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Globotriaosylceramide isoform profiles in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

Globotriaosylceramide (GL3) is a heterogeneous glycosphingolipid that is elevated in the blood plasma of patients diagnosed with Fabry disease. GL3 consists of numerous isoforms, some of which are distinctly specific to human plasma. An electrospray-ionization LC/MS/MS method has been developed that has the capacity to monitor the GL3 isoform profiles in plasma extracts. Total GL3 is extracted from human plasma via chloroform/methanol liquid–liquid extraction, purified by C_{18} solid-phase extraction and analyzed by multiple reaction monitoring LC/MS/MS. The relative responses of eight selected isoforms are calculated on the basis of the total GL3 response and the isoform responses are subsequently utilized to construct isoform profile plots.

Keywords: Globotriaosylceramide; Lipids

1. Introduction

Fabry disease (Anderson-Fabry disease) is a rare inherited lysosomal storage disorder caused by partial or complete deficiency of the lysosomal enzyme alpha-galactosidase A (EC 3.2.1.22). Because the disease is X-linked, it predominantly affects males (hemizygotes) even though females (heterozygotes) are known carriers. Deficiency of alpha-galactosidase A results in the progressive accumulation of neutral glycosphingolipids, specifically globotriaosylceramide (GL3, Fig. 1A), in the vascular endothelium and visceral tissues throughout the body. As GL3 accumulates in the body, affected individuals suffer from chronic pain, renal failure, stroke and cardiovascular complications. Clinical manifestations of disease progression in Fabry patients are commonly marked by the finding of increased GL3 levels in the peripheral blood system (plasma) and nonneural tissues (kidney, heart, skin, etc.).

Recent progress in the treatment of Fabry disease has shown that elevated GL3 levels can be significantly decreased in patients receiving enzyme replacement therapy (ERT) [1–3], suggesting that GL3 could function as a surrogate biomarker. However, the accurate measurement of GL3 in biological samples, i.e., plasma, is not trivial due to the inherent heterogeneity and amphiphilic nature of the GL3 molecule (Fig. 1A). The structure of both the sphingoid base region (long-chain base), as well as the fatty-acyl chain region, can exhibit heterogeneity which increases the overall complexity of GL3 measurements [4]; the additional measurement complexity is attributed to the need to measure a large number of possible GL3 isoforms. Current analytical methods typically reduce the complexity of GL3 determinations by measuring "total GL3" in a one step summation of all GL3 isoforms. Methods such as thin-layer chromatography (TLC) [5] and enzyme-linked immunosorbent assay (ELISA) [6] measure total GL3 in this manner. Other methods, such as liquid chromatography with ultraviolet detection (LC/UV) [7–11] or gas chromatography with flame ionization detection (GC-FID) [12], measure total GL3 by measurement and summation of the individual isoforms. However, both the LC and GC methods require lengthy derivatization schemes that produce indirect measurements of the GL3 isoforms. Most recently, electrospray-ionization tandem mass spectrometry (ESI/MS/MS) in flow-injection

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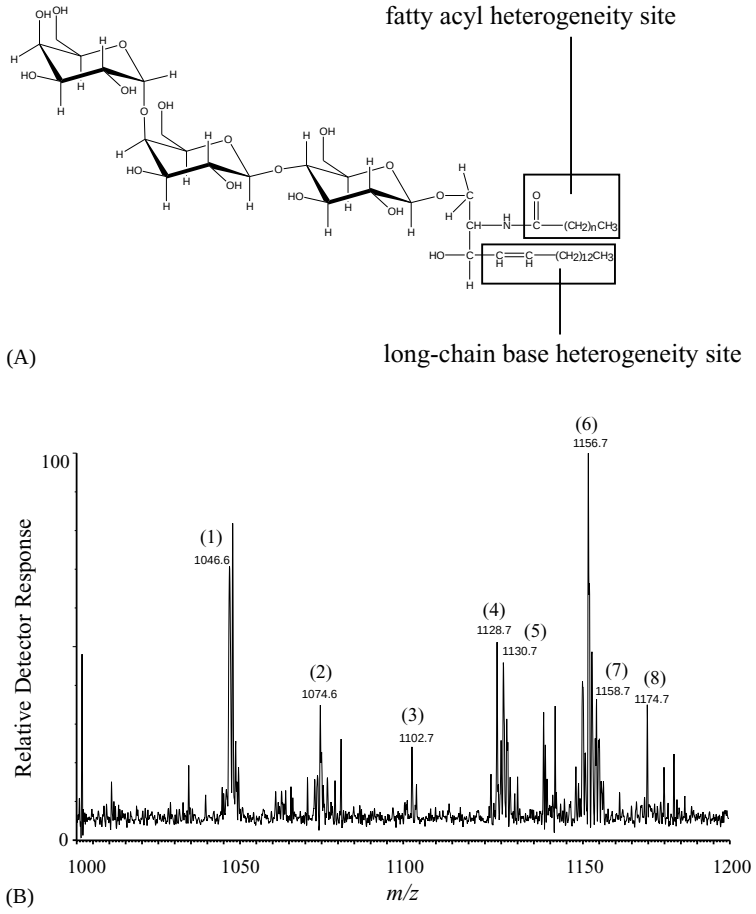


Fig. 1. (A) General molecular structure of GL3 showing the two sites of structural heterogeneity. The N-linked fatty-acyl region typically contains saturated and mono-unsaturated species with chain lengths from C_{14} to C_{26}; hydroxylated derivatives of these fatty-acyl species also exist. The long-chain base region typically contains dihydroxylated sphingosine species (d18:1) in mammalian tissues; however, other species such as dihydroxylated dihydrosphingosine (d18:0) and dihydroxylated dihydrosphingosine (d18:2), etc., are present at reduced levels. (B) Characteristic constant neutral loss mass spectrum of human plasma GL3 isoforms obtained by LC/MS/MS analysis. The plasma was prepared, extracted and analyzed using the conditions described in Section 2. The identity of the eight isoforms (monosodiated precursor ions) are listed in Table 1.

mode [13,14] has been applied to the measurement of total GL3 in plasma. MS/MS analyses, besides providing increased analytical sensitivity, allow for the direct measurement of total GL3, as well as of the individual GL3 isoforms.

There exists little information regarding the relative distribution of GL3 isoforms in the plasma of patients undergoing ERT. The monitoring of individual isoforms or specific isoform ratios [13], in addition to the quantification of total GL3, could potentially yield a better insight into the overall effectiveness of ERT. A high-throughput LC/MS/MS method has been developed to monitor the GL3 isoform profiles in human plasma. The method was optimized to permit rapid multiple-reaction-monitoring (MRM) of eight predominant GL3 isoforms in plasma (Table 1). Briefly, total lipids were extracted from plasma via chloroform/methanol liquid–liquid extraction and GL3 was purified from the total lipid extract by C_{18} solid-phase extraction (SPE) [6,12]. The extracted GL3 was then solubilized in dimethyl sulfoxide (DMSO) and the specified isoforms were monitored by LC/MS/MS.

2. Experimental

Note: All lipid powders, lipid solutions and plasma lipid extracts were stored at $\leq -20\,^{\circ}C$, unless noted otherwise.

Table 1
Plasma GL3 isoforms[a]

#	GL3 isoform	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)
1	C16:0	1046.7	884.6
2	C18:0	1074.7	912.6
3	C20:0	1102.7	940.7
4	C22:1	1128.7	966.7
5	C22:0	1130.8	968.7
6	C24:1	1156.8	994.7
7	C24:0	1158.8	996.7
8	C24:0-OH	1174.8	1012.7
IS[b]	C17:0	1060.7	898.6

[a] Precursor ions are mono-sodiated. The listed product ions result from the neutral loss of a single galactosyl fragment (162.1 Da) from the precursor ions.

[b] C17:0-GL3 was utilized as an internal standard (IS) compound.

C/M refers to a freshly prepared solution of 2:1 (v/v) chloroform/methanol.

2.1. Materials

Porcine globotriaosylceramide (GL3) and porcine globotriosylsphingosine A (*lyso*-GL3) were obtained from Matreya (State College, PA). C16:0-GL3 and C17:0-GL3 were enzymatically synthesized from *lyso*-GL3 at Genzyme Pharmaceuticals (Liestal, Switzerland) using published procedures [13]. C16:0-fortified-GL3 was prepared by gravimetrically combining C16:0-GL3 and GL3 in a 9:25 g/g mass ratio. The C16:0-fortified-GL3 compound was then redissolved in C/M and aliquoted into glass autosampler vials so that each vial contained 10 μg of C16:0-fortified-GL3 after evaporation of the solvent. Heparin stabilized pooled human plasma was obtained from Interstate Blood Bank (Memphis, TN). HPLC grade methanol, water and chloroform were obtained from Honeywell Burdick & Jackson (Muskegon, MI). DMSO was obtained from Sigma (St. Louis, MO). LiChrolut RP-18 SPE columns (500 mg) were obtained from EM Science (Gibbstown, NJ). All other chemical reagents and solvents were ACS reagent grade unless stated otherwise.

2.2. Preparation of GL3 stock solutions

Glass autosampler vials containing known amounts (μg) of C16:0-GL3, C17:0-GL3 or C16:0-fortified-GL3 were reconstituted with appropriate volumes of DMSO as needed. The reconstituted standards were then vortex mixed (30 s) and sonicated (3 min, 37 °C) in a water bath before use.

2.3. Extraction and purification of total GL3 from human plasma

2.3.1. GL3 extraction

GL3 was extracted from human plasma on the basis of the Folch liquid–liquid partitioning procedure [15]. Note: plasma samples were clarified before analysis by centrifuging (14,000 rpm, 5 min, room temperature) to remove particulates and other insoluble matter. Briefly, 1200 μl of C/M solution + 60 μl of clarified plasma + 48 μl of 5 μg/ml C17:0-GL3 (240 ng of C17:0-GL3) were pipetted (in the order listed) into a 2 ml Eppendorf tube and the sample was vortex mixed (15 min) at room temperature. C17:0-GL3 was utilized as an internal standard compound in all procedures. The sample was then centrifuged (14,000 rpm, 5 min, room temperature) to pellet the proteins and the supernatant (1000 μl) was transferred into a clean Eppendorf tube. Two hundred microliters of distilled/deionized water was added to the supernatant, the supernatant and water were vortex mixed (30 s) and centrifuged (14,000 rpm, 5 min, room temperature) to promote liquid–liquid phase separation. The organic phase (bottom layer) containing the total extracted lipids was removed from the tube and transferred into a glass centrifuge tube (12 mm × 75 mm). The organic phase was then evaporated to dryness under N_2 and stored at −20 °C or reconstituted with 1000 μl of chloroform and purified as described in the next section (GL3 purification). Plasma calibrants (60 μl) were prepared in an identical manner as the samples, except that sequentially increasing amounts of a 40 μg/ml C16:0-fortified-GL3 stock solution were added to samples that contained a constant amount (48 μl of 5 μg/ml = 240 ng) of C17:0-GL3 internal standard stock solution. The preparation and use of C16:0-fortified-GL3 was rationalized based on the fact that normal human plasma contains an large abundance of the C16:0 isoform [13]; however, commercial porcine GL3 is notably deficient in the C16:0 isoform. In order to more closely mimic the C16:0 isoform level in human plasma, the porcine GL3 standard was accordingly fortified with the C16:0 synthetic isoform. The sequential GL3 concentrations corresponded to [0 (calibrant blank), 5, 10, 15, 20, 25, 30, 35 and 40] μg/ml GL3 and the C17:0-GL3 level corresponded to a constant 240 ng in each of the respective calibrants.

2.3.2. GL3 purification

GL3 was purified from total lipid extracts using commercial C_{18} SPE columns [6,12] as follows (all extractions were completed with a 12-port vacuum manifold system): (1) the column was preconditioned by rinsing (via gravity) with 1 ml of chloroform; (2) the column flow was sealed off and 0.5 ml of chloroform was added to the column bed; (3) the total lipid extract was applied to the top of the column and allowed to flow through (via gravity); (4) the column was washed with 1 ml of chloroform (5) and finally, GL3 was eluted directly into a glass autosampler vial by rinsing (via gravity) the column with 1 ml of 9:1 (v/v) acetone/methanol. The purified GL3 was evaporated under N_2, reconstituted with 75 μl of DMSO and injected (20 μl) onto the LC/MS/MS system.

2.4. Liquid chromatography

The LC system consisted of a Waters Alliance 2795 autosampler (25 °C) and pump. Samples were analyzed using 2-Luna C_8 guard columns connected in series (3 mm i.d. × 8 mm total length, 5 μm particle size). LC analysis of GL3 was performed using the following step-gradient elution conditions: mobile phase A = 2 mmol/l ammonium acetate + 0.1% formic acid in water, mobile phase B = 2 mmol/l ammonium acetate + 0.1% formic acid in methanol, mobile phase C = 2:1 (v/v) chloroform/methanol; time program = 0 min, 50% A/50% B/0% C; 0.5 min, 0% A/100% B/0% C; 6 min, 0% A/100% B/0% C; 9 min, 0% A/0% B/100% C; 10 min, 50% A/50% B/0% C; flow rate = 500 μl/min; column temperature = 45 °C.

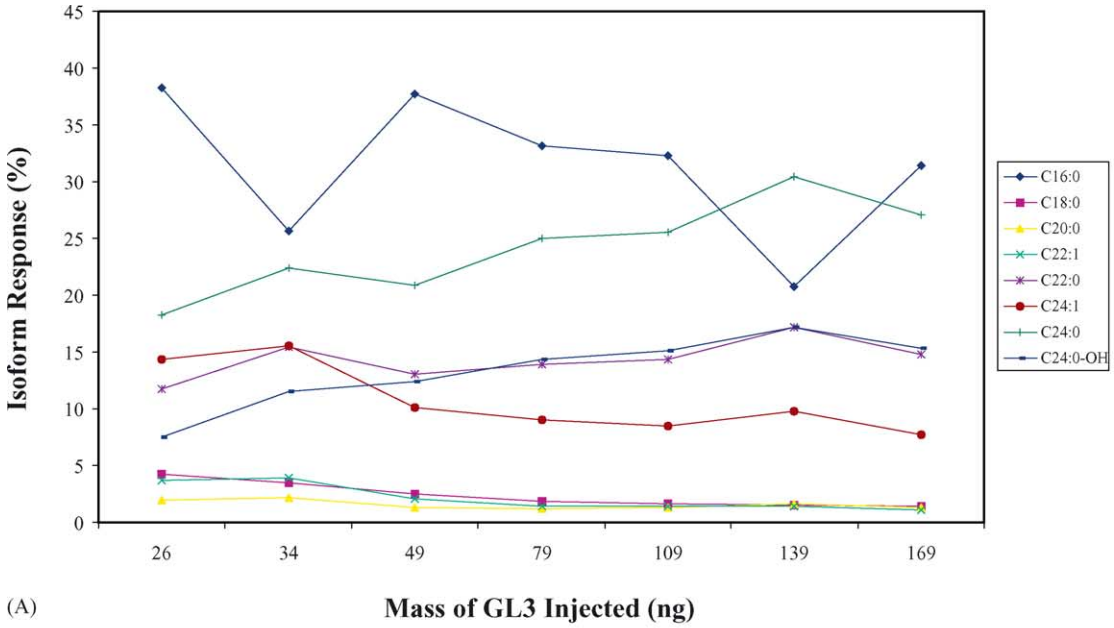
2.5. Mass spectrometry

MS/MS analyses were performed on a Micromass Quattro Micro benchtop tandem quadrupole mass spectrometer

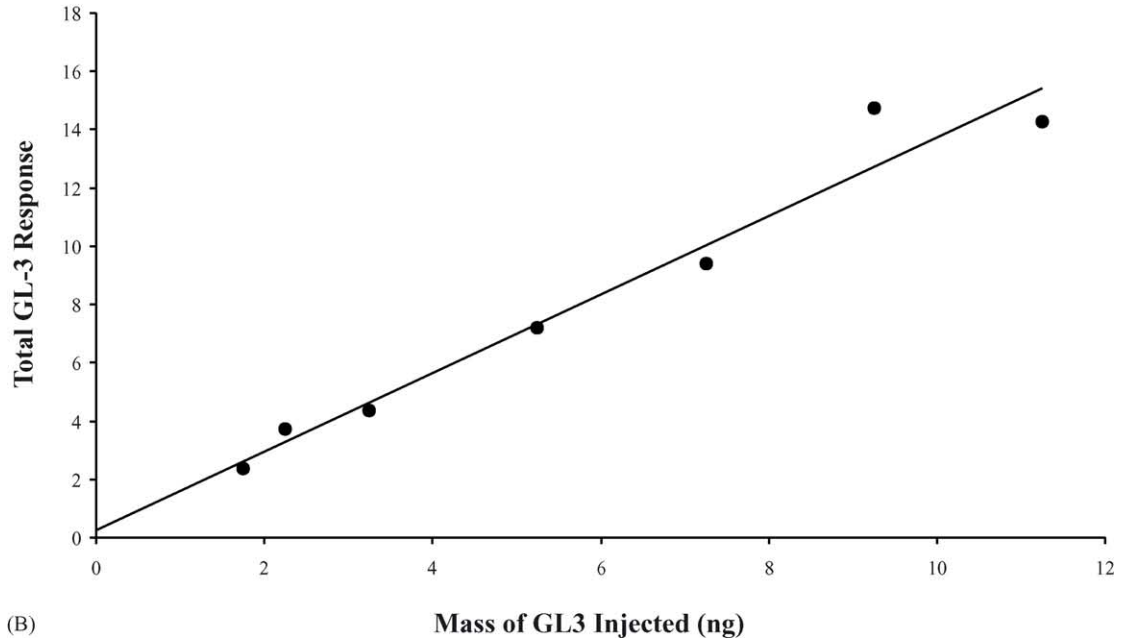
in positive ESI mode. Instrument parameters for LC/MS/MS analyses were as follows: dwell time = 0.1 s; interchannel delay = 0.03 s; interscan delay = 0.03 s; capillary voltage = 3.50 kV; cone voltage = 90 V; extractor voltage = 2.0 V; RF lens voltage = 0.2 V; source temperature = 130 °C; desolvation temperature = 350 °C; cone gas flow = 62 l/h; desolvation gas flow = 661 l/h; collision energy = 63 eV; multiplier setting = 650 V. Direct infusion parameters were identical to LC/MS/MS parameters except: source temperature = 100 °C, desolvation temperature = 150 °C, desolvation gas flow = 200 l/h. The relevant MRM transitions for the GL3 isoforms are listed in Table 1.

3. Results and discussion

Routine monitoring of plasma GL3 isoforms by LC/MS/MS requires the reproducible extraction, purification and concentration of total plasma GL3. Monitoring of



(A)



(B)

Fig. 2. (A) Typical isoform profile of extracted plasma calibrants using the unmodified MRM mode LC/MS/MS method. The calibrants were prepared, extracted and analyzed as described in Section 2 except for the following items: the initial plasma pool was not clarified, the final extracts were not solubilized in DMSO, the LC method did not incorporate a C/M rinse and the LC separation did not utilize two C_8 columns in series. (B) GL3 response linearity of extracted plasma calibrants based upon internal standard calibration ($y = 1.35x + 0.25$).

GL3 isoforms is complicated by the presence of other types of neutral glycosphingolipids (glucosylceramide, lactosylceramide, globotetraosylceramide) [9,16] in the plasma matrix and by the extensive heterogeneity of the GL3 fatty-acyl and long-chain base side groups [17,18]. However, previous researchers have shown that it is possible to selectively monitor the neutral loss of galactosyl groups (162.1 Da) from mono-sodiated GL3 isoform adducts $[M + Na]^+$ using appropriate collision-induced-dissociation (CID) MS/MS conditions [13,14]. Building upon this foundation, we have combined a reversed-phase LC separation step with selective mass spectrometric detection in order to develop a method capable of both concentrating and monitoring the GL3 isoforms. As detailed in Section 2, a GL3 standard or plasma extract is injected onto the LC system and eluted using a methanol-based step-gradient. Application of the step-gradient allows the removal of interfering contaminants and also allows the GL3 sample to elute as a concentrated plug for higher sensitivity detection. A typical LC/MS/MS analysis employing constant neutral loss (CNL) scanning of a plasma extract is shown in Fig. 1B. CNL MS/MS is based on monitoring the loss of identical neutral fragment(s) from a mixture of structurally similar compounds by scanning across a window of ions within a selected mass range using quadrupole one (Q1) and quadrupole three (Q3) simultaneously. To effect a CNL scan, Q1 is offset from Q3 by a fixed *m*/*z*, which corresponds to a structure specific neutral loss, i.e. 162.1 Da. Q1 is instrumentally set to a higher mass value than Q3. Precursor ions which produce the designated neutral fragments are the only ions transmitted to the detector. A total of eight GL3 isoforms (Table 1) were detected and identified based upon published data [13] and upon in-house CID MS/MS experiments (data not shown). The principal GL3 isoforms detected in the plasma extract were C16:0 (*m*/*z* 1046.6) and C24:1 (*m*/*z* 1156.7). However, reproducible monitoring of the isoform distribution using CNL MS/MS is not possible due to insufficient isoform detection sensitivity. Some of the isoforms, such as C18:0 (*m*/*z* 1074.6) and C20:0 (*m*/*z* 1102.7), are too low in absolute abundance to be detected reproducibly. However, MRM MS/MS utilizes specific ion transitions for the detection of individual molecular species, and as such, is inherently more selective and more sensitive than CNL MS/MS.

Development of an MRM LC/MS/MS monitoring method was initiated based on the analysis of dried GL3 standards and plasma calibrants solubilized in methanol. Both the standards and extracted plasma calibrants, spiked to increasing total concentrations with a C16:0-fortified-GL3 stock solution, exhibited isoform variability for five out of the eight monitored isoforms. Fig. 2A shows a typical profile of the relative isoform distribution for a set of extracted plasma calibrants; C16:0 and C24:0 are the predominant isoforms. The absolute GL3 concentrations for each data point have been converted to the actual mass (ng) of analyte injected on-column for purposes of clarity. Distinct cross-over points and fluctuations (increases and decreases) in the measured

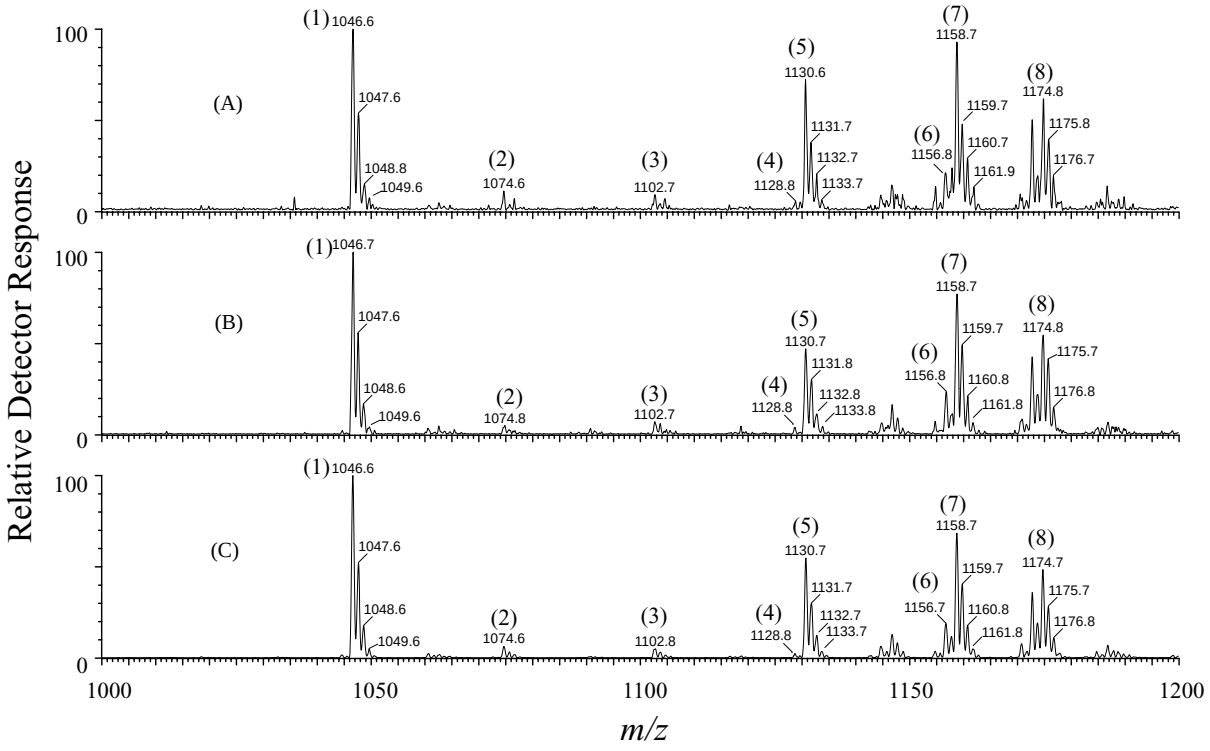


Fig. 3. Characteristic CNL (*m*/*z* 162) mass spectra of GL3 isoforms in C16:0-fortified-GL3 standards (10 μg/ml) solubilized in different organic solvents. All spectra were acquired via direct infusion (10 μl/min) of each standard and represent the average of 30 scans. The identity of the eight isoforms are listed in Table 1. (A) DMSO; (B) C/M; (C) methanol.

GL3 responses for individual isoforms across the calibration range were evident. A least squares plot (Fig. 2B) of the total GL3 response versus injected mass produced a non-linear ($r^2 = 0.949$) calibration line. The variability in the total GL3 response and mass have been corrected by the C17:0 internal standard response and mass.

Three main factors were identified as potential sources of the variability in the isoform profiles: (1) reduced solubility of GL3 in methanol, (2) reversible adsorption of GL3 to the LC tubing and to the LC column and (3) overload of GL3 onto the LC column. It is known that GL3 has limited solubility in alcohols (i.e., methanol) [19], but moderate solubility in mixtures of chloroform/methanol [19]. However, chloroform and methanol are hazardous chemicals, therefore DMSO was evaluated as an alternative solvent since it has minimal toxicity and been demonstrated to be a good solvent for certain glycosphingolipids [20]. Calibration curves of C16:0-fortified-GL3 standards prepared in methanol, C/M and DMSO were generated using the MRM mode LC/MS/MS conditions (using a single LC column) given in Section 2. Analysis of the results indicated that the maximum solubility (~80 μg/ml) of GL3 in

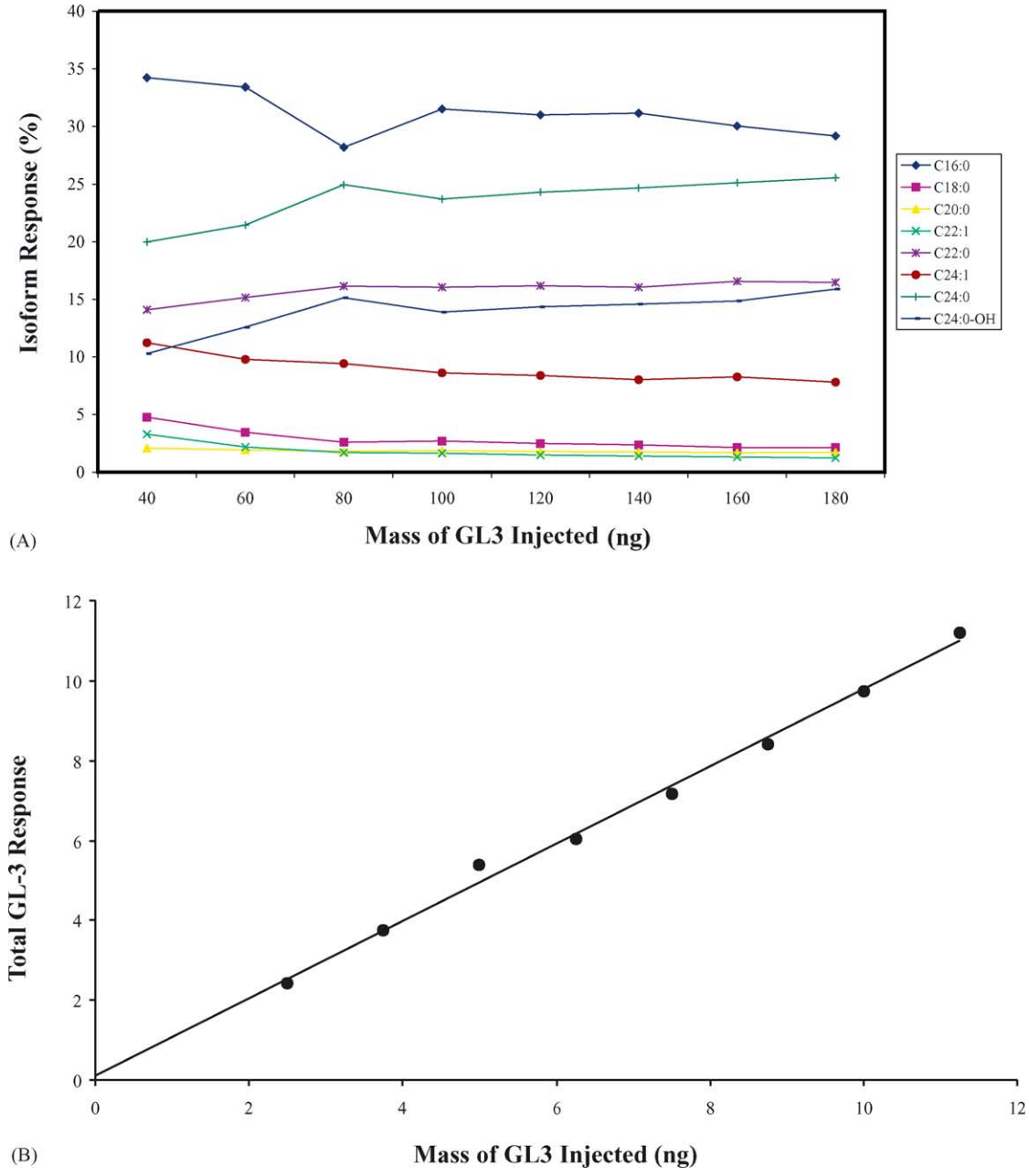


Fig. 4. (A) Typical isoform profile of extracted plasma calibrants using the modified MRM mode LC/MS/MS method. The calibrants were prepared, extracted and analyzed as described in Section 2. (B) GL3 response linearity of extracted plasma calibrants based upon internal standard calibration ($y = 0.97x + 0.12$).

methanol was substantially less than the maximum solubility (>100 μg/ml) of GL3 in C/M or DMSO. Standards of C16:0-fortified-GL3 (10 μg/ml) were also solubilized in the three solvents and analyzed by direct infusion CNL MS/MS. The eight mono-sodiated isoforms were readily observed in each of the standards (Fig. 3) and the CNL spectra in the three different solvents were virtually identical with good isotopic resolution for each of the most abundant isoforms (C16:0, *m/z* 1046.6; C22:0, *m/z* 1130.7; C24:0, *m/z* 1158.7; C24:0-OH, *m/z* 1174.7). The results from the solubility study indicated that DMSO was similar to C/M in its capacity to solubilize higher levels of GL3 than methanol. The results from the CNL isoform study showed that there exists no apparent bias among the solvents for the different isoforms at 10 μg/ml. Based on both sets of experimental results and factoring in the low toxicity of DMSO, the solvent for the preparation of GL3 standards and calibrants was changed from methanol to DMSO.

GL3 isoforms with long-chain fatty-acyl groups tend to adhere to PEEK LC tubing and to reversed-phase LC column packings due to hydrophobic adsorption. Decreases and increases in the relative detector response for the C22:0, C24:0 and C24:0-OH isoforms were readily observed by simply varying the inner diameter and/or length of the PEEK tubing connecting the LC sample injector to the electrospray source. The relative detector response for the long-chain fatty-acyl isoforms also varied from injection to injection when using the same tubing. Adsorption of the isoforms was likely occurring throughout the LC system and was undoubtedly a significant contributing factor leading to variability in isoform profiles. In order to reduce and/or prevent the adsorption of the isoforms throughout the LC system, a rigorous rinsing procedure, following each analysis of GL3, was developed based on the use of a C/M solution. A 1 min rinse with C/M solution after elution of GL3 from the LC column was effective in removing >99% of the GL3 retained on the LC system.

Short-chain fatty-acyl isoforms (C16:0, C18:0) injected at high levels (≥80 ng GL3 on-column) onto a single C_8 LC column tend to overload the column and partially elute within the LC system void volume. MRM LC/MS/MS analysis of calibration standards (0–100 ng) demonstrated that as the level of GL3 injected onto the column increased above ~80 ng, the percentage of each short-chain isoform eluting within the system void volume steadily increased. Overloading of the LC column was another distinct contributor to the fluctuation of the isoform profiles, specifically for the C16:0 isoform (Fig. 2A). Overloading of the LC column was readily corrected by increasing the length of the stationary phase from 4 to 8 mm (two 4 mm LC columns were connected in series). This modification permitted increased on-column loading of GL3 and extended the upper range for isoform analysis from ~100 to ~200 ng total GL3 on-column.

After incorporation of the described method modifications, the isoform profiles were reexamined by monitoring the GL3 isoforms in DMSO-based standards. Consistently flat isoform profiles (data not shown) were generated over the range of 20–200 ng GL3 (injected on-column), indicating that the method modifications were successful. The method modifications were further tested by preparing, extracting and analyzing a set of nine plasma GL3 calibrants reconstituted in DMSO. The calibrants produced relatively flat isoform profiles from 40 to 180 ng GL3 injected on column (Fig. 4A). The 20 ng GL3 calibrant was excluded from the profile because this sample, with no addition of exogenous C16:0-fortified-GL3, was not representative of the isoform profile characteristic of the analytical calibrants. The isoform profile from the calibrants contained only one minor cross-over point and the resulting internal standard calibration plot (Fig. 4B) showed satisfactory ($r^2 = 0.994$) response linearity.

4. Conclusions

The data generated by this study suggest that mass spectrometry has the potential to selectively monitor changes in the GL3 isoform profile in human plasma extracts. The sensitivity and reproducibility of the LC/MS/MS method for monitoring individual isoforms in actual Fabry patient samples are currently under investigation.

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