regarded as very specific (1, 6). Because of this specificity, a proportion of patients who are investigated for vitamin B<sub>12</sub> deficiency will end up being tested for the presence of IFABs, regardless of the diagnostic cascade favored by the investigating physician(s) (1, 2). In referral laboratories, this may lead to substantial test volumes of labor-intensive manual RIAs, with all the associated problems. These range from an increased risk of sample mix-ups or analytical mishaps attributable to multiple manual sample-handling steps, to difficulties in maintaining consistent performance, to the need to handle and dispose of radioactive, and to lower analytical precision and increased turnaround time compared with automated assays. The new automated IFAB assay addresses all of these problems while maintaining comparable diagnostic accuracy. Moreover, both vitamin B<sub>12</sub> measurements and IFAB measurements can be performed on the same instrument, with the possibility to tag IFAB testing to individual vitamin B<sub>12</sub> samples, conditional on the measured cobalamin concentration, a further improvement in workflow. We therefore believe that the availability of this new IFAB assay provides an opportunity for increased and more expeditious testing and diagnosis of pernicious anemia.

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References


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Diagnosis of Methylmalonic Acidemia from Dried Blood Spots by HPLC and Intramolecular-Excimer Fluorescence Derivatization, Osama Y. Al-Dirbashi,1 Minnie Jacob,2 Zuhair Al-Hassanan, Fahad El-Badaoui,3 and Mohamed S. Rashd1,2 (Departments of 1Genetics and 2Medical Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; * address correspondence to this author at: Department of Genetics, King Faisal Specialist Hospital and Research Center, PO Box 3354, Riyadh 11211, Saudi Arabia; fax 966-1-442-4546, e-mail rashed@kfshrc.edu.sa)

Methylmalonic acidemias, a group of heterogeneous disorders, are characterized by accumulation of methylmalonic acid (MMA) and its byproducts in biological fluids (1, 2). Methylmalonic acidemia is now included in all tandem mass spectrometry (MS/MS)-based newborn screening programs (3–5). Detection is based on the finding of increased propionylcarnitine and/or increased propionylcarnitine-to-acetyl carnitine ratio in dried blood spots (DBS) by MS/MS. These markers, however, are not specific because they are increased in propionic acidemia and, possibly, in multiple carboxylase deficiency (3). In most programs, newborns or patients with initial positive results are recalled for a second blood spot, and a urine sample is collected for organic acid analysis to differentiate among the three disorders.

In the present study, we used the intramolecular-excimer fluorescence derivatization approach of Nohta and coworkers (6, 7) to form a fluorescent derivative of MMA. This would allow the detection of MMA in DBS samples from affected neonates, leading to a conclusive diagnosis with the remains of the DBS within a short time, often the same working day.

From a DBS, four 3.2-mm discs were punched and extracted into 250 μL of methanol containing 20 μmol/L malonic acid (MA) as internal calibrator by vortex-mixing for 30 s and standing at room temperature for 1 h. After evaporation and reconstitution of the residue in 50 μL of water, we successively added 25 μL of 0.5 mol/L 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) in water, 25 μL of 400 g/L pyridine in dimethylsulfoxide, and 50 μL of 15 mmol/L 1-pyrenebutyric hydrazide (Fluka Chemie) in dimethylsulfoxide, tightly capped and vortex-mixed the vials, and left them to stand at room temperature for 5 h. The injection volume was 5 μL, but injections up to 20 μL were possible without adverse effects on the resolution. Moreover, in case of a limited DBS sample, two punches can be used with the proportionate reduction in reagent volumes described above.

The labeling of MMA was confirmed by MS analysis (Quattro micro API; Micromass) with purified derivatives obtained by fractional collection. Two abundant ions at m/z 687 and m/z 709 appeared in the spectrum, which correspond to [M+H]<sup>+</sup> of pyrene-dilabeled MMA derivative and the [M+Na]<sup>+</sup> adduct, respectively.

Chromatography was performed on a Waters Breeze HPLC System, a model 2475 multi λ fluorescence detector (Waters) and a C8 Symmetry column [3.9 × 150 mm (i.d.),
5 μm bead size; Waters]. Mobile phases A and B were a mixture of acetonitrile–water (2:1 by volume) and acetonitrile, respectively, at a flow rate of 1 mL/min. The gradient was as follows: 0–5 min, 100% A; 5–8 min, 100% A to 10% A; 8–10 min, 10% A; 10–11 min, 10% A to 100% A; and 11–15 min, 100% A.

The maximum fluorescence values for the target analytes were obtained at an emission wavelength of 475 nm (excitation at 345 nm), with no interference observed from the monomeric fluorescing compounds, which emit at 385 nm. Fig. 1 shows three chromatograms obtained for a calibrator solution of MMA and internal calibrator and for extracts of DBS samples from known patients with methylmalonic acidemia. As shown in Fig. 1, a single, well-resolved peak was obtained for each of the pyrene derivatives of internal calibrator and MMA at 4.5 and 5.5 min, respectively. No interference was observed from other components coextracted from the DBS for more than 100 different DBS samples analyzed or in pooled blood samples from healthy adults. Furthermore, the dicarboxylic acid isomer succinic acid, a potentially interfering factor, eluted earlier in the chromatogram at 3.9 min, whereas the higher homolog ethylmalonic acid eluted at 6.1 min (data not shown).

The usefulness of the proposed method in giving a conclusive diagnosis for methylmalonic acidemia was assessed by a blinded retrospective study on DBS samples (n = 100) from known patients with methylmalonic acidemia (n = 33) or propionic acidemia (n = 33). The remaining DBS samples (n = 34) belonged to healthy infants. These samples were randomized, and the study was carried out with the analysts blinded to sample details. DBS samples prepared from MMA-supplemented blood served as calibrators for quantification. All samples from patients with methylmalonic acidemia gave a substantial peak corresponding to the pyrene derivative of MMA. On the other hand, no peak at the retention time of MMA was detectable in samples from healthy infants or from patients with propionic acidemia.

As shown in Table 1, three groups of specimens from confirmed methylmalonic acidemia cases were analyzed: asymptomatic newborn screening specimens; symptomatic neonate specimens submitted for metabolic screening; and specimens from known patients. All three groups showed substantially increased MMA regardless of the age of the sample.

In this report, we present the first clinical application of the intramolecular-excimer fluorescence derivatization approach for the determination of dicarboxylic acids in DBS specimens. Our main objective was to develop a second-tier test that would permit definitive diagnosis of methylmalonic acidemia from the same DBS sample received for MS/MS-based newborn screening. Thus, a newborn screening specimen flagged as abnormal because of high propionylcarnitine can be tested immediately for methylmalonic acidemia. The data presented for

![Fig. 1. Typical chromatograms with intramolecular-excimer fluorescence detection.](A), mixture of MMA and internal calibrator at 20 μmol/L each; (B), DBS sample from an infant (MMA concentration, 230.1 μmol/L); (C), DBS sample from a 7-year-old known case of methylmalonic acidemia with the lowest concentration of MMA obtained in this study (17.4 μmol/L). Peak 1, internal calibrator; peak 2, MMA.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>MMA concentration, μmol/L</th>
<th>Date of specimen, a day</th>
<th>Date of assay, b month</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn screening (n = 5)</td>
<td>232.0</td>
<td>1–5</td>
<td>3–104</td>
</tr>
<tr>
<td>Symptomatic neonates (n = 17)</td>
<td>133.4</td>
<td>2–8</td>
<td>3–106</td>
</tr>
<tr>
<td>Known case (n = 11)</td>
<td>268.7</td>
<td>5–2920</td>
<td>0–107</td>
</tr>
</tbody>
</table>

a Date of collection of specimen from neonate or patient.

b Age of sample when assayed by current method.

c All newborn screening specimens were from asymptomatic infants; one specimen was from a high-risk family.
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.

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References


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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,2 Crystal C.C. Sung,1† Shaparak Arogh,1 Dennis Wilkens,2 X. Kate Zhang,3 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 globotriaosylphosphoglycerine 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 Fabrazyme™ from the onset of the trial, whereas the other set received placebo for 5 months and was then given Fabrazyme. 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