asymptomatic and symptomatic neonates show that this is possible.

Our results indicate that this method can be diagnostic for methylmalonic acidemia in DBS samples >100 months old, which suggests that this compound is relatively stable for many years if the DBS is stored at room temperature under relatively dry conditions. The method may therefore find use in retrospective screening of archived DBS specimens for previously undiagnosed cases suspected with methylmalonic acidemia. In this case, MS/MS analysis may be less useful because of the limited stability of carnitine esters (3).

To our knowledge, this is the first time that methylmalonic acidemia was definitively diagnosed from a DBS. Unfortunately, the method did not allow for the detection of control values for MMA, which are quite low (0–0.4 μmol/L). However, the same method may be useful if applied to serum or plasma samples and may serve as an alternative test for MMA in cobalamin deficiencies.

We gratefully acknowledge Prince Salman Center for Disability Research for funding this research.

References


Liquid Chromatography–Tandem Mass Spectrometry Quantification of Globotriaosylceramide in Plasma for Long-Term Monitoring of Fabry Patients Treated with Enzyme Replacement Therapy, Thomas P. Roddy,1 Bryant C. Nelson,1 Crystal C.C. Sung,1 Shaparak Araghi,1 Dennis Wilkens,1 X. Kate Zhang,2 John J. Thomas,2 and Susan M. Richards1 (1 Clinical Laboratory Science, and 2 Protein Characterization & Modification, Genzyme Corporation, One Mountain Road, Framingham, MA 01701-9322; * author for correspondence: fax 508-820-7664, e-mail Crystal. Sung@genzyme.com)

Fabry disease is a rare X-linked lysosomal storage disorder resulting from a deficiency in the α-galactosidase A enzyme. Deficiency in the activity of this enzyme causes an accumulation of neutral glycosphingolipids, predominantly globotriaosylceramide (GL-3), in most nonneural tissues and in body fluids (1). Recent clinical studies indicate that tissue and plasma GL-3 concentrations in Fabry patients can be significantly reduced by enzyme replacement therapy (2–5).

GL-3 (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue1/) exists as a mixture of structural isoforms containing acyl chains ranging from 16 to 24 carbons in length with various degrees of saturation and hydroxylation. These variations make the extraction and quantification of GL-3 challenging (6, 7). Moreover, no well-characterized reference standards of known purity are available.

GL-3 in tissues and plasma has been measured by thin-layer chromatography (8, 9), liquid chromatography (LC) (10–15), and gas chromatography (16), but the methods are labor-intensive and slow. An enzyme-linked immunosorbent assay (17) requires recombinant verotoxin B and polyclonal rabbit anti-verotoxin B. To date, the most rapid quantitative assays for total GL-3 have used flow-injection tandem mass spectrometry (MS/MS) (18, 19). We now report the development and use of a rapid LC/MS/MS method for the quantitative determination of total plasma GL-3.

Porcine GL-3 and porcine globotriaosylphosphinosine A (lyso-GL-3), from Matreya, were ≥98% pure by thin-layer chromatographic analysis. C16:0-GL-3 and C17:0-GL-3 were enzymatically synthesized from lyso-GL-3 at Genzyme Pharmaceuticals (18). C16:0-enriched GL-3 was prepared by gravimetrically combining C16:0-GL-3 and porcine GL-3 in a 9:25 g/g mass ratio. Methanol, water, and chloroform were HPLC grade.

Normal heparin-plasma samples from 104 men and 101 women were obtained from Interstate Blood Bank (Memphis, TN), ProMedDx LLC, and internal Genzyme sources. Heparin-plasma samples were also collected randomly from 57 Fabry patients enrolled in a clinical trial (3). Two sets of samples were collected: one set was from a group of patients who received intravenous Fabryzyme™ from the onset of the trial, whereas the other set received placebo for 5 months and was then given Fabryzyme. All patients provided informed consent. The procedures were approved by the Institutional Review Boards and/or Ethics Committees of all participating centers. Pooled normal plasma for method development and validation was from 50 healthy donors. Quality-control (QC) materials were prepared by combining plasma from healthy donors or Fabry patients.

Briefly, 1.2 mL of chloroform-methanol, 60 μL of plasma, and 48 μL of 5 mg/L C17:0-GL-3 were pipetted
into a 2-mL microcentrifuge tube. The sample was subsequently extracted (20, 21) and purified by solid-phase extraction as described recently (6, 7). Purified samples were reconstituted with methanol (75 μL), vortex-mixed (30 s), and sonicated (3 min at 37 °C) before analysis by LC/MS/MS.

Plasma calibrants were prepared in bulk quantities based on previously reported procedures (6, 7). Purified calibrants were reconstituted with methanol and analyzed by LC/MS/MS as described above.

LC/MS/MS analyses were performed on a Waters Alliance HPLC separations module coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer operating in positive electrospray ionization mode.

Purified extracts were injected (20 μL) on two Luna C8 guard columns [8 × 3 mm (i.d.); 5 μm particle size] set at 45 °C and connected in series and were eluted (500 μL/min) with the gradient mobile phase conditions given in Table 1 of the online Data Supplement. Mobile phase A was 2 mmol/L ammonium acetate plus 1 mL/L formic acid in water, mobile phase B was 2 mmol/L ammonium acetate plus 1 mL/L formic acid in methanol, and mobile phase C was 2:1 (by volume) chloroform-methanol.

The MS/MS operating and detection conditions have been described in other reports (6, 7). Neutral loss scan spectra were collected for both plasma extracts and calibrators. The GL-3 isoforms were detected and summed based on the multiple-reaction monitoring transitions specified in Table 1. The total GL-3 value (mg/L) was calculated by summing the responses from 10 individual isoforms (Fig. 2, a and b, in the online Data Supplement).

Commercial reference standards for human plasma GL-3 do not exist. Porcine GL-3 (Fig. 1A) was found to be similar to human GL-3 (Fig. 1B), but porcine GL-3 lacked the C16:0-GL-3 isoform usually found in high concentrations in human plasma GL-3. Porcine GL-3 was subsequently fortified with an appropriate amount of synthetic C16:0-GL-3 isoform to make the porcine material suitable for quantifying total GL-3 extracted from human plasma (Fig. 3a in the online Data Supplement). C17:0-GL-3 was also synthesized and used as an internal standard because this isoform is not a common isoform found in human plasma (Fig. 3b in the online Data Supplement).

The instrumental limit of detection for GL-3 was <0.01 mg/L (signal-to-noise ratio = 151). Repeat injection (n = 189) of a 5 mg/L GL-3 calibrator had area response and retention time CVs of 4.9% and 0.6%, respectively. The method limit of quantification was 0.5 mg/L with an injection imprecision of 15%. The bias in the determina-

### Table 1. Plasma GL-3 isoforms.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GL-3 isoform</th>
<th>Precursor ion, m/z</th>
<th>Product ion, m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C16:0</td>
<td>1046</td>
<td>884</td>
</tr>
<tr>
<td>2</td>
<td>C17:0</td>
<td>1060</td>
<td>898</td>
</tr>
<tr>
<td>3</td>
<td>C18:0</td>
<td>1074</td>
<td>912</td>
</tr>
<tr>
<td>4</td>
<td>C20:0</td>
<td>1102</td>
<td>940</td>
</tr>
<tr>
<td>5</td>
<td>C22:1</td>
<td>1128</td>
<td>966</td>
</tr>
<tr>
<td>6</td>
<td>C22:0</td>
<td>1130</td>
<td>968</td>
</tr>
<tr>
<td>7</td>
<td>C22:0-OH</td>
<td>1146</td>
<td>984</td>
</tr>
<tr>
<td>8</td>
<td>C24:1</td>
<td>1156</td>
<td>994</td>
</tr>
<tr>
<td>9</td>
<td>C24:0</td>
<td>1158</td>
<td>996</td>
</tr>
<tr>
<td>10</td>
<td>C24:0-OH</td>
<td>1174</td>
<td>1012</td>
</tr>
<tr>
<td>11</td>
<td>C26:0</td>
<td>1186</td>
<td>1024</td>
</tr>
</tbody>
</table>

* Precursor ions are monosodiated. The listed product ions result from the neutral loss of a single galactosyl fragment (162 Da) from the precursor ions.
tion at 0.5 mg/L was 14.7% (n = 10), and the measured value was different from the matrix signal with 97% confidence (Student t-test). The area response injection precision of QC materials was ~6% over 10 testing days.

Method accuracy was assessed based on 10 days of analysis. Measured results across the calibration range were within 94–114% of the expected values. The interassay imprecision (CV) of the calibration points (2–40 mg/L) was 6.0–14% (n = 10), whereas the intrassay imprecision of the points was 4.1% (n = 3). The solid-phase extraction procedure had a CV of 14% based on a 1-day analysis of 36 extractions performed by three operators over 2 months. The overall assay (including analyte extraction and LC/MS/MS analysis) had a CV of 17% based on 10 QC samples analyzed over a 2-month period.

The distributions of GL-3 concentrations in the plasma of 205 healthy donors and 57 Fabry patients are shown in Fig. 1C. Healthy donors had a mean of 3.5 mg/L, a median of 3.3 mg/L, and a range of <2 to 11.2 mg/L for total GL-3. Fabry patients had a mean of 10.1 mg/L, a median of 9.4 mg/L, and a range of 3.6–16.7 mg/L for total GL-3. The difference between the healthy donors and Fabry patients was significant (P <0.0001) by the Student t-test. A decrease in plasma GL-3 concentration was observed in Fabrazyme-treated patients. In general, after 3 months of receiving Fabrazyme, plasma GL-3 concentrations were reduced to within the reference interval and essentially remained within the reference interval for the rest of the study (Fig. 4, a and b, in the online Data Supplement).

The first step in developing this method was to locate a well-characterized GL-3 reference standard for the preparation of GL-3 calibration curves. Unfortunately, at this time, no defined standards exist for the quantification of GL-3 calibration curves. In addition to providing a tool for characterizing GL-3 isoforms (6, 7), LC/MS/MS analysis allows for the sensitive quantification of low concentrations of total GL-3. The GL-3 limit of quantification (0.5 mg/L) for this method, including extraction and purification, is well within the detection limits of the MS system.

Comparison of this new method with previously reported methods in terms of accuracy, reproducibility, and analyst effort is not straightforward. Differences involved in the multiple steps of the extraction methods, selection of Fabry patients and healthy donors, preparation and sources of calibrators, and principles of methodologies will contribute to the variances inherent to GL-3 determinations. Zeidner et al. (17) used an ELISA method to determine total GL-3; the mean plasma GL-3 concentrations in male and female Fabry heterozygotes were reported as 12.6 and 1.1 mg/L, respectively, whereas the concentration in healthy individuals was 0.9 mg/L. In contrast, Mills et al. (18) reported that the mean total GL-3 concentrations, measured by flow-injection MS/MS in Fabry and normal plasma, were 29.1 and 8.4 mg/L, respectively. Schifflmann et al. (10) reported mean plasma GL-3 values in Fabry (placebo and treated) of 10.96 and 12.14 nmol/mL (equivalent to 11.6 and 12.8 mg/L).

Longitudinal studies of biomarkers such as GL-3 require that the technique is reproducible. To ensure long-term reproducibility of the method, QC materials were used to evaluate the efficiencies of different operators and reagents and other key factors in the method. The reproducibility of the extraction and of the overall method demonstrates that the method is rugged for individual analysts and reagents, especially considering the overall diversity of GL-3 molecules, the complexity of a plasma matrix, and the number of steps involved in the analysis.

In summary, we have developed a rapid LC/MS/MS method to determine and monitor changes in the concentration of total GL-3 in human plasma. The method provides a sensitive, reliable, and reproducible technique to monitor GL-3 in patients with Fabry disease treated by various therapeutic modalities.

We acknowledge Carole Elbin and William Chung for their initial guidance in lipid extraction. In addition, Amy Cotsonas and Christian Braithwaite participated in the long-term reproducibility studies. We would also like to acknowledge Fei Wang, Jim MacDougal, and Biomedical Operations at Genzyme for contributions to the statistical analysis.

References


Previously published online at DOI: 10.1373/clinchem.2004.038323