from Waters(USA). The column temperature was set to 40℃ . The chromatograms of Lyso-GB3 and GSG are shown in Figure 2.

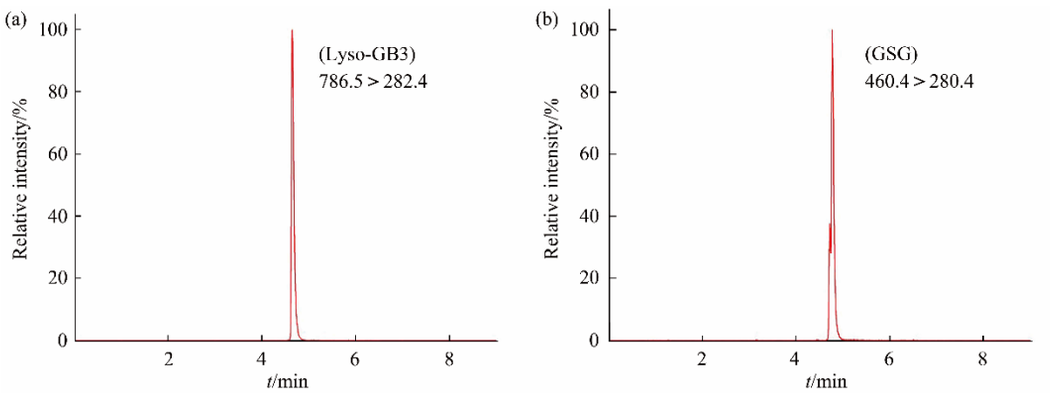

Fig. 2　HPLC−ESI−MS/MS analysis of Lyso−GB3 and GSG

(a) Ion chromatogram of Lyso-GB3 (concentration: 20 μg/L; retention time: 4.65 min); (b) Ion chromatogram of GSG (concentration: 20 μg/L; retention time: 4.77 min).

1.7　Statistical analysis

Statistical analysis was performed using software SPSS Statistics 17.0. The test results were represented as mean±standard deviation($\bar{x}$±s). When each variable was analyzed, a *t*-test was applied to evaluate the difference between the baseline values and the values under different predetermined conditions. The difference was statistically significant when $P<0.05$. The receiver operating characteristic curve(ROC) analysis was performed using IBM SPSS Statistics 22 software.

2　Results

2.1　Assay verification

The ratio of the peak area of Lyso-GB3 to the peak area of GSG was plotted against the concentration of Lyso-GB3(linear range: 0.244−500 μg/L) and analyzed by linear regression analysis (Figure 3). The regression equation "y=0.0181x+0.00103" was obtained by setting the weight at "1/x". The calculated result of regression coefficient(R^2) was 0.9989(n=3), and the LOD and LOQ were determined to be 0.122 μg/L and 0.244 μg/L, respectively. Table 1 summarizes the intra- and inter-day accuracy and imprecision of low, medium, and high QC samples, all of which are less than 15%. No carryover was

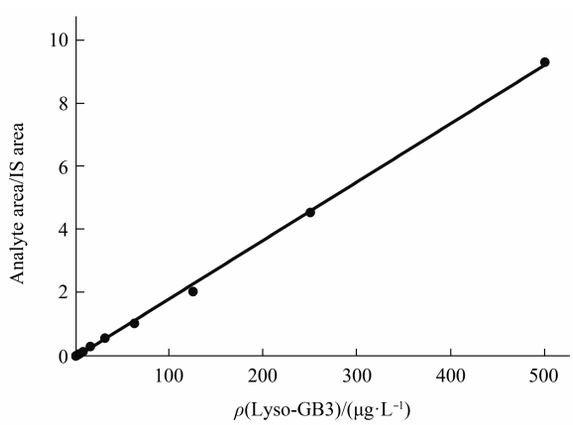

Fig. 3　The calibration curve of Lyso−GB3

Table 1　Intra− and inter−day accuracy and imprecision of Lyso−GB3 spiked quality controls （QCs）

Plasma Lyso-GB3/（μg/L）	Intraday（n=5）		Interday（n=3）	
	Accuracy（Bias%）	Imprecision（CV%）	Accuracy（Bias%）	Imprecision（CV%）
1	2.2	2.95	2.43	2.91
30	1.06	2.04	1.44	2.7
300	−1.6	2.28	−1.49	1.86

detected between the injections(P>0.05), the detailed data are summarized in supplementary material. Together, these results showed that the method is sufficient for use in analysis of Lyso-GB3.

2.2 Effect of storage temperature and time on plasma samples

In order to study the effects of storage time at different temperatures on plasma Lyso-GB3 level, we measured a total of 93 aliquots of plasma. The detailed data are summarized in supplementary material. As shown in Figure 4, the baseline value was detected as (177.3±5) μg/L(Storage time: 0 day). We failed to find statistically significant difference(P> 0.05) in Lyso-GB3 concentrations at any time point of this analysis (12 h, 1 d, ..., 28 d), in comparison to the baseline value, when the storage temperatures were -20℃ and 4℃. No statistically significant alterations were observed in Lyso-GB3 concentrations at storage conditions at 20℃ for 20 d (P>0.05). However, we found statistically significant differences between the baseline value and the Lyso-GB3 concentrations post 20 d (P<0.05), and the Lyso-GB3 values decreased by 6.5% and 9.6%, respectively. All the CVs of measurements in this study were lower than 15%. Together, these results suggested that Lyso-GB3 was stable in plasma samples stored at −20℃ and 4℃ for about 28 d. When stored at room temperature(20℃), the FD plasma Lyso-GB3 level gradually decreased post 4 d. The quality of FD samples was stable for about 20 d at storage conditions at 20℃.

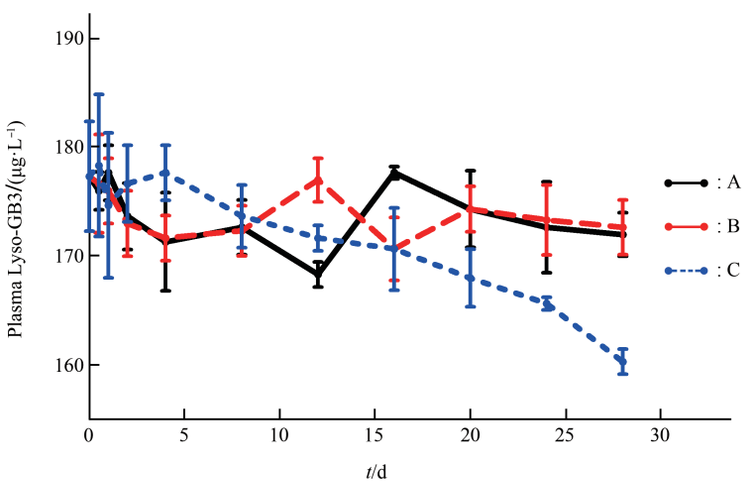

Fig. 4　The effect of storage temperature and time on the plasma Lyso−GB3 assay

The x-axis represents the time（days）. The y-axis shows Lyso-GB3 values（mean±sd）. Line A, B and C represent plasma Lyso-GB3 values at storage temperature of −20℃, 4℃ and 20℃, respectively. All comparisons were made relative to the baseline result.

2.3 Effect of freeze−thaw cycles on plasma samples

In order to study the effects of freeze-thaw cycles on plasma Lyso-GB3 level, we measured a total of 15 aliquots of plasma. Figure 5 shows minor fluctuations of Lyso-GB3 levels at different freeze-thaw cycles. The baseline value was measured as (177.3±5) μg/L (Freeze-thaw cycle: 0). No statistically significant change(P>0.05) was measured for plasma Lyso-GB3 throughout this process. All CVs in this study were lower than 15%. Together, these results suggested that freeze-thaw cycle has little impact on the stability of plasma samples.

2.4 Effect of hemolysis on samples

In order to study the effects of hemolysis on plasma Lyso-GB3 level, we selected a total of 18 aliquots of whole blood. These samples were centrifuged and the supernatants analyzed using a BC-5310 automated blood cell analyzer to measure the hemoglobin(Hb) concentration. The average Hb concentrations in samples of level 1, 2, 3, 4, and 5 were 0, 1, 3, 10, 49 g/L, respectively(n=3). As shown in Figure 6, the detected baseline value was (88.5 ± 2.8) μg/L(no hemolysis). When the degree of hemolysis was at level 1(Hb: 0 g/L), level 2(Hb: 1 g/L), and level 3(Hb: 3 g/L), respectively, we found

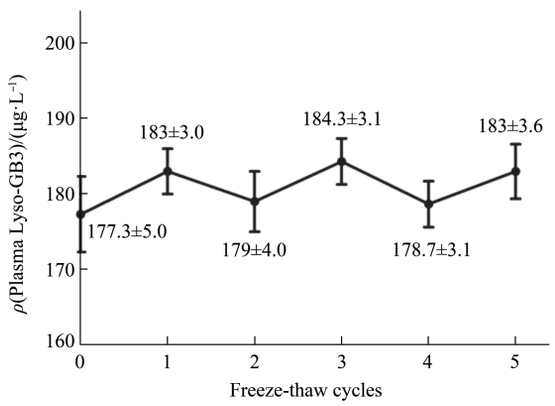

Fig. 5　The effect of freeze−thaw cycles on the plasma Lyso−GB3 assay

The *x*-axis represents the freeze-thaw cycles of plasma samples. The *y*-axis shows the Lyso-GB3 values（mean±*sd*）. All comparisons are made relative to the baseline result.

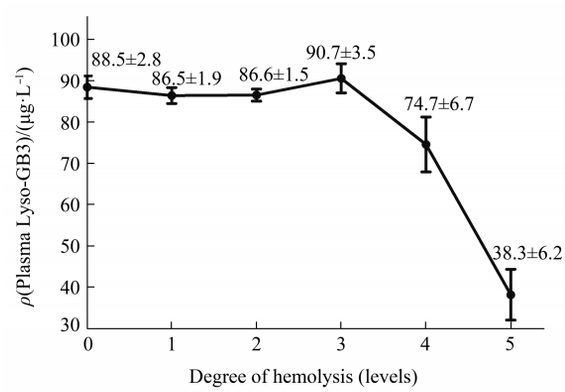

Fig. 6　The effect of hemolysis on the plasma Lyso−GB3 assay

The x-axis represents the degree of hemolysis of plasma samples. The y-axis shows the Lyso-GB3 values（mean±*sd*）. All comparisons are made relative to the baseline result.

no statistically significant differences(P>0.05) in Lyso-GB3 values in comparison to the baseline value. However, when the degree of hemolysis was at level 4 (Hb: 10 g/L), the Lyso-GB3 value decreased to 74.7 μg/L and the CV increased to 8.9%. When the degree of hemolysis was at level 5(Hb: 49 g/L), the Lyso-GB3 value dramatically decreased to 38.3 μg/L and the CV increased to 16.1%. The degree of hemolysis at level 4 and 5 resulted in statistically significant changes(P<0.01) in Lyso-GB3 values. Together, these results suggested that moderate (Hb: 10 g/L) and severe(Hb: 49 g/L) hemolysis can affect the accuracy and imprecision of the detection significantly.

2.5　Effect of storage temperature and time on whole blood samples

In order to study the effect of storage temperature and time on whole blood samples, we measured a total of 36 aliquots of whole blood. Detailed results were summarized in supplementary material. As shown in Figure 7, the detected baseline value was (88.5±2.8) μg/L(Storage time: 0 h). The Lyso-GB3 level at any of the 6 moments of the analysis (12 h, 24 h, ..., 72 h) changed little when compared to the baseline result, regardless of the storage temperatures chosen, as supported by our statistical analysis(P>0.05). All CVs in this study were below 15%. Together, these results suggested that storage temperature and time has little impact on the stability of whole blood samples.

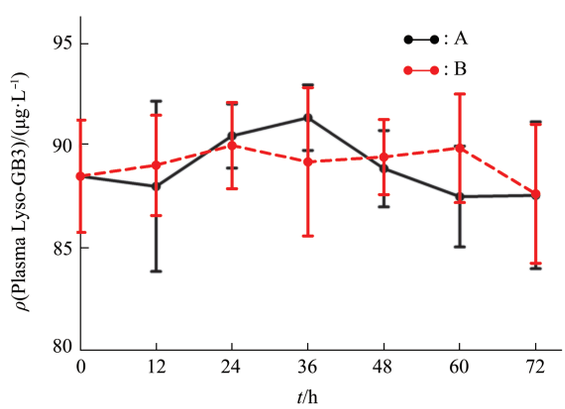

Fig. 7　The effect of storage temperature and time on the whole blood Lyso−GB3 assay

The *x*-axis represents the time. The *y*-axis shows the Lyso-GB3 values（mean±sd）. Line A and B represent the whole blood Lyso-GB3 values at storage temperature of 4℃ and 20℃, respectively. All comparisons are made relative to the baseline result.

2.6　Effect of centrifugation temperature on samples

In order to study the effect of centrifugation temperature on whole blood samples, we measured a total of 9 aliquots of whole blood. Figure 8 shows the relatively smooth fluctuations of Lyso-GB3 concentrations when whole blood was centrifuged at different temperatures. The baseline value was measured as (88.5±2.8) μg/L (centrifugation

temperature: 4℃). Regardless the chosen centrifugation, the Lyso-GB3 concentrations exhibited no statistically significant changes(P>0.05). All CVs in this study were below 15%. Together, these results suggested that centrifugation temperature has little impact on the stability of whole blood samples.

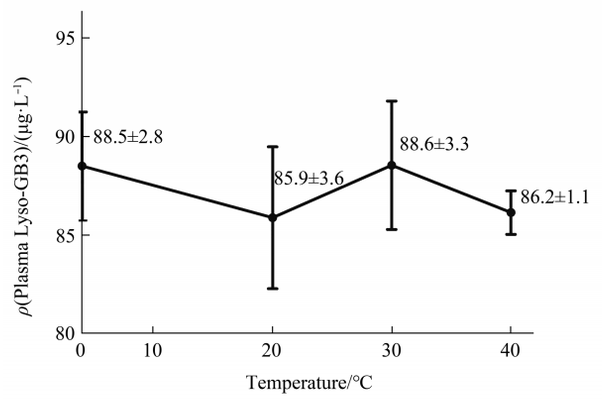

Fig. 8 The effect of centrifugation temperatures on the plasma Lyso−GB3 assay

The *x*-axis represents the centrifugation temperatures (4, 20, 30 and 40℃). The *y*-axis shows the Lyso-GB3 values(mean±sd). All comparisons are made relative to the baseline result.

2.7 Sensitivity and specificity

After the assay verification was carried out and the conditions for guaranteeing the quality of the FD sample were determined, in order to verify the sensitivity and specificity of the method, we collected 86 FD samples and 100 healthy human samples over a two-year period following the conditions we found for guaranteeing the quality and analyzed these samples with LC-MS/MS. As shown in Table 2, the mean level of Lyso-GB3 was 95.3 μg/L in 75 male patients (range: 1.15−381 μg/L) and 9.2 μg/L in 11 female patients(range: 0.91−23.9 μg/L). The mean level of Lyso-GB3 was 0.44 μg/L in 50 normal males(range: 0.27−0.78 μg/L) and 0.462 μg/L in 50 normal females (range: 0.29−0.72 μg/L). With a cutoff value of 0.81 μg/L of the Chinese population[13], patients with Lyso-GB3 concentration higher than 0.81 μg/L were considered to be FD patients. The sensitivity and specificity were both 100%(Figure 9). These results demonstrate that our LC/MS-MS method can accurately screen FD samples.

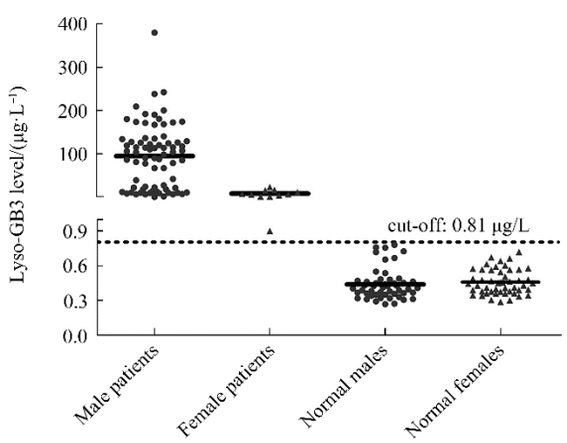

Fig. 9 Plasma Lyso−GB3 levels in male patients (*n*=75), female patients (*n*=11), healthy males (*n*=50) and healthy females (*n*=50)

Solid lines represent the mean value in each group. The dot line represents the cut-off value for normal population.

Table 2 Details of 86 FD patients and 100 normal people

Parameter	FD patients (*n*=86)				Normal human (*n*=100)	
	Males	Females	Classical	Non-Classical	Males	Females
Number (*n*)	75	11	69	17	50	50
Age (mean±*sd*)	33.3±9.3	41.1±7.8	33.3±9.8	38.4±6.5	32.7±11.2	37.7±13.7
Lyso-GB3 level range	1.15−381	0.91−23.9	0.91−381	1.72−42.8	0.27−0.78	0.29−0.72
Lyso-GB3 level (mean±*sd*)	95.3±74.1	9.2±7.4	101.3±74.2	15±10.3	0.44±0.13	0.46±0.11

86 FD patients were classified according to gender and disease type. 100 normal people are classified by gender. All values of Lyso-GB3 are given in μg/L.

2.8 Lyso−GB3 level and disease classification of FD

According to clinical manifestations and α-Gal A enzyme activity, Fabry disease can be classified into classical and non-classical types. To investigate whether Lyso-GB3 concentration is related to disease classification, we performed scatter plot analysis and ROC curve analysis. As shown in Table 2 and Figure 10a, of the 86 patient samples, 69 were classical, with an average age of 33.3, and 17 were non-classical,

with an average age of 38.4. The mean level of Lyso-GB3 in the classical patients was 101.3 μg/L, and that in the non-classical patients was 15 μg/L. The mean level of Lyso-GB3 in the classical patients was much higher than that in the non-classical patients(P< 0.001). The ROC curve was plotted with the state variables set to classical and non-classical Fabry disease(Figure 10b), the AUC(area under curve) value is 0.83. When the cut-off value was set to 55.15 μg/L, the Youden index(sensitivity+specificity-1) was the largest, and the sensitivity and specificity were 71% and 100%, respectively. It indicates that the Lyso-GB3 level is an ideal reference for the classification of FD.

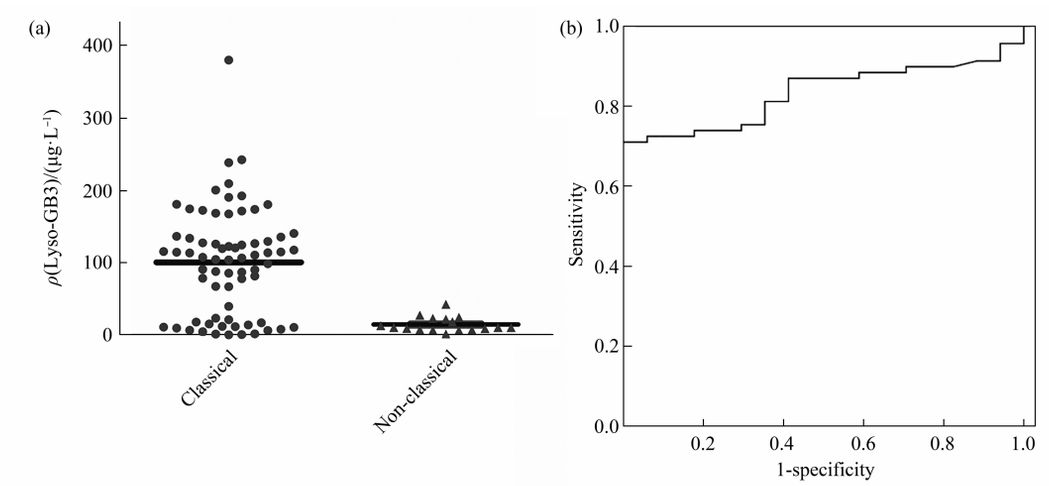

Fig. 10 Plasma Lyso−GB3 levels in classical and non−classical type of FD（a）and ROC analysis（b）

(a) The *x*-axis represents the classification of FD, the *y*-axis shows the plasma Lyso-GB3 levels. (b) A receiver operating characteristic curve of diagnostic value of Lyso-Gb3 between classical FD patients and non-classical FD patients.

3 Discussion

In summary, we found that freeze-thaw cycles, storage temperature and time of whole blood samples, and centrifugation temperature had little impact on FD samples' quality. However, plasma Lyso-GB3 level decreased post 4 days at storage condition at 20℃ . In addition, moderate and severe hemolysis could adversely affect the quality of FD samples, further influencing the accurate quantification of Lyso-GB3.

Hemolysis is believed to be the most common cause of unqualified specimens in clinical laboratories (40%−70%)[27]. Several factors can lead to hemolysis, including improper operation during blood drawing, the patient's own hemolytic disease, and inappropriate management of samples after blood was taken. Although the effects of hemolyzed samples on clinical chemistry and immunochemistry have been widely reported[28-29], few reports studied the potential influence of sample hemolysis on LC-MS/MS testing.

The effects of hemolysis on the Lyso-GB3 value may be speculated as follows: (1) Generally, after red blood cells rupture, various substances(iron, potassium, carbohydrates, aspartate aminotransferase, lactate dehydrogenase, *etc*.) enter the plasma, which may ultimately change the concentration of Lyso-GB3 by direct interference, absorption and degradation[30]. (2) For our study, we used solid phase extraction to remove the inferences in plasma like proteins to improve the sensitivity of Lyso-GB3 detection by LC-MS/MS. When the degree of hemolysis was at level 4 and 5, the amount of hemoglobin in plasma was too high which exceeded the processing capacity of the solid phase extraction column. The unremoved hemoglobin, after entering the mass spectrometer, can suppress the signal of the target analyte in mass spectrometry.

How to minimize the influence of hemolysis on sample testing has been a common problem faced by medical laboratories around the world: minimizing the effect of hemolysis on samples has been the focus of much work, as this would greatly advance the accurate quantification of target analyte. In addition to

optimizing chromatographic conditions and mass spectrometry parameters, Hughes *et al.*[31] believe that adding an extra step for protein precipitation during sample preparation should help to attenuate the interference of hemolysis. In addition, a number of reports suggested that using isotopically labeled internal standards instead of the non-isotopically labeled internal standards can more efficiently reduce the influences of matrix effects[23,32]. Because the isotope internal standard of Lyso-GB3 was not commercially available, we could only use a non-isotopically labeled internal standard(GSG) in our study.

Our laboratory has established a method for detecting Lyso-GB3, which is highly specific, accurate and precise, conformed with the requirements of LC-MS/MS assay verification. Many reports indicated that the majority of errors in medical laboratories occur in the pre-analytical phase[27,33]. In particular, the improper collection of samples and unsuitable mode of transport of samples may affect the quality of the samples badly and bring errors to routine laboratory analysis. Our study greatly enhanced a detailed understanding the effects of pre-analytical variables on LC-MS/MS detection of Lyso-GB3 during the periods of blood collection, centrifugation, sample transport and storage. The cold chain transport of plasma samples is preferred, but if cold chain transport is impossible, plasma samples should be transported to center institute within 4 days to avoid the decrease of stability of Lyso-GB3. To avoid hemolysis, care should be taken to ensure that the blood collection process is correct and no violent shocks and sudden freezing of collection tubes. When hemolyzed samples(Hb>3 g/L) were finally sent to the center institute, the LC-MS/MS method should be further optimized to reduce the interferences from hemolysis.

Our LC-MS/MS method for detecting Lyso-GB3 shows great sensitivity and specificity toward diagnosis of FD. We found that although there were not much difference of plasma Lyso-GB3 level between healthy males and females, the Lyso-GB3 concentrations in male FD patients were much higher than those in female FD patients. This is in agreement with the nature of FD, a X-linked recessive hereditary disease. Furthermore, we can effectively distinguish between classical and non-classical types by measuring plasma Lyso-GB3 Level, which provides a powerful tool for clinicians to determine the Chinese FD patient classification.

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液相色谱串联质谱法检测法布雷病样本的质量控制研究*

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摘要　法布雷病（FD）是一种罕见的X连锁隐性遗传的溶酶体贮积病. 用于检测FD的常用生物学标志物之一是Lyso-GB3，它是一种脱乙酰基的神经酰胺三己糖苷，通常通过高灵敏和特异性的液相色谱-串联质谱法（LC-MS/MS）进行分析. 由于LC-MS/MS技术的复杂性，中国大多数医院仍无法自行建立LC-MS/MS对FD进行高效可靠的诊断. 患者的样本需要在当地医院采集，然后送到中心实验室进行LC-MS/MS分析. 样本经过漫长的运输和储存过程，可能会影响样本质量，从而影响诊断的准确性. 我们首先建立并验证了一种新的LC-MS/MS方法以保证FD样本质量，研究了分析前变量对Lyso-GB3检测的影响. 这些分析前变量包括血浆的储存温度和时间、血浆的冻融循环、血浆的溶血程度、全血的储存温度和时间，以及离心温度. 最后，我们分析了86个FD样本和100个正常人样本，并对Lyso-GB3浓度和与FD疾病分型的关系进行了相关性研究. 结果显示，当血浆在20℃下储存时，Lyso-GB3浓度在4 d后开始下降. 此外，溶血会显著影响FD样品的质量，重度溶血导致Lyso-GB3浓度下降了57.8%，其他分析前变量对FD样品质量影响不大. 本方法在使用分析前标准化操作流程下，筛查FD的灵敏度和特异度为100%，确保了检测的正确性；当Lyso-GB3浓度的cut-off值为55.15 μg/L，鉴别经典型、非经典型患者的灵敏度和特异度为71%和100%，ROC曲线下面积为0.83. 我们的研究结果有助于医学实验室操作程序的标准化，以保证FD样本的质量，并为临床判定中国法布雷病的分型提供依据.

关键词　法布雷病，脱乙酰基的神经酰胺三己糖苷，液相色谱串联质谱法，分析前因素，质量控制，疾病分型

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