Determination of Total Homocysteine, Methylmalonic Acid, and 2-Methylcitric Acid in Dried Blood Spots by Tandem Mass Spectrometry

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BACKGROUND: Newborn screening (NBS) for inborn errors of propionate, methionine, and cobalamin metabolism relies on finding abnormal concentrations of methionine and propionylcarnitine. These analytes are not specific for these conditions and lead to frequent false-positive results. More specific markers are total homocysteine (tHCY), methylmalonic acid (MMA), and methylcitric acid (MCA), but these markers are not detected by current NBS methods. To improve this situation, we developed a method for the detection of tHCY, MMA, and MCA in dried blood spots (DBSs) by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

METHODS: The analytes were extracted from a single 3/16" DBS punch with acetonitrile:water:formic acid (59:41:0.42) containing dithiothreitol and isotopically labeled standards (d₃-MMA, d₃-MCA, ²H₈-homocystine). The extract was dried and treated with 3 N HCl in n-butanol to form butylesters. After evaporation of the butanol, the residue was reconstituted and centrifuged and the supernatant was subjected to LC-MS/MS analysis. Algorithms were developed to apply this method as an efficient and effective second-tier assay on samples with abnormal results by primary screening.

RESULTS: The 99th percentiles determined from the analysis of 200 control DBSs for MMA, MCA, and HCY were 1.5, 0.5, and 9.8 μmol/L, respectively. Since 2005, prospective application of this second-tier analysis to 2.3% of all NBS samples led to the identification of 13 affected infants.

CONCLUSIONS: Application of this assay reduced the false-positive rate and improved the positive predictive value of NBS for conditions associated with abnormal propionylcarnitine and methionine concentrations.

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β-cystathionine synthase deficiency (Homocystinuria, OMIM +236200) has an incidence of 1 in 1800 live births, a separate measurement of tHcy by a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method can be affordable and even more effective than molecular genetic testing (7, 8). In less homogeneous and larger populations, this approach cannot be justified, which led several groups, including ours, to develop second-tier methods to improve NBS performance (9, 10). Here, we describe a method to simultaneously measure tHcy, MMA, and MCA in dried blood spots (DBSs) by LC-MS/MS and our experience with the prospective application of this method as a second-tier test after the detection of abnormal concentrations of C3-AC and/or Met.

### Materials and Methods

We purchased MMA and HCY from Sigma-Aldrich and obtained dithiothreitol from Amresco. We purchased MCA from CDN Isotopes. Isotopically labeled MMA and homocystine standards were purchased from Cambridge Isotope Laboratories and isotopically labeled MCA from CDN Isotopes. The 3 mol/L HCl in n-butanol was purchased from Regis Chemical. All other chemicals and solvents were of the highest purity available from commercial sources and were used without further purification.

#### PREPARATION OF CALIBRATORS AND CONTROLS

We prepared DBSs for calibration, recovery, stability, and imprecision studies as follows: aliquots of pooled whole blood were spiked with HCY, MMA, and MCA in dried blood spots (DBSs) by LC-MS/MS and our experience with the prospective application of this method as a second-tier test after the detection of abnormal concentrations of C3-AC and/or Met.

#### SAMPLES

With approval from the Mayo Clinic’s Institutional Review Board, for the validation of this method, we analyzed a total of 200 leftover NBS blood spots that were initially submitted to the Mayo Clinic’s supplemental NBS program. These blood spots did not suggest a screened condition, including inborn errors of propionic acid, Met, or Cbl metabolism, or suggest nutri-
tional vitamin B12 deficiency. We used these samples to determine reference ranges for tHCY, MMA, and MCA. Samples obtained from the original NBS specimen confirmed cases with β-cystathionine synthase deficiency (n = 4), propionyl-CoA carboxylase deficiency (n = 2), methylmalonyl-CoA mutase deficiency (n = 4), Cbl C deficiency (n = 7), various remethylation disorders (MTHFR, n = 3; Cbl G, n = 3; Cbl D variant 1, n = 1), and maternal vitamin B12 deficiency (n = 8) were identified prospectively during routine screening or made available by various screening laboratories with informed consent or submitted for routine second-tier testing. Data from the analysis of these samples as well as disease ranges obtained from the Region 4 Collaborative Project (www.region4genetics.org) were used to determine cutoffs for each analyte and analyte ratio.

METHODS
A 3/16" disc was punched from each control and sample DBS and transferred to a 12 × 55 mm glass culture tube. A 250-μL volume of a solution containing a mix of isotopically labeled standards (1 μmol/L 2H3-MMA, 1 μmol/L 2H2-MCA, 0.5 μmol/L 2H2-homocystine) and dithiothreitol (30 mmol/L) in acetonitrile:water:formic acid (59:41:0.42) was added to each tube. The tubes were capped, and the discs were eluted by mixing using an orbital rotator for 60 min at 120 rpm. The eluates were then transferred to 1-mL reaction vials and dried under a stream of nitrogen at 40 °C (approximately 15–20 min). A 100-μL volume of 3 mol/L HCl in n-butanol was added to the dried residues, which were then capped and incubated for 15 min at 65 °C. After incubation, excess reagent was evaporated to dryness (approximately 5–7 min) under heated nitrogen (40 °C), and the vials containing butylesters of MMA, MCA, and HCY were reconstituted into 100 μL mobile phase A (aqueous 0.1% formic acid) and mobile phase B (acetonitrile:water:formic acid, 80:20:0.1%) as follows: 0% B to 10% B in 8 min, 10% B to 60% B in 0.1 min, 60% B to 75% B in 4.9 min, 75% B to 100% B in 0.5 min plus an additional 1 min at 100% B, then back to 0% B in 0.1 min and re-equilibration for 1 min. We used a Varian Polaris C18-A 3 μm, 50 × 2.1 mm, heated at 40 °C, as the primary chromatographic column. Two other chromatographic columns were validated as suitable secondary columns: a Phenomenex Synergy 4 μm, 50 × 2.0 mm, and a Waters Atlantis T3, 3 μm, 50 × 2.1 mm, both used at ambient conditions. The analysis time per sample was 15.6 min.

RESULTS
LINEARITY AND IMPRECISION
Blood spot calibrators of MMA, MCA, and HCY at six different concentrations of spiked analyte (0, 5, 10, 50, 100, and 200 μmol/L) showed detectable and reproducible signals with a linear response (n = 5 for each analyte; MMA R² = 0.9994; MCA R² = 0.9995; HCY R² = 0.9972). Intraassay imprecision for MMA, MCA, and tHCY was determined at three concentrations (Table 2), and interassay imprecision was determined at two concentrations by the analysis of quality control samples that were analyzed by five different technologists over a period of 2 months.

STABILITY
The stability of extracted and prepared specimens was assessed by analysis of two controls enriched with MMA, MCA, and HCY at 5.5, 9.5, and 23.2 μmol/L (n = 3) and 25.1, 29.9, and 45.7 μmol/L (n = 3), respectively, before and after 24 h under ambient conditions. Prepared specimens yielded their expected concentrations within 0.8% for MMA, 1.3% for MCA, and 5.5% for tHCY. Blood spot stability at ambient conditions was assessed by the analysis of a control enriched with MMA, MCA, and HCY at 56.3, 61.0, and 78.5 μmol/L. The percent change in concentration for MMA, MCA, and HCY was −2.3%, −9.8%, and
−19.0%, respectively, after 7 days of ambient (22 °C) storage. Frozen blood spot stability was assessed by the analysis of two controls enriched with MMA, MCA, and HCY at 7.8, 9.3, and 16.4 μmol/L and 26.4, 29.4, and 37.6 μmol/L, respectively, before and after 2 years of frozen (−20 °C) storage. The percent change for MMA, MCA, and HCY was 33.2%, 19.4%, and 48.8% in the first control and 17.0%, 7.5%, and 6.4% in the second control, respectively.

**METHOD COMPARISON**

Using plasma spotted on filter paper, the method was compared to other LC-MS/MS assays for plasma MMA and tHCY analysis (11, 12). An X-Y plot demonstrated excellent concordance between these methods for MMA (n = 14 patients; range 1.6–24.0 μmol/L; $R^2 = 0.9868; m = 1.0579; b = -0.1979$) and tHCY (n = 20 patients; range 6–32 μmol/L; $R^2 = 0.9151; m = 1.1179; b = -2.0294$).

**RECOVERY**

Recovery was evaluated by the analysis of DBSs spiked with MMA, MCA, and tHCY at two concentrations (5 and 50 μmol/L). Recovery was defined as (final concentration-endogenous concentration)/added concentration. The recoveries, which ranged from 64.7% to 111.0%, are shown in Table 3.

**LIMIT OF DETECTION**

The limits of detection for MMA, MCA, and tHCY were determined to be 0.13, 0.02, and 0.25 μmol/L, respectively, as defined by the mean + 3 standard deviations of the concentration measured in a blank filter paper punch containing internal standard but no blood.

**MATRIX EFFECTS**

MS signal suppression and/or enhancement was evaluated by the post-column infusion of MMA, MCA, and HCY (13). Signal suppression was observed between 0.2 and 0.3 min and 8.4 and 8.7 min with no signal enhancement present. HCY, MMA, and MCA did not elute during either region of signal suppression (Fig. 1).

**REFERENCE RANGES**

Reference ranges for Met, C3-AC, and relevant analyte ratios were based on 469 279 newborn screening samples analyzed in our laboratory between June 2004 and December 2009 (Table 4). The concentration range (mean) of tHCY, MMA, and MCA in 200 random NBS

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**Table 2. Imprecision for MMA, MCA, and tHCY.**

<table>
<thead>
<tr>
<th></th>
<th>Intraassay imprecision (n = 10)</th>
<th>Interassay imprecision (n = 20)</th>
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<tbody>
<tr>
<td>MMA</td>
<td>6.6% (6.1)</td>
<td>6.9% (100.0)</td>
</tr>
<tr>
<td>MCA</td>
<td>3.9% (9.3)</td>
<td>6.7% (101.2)</td>
</tr>
<tr>
<td>tHCY</td>
<td>6.6% (17.9)</td>
<td>7.5% (112.9)</td>
</tr>
</tbody>
</table>

* Data are % CV (concentration), and all concentrations are given as μmol/L.

**Table 3. Recovery of MMA, MCA, and tHCY from spiked DBS specimens.**

<table>
<thead>
<tr>
<th></th>
<th>Enrichment (μmol/L)</th>
<th>Endogenous concentration (μmol/L)</th>
<th>Final concentration (μmol/L)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1</td>
<td>5</td>
<td>2.5</td>
<td>5.7</td>
<td>64.7</td>
</tr>
<tr>
<td>Level 2</td>
<td>50</td>
<td>2.5</td>
<td>55.0</td>
<td>105.0</td>
</tr>
<tr>
<td>MCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1</td>
<td>5</td>
<td>6.7</td>
<td>10.2</td>
<td>70.8</td>
</tr>
<tr>
<td>Level 2</td>
<td>50</td>
<td>6.7</td>
<td>58.6</td>
<td>103.8</td>
</tr>
<tr>
<td>tHCY</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1</td>
<td>5</td>
<td>15.6</td>
<td>20.1</td>
<td>89.2</td>
</tr>
<tr>
<td>Level 2</td>
<td>50</td>
<td>15.6</td>
<td>71.1</td>
<td>111.0</td>
</tr>
</tbody>
</table>

* n = 5 interassay analyses at each concentration.
samples was 0.8–10.9 μmol/L (5.3 μmol/L), 0.2–2.0 μmol/L (0.6 μmol/L), and undetectable to 0.7 μmol/L (0.2 μmol/L), respectively. Comparison of control and disease ranges for relevant analytes and analyte ratios as determined by primary newborn screening and second-tier testing provided the basis for the development of algorithms for the decision of which samples need to be submitted for second-tier testing (Fig. 2). Further comparison of the percentile range in negative controls and observations in true-positive samples was used to select the cutoff concentrations for tHCY, MMA, and MCA at 15.0, 5.0, and 1.0 μmol/L, respectively, which allowed clear discrimination of the control population from affected patients (Table 4).

Fig. 1. Multiple reaction monitoring chromatograms of eluents from DBS samples of a newborn with propionic acidemia (A), Cbl C deficiency (B), homocystinuria (C), and a healthy control (D).

1, d4-homocystine; 2, tHCY; 3, succinic acid; 4, d3-MMA (internal standard); 5, MMA; 6, DL-2-methyl-d3-citric acid (internal standard); 7, MCA.
Table 4. Medians and ranges of analyte concentrations and ratios in primary and second-tier NBS tests as observed in the control populations and patients with various inborn errors of propionic acid, Cbl, and Met metabolism, as well as newborns of mothers with vitamin B12 deficiency.

<table>
<thead>
<tr>
<th></th>
<th>Primary screening result</th>
<th>Second-tier test results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C3* (μmol/L)</td>
<td>C3/C2</td>
</tr>
<tr>
<td>Controls</td>
<td>1.9 (0.6–5.1)b</td>
<td>0.08 (0.04–0.19)b</td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase deficiency (n = 2)</td>
<td>25.8 (17.3–34.4)</td>
<td>0.96 (0.81–1.10)</td>
</tr>
<tr>
<td>Methylmalonyl-CoA mutase deficiency (n = 4)</td>
<td>8.9 (6.0–119.0)</td>
<td>0.43 (0.29–5.40)</td>
</tr>
<tr>
<td>Cystathionine β-synthase deficiency (n = 4)</td>
<td>0.9 (0.5–2.1)</td>
<td>0.10 (0.06–0.12)</td>
</tr>
<tr>
<td>Cbl C deficiency (n = 7)</td>
<td>5.5 (0.3–7.7)</td>
<td>0.22 (0.17–0.52)</td>
</tr>
<tr>
<td>Cbl D deficiency (n = 3)</td>
<td>1.6 (1.4–1.9)</td>
<td>0.08 (0.05–0.09)</td>
</tr>
<tr>
<td>Cbl D variant 1 deficiency (n = 1)</td>
<td>1.7</td>
<td>0.05</td>
</tr>
<tr>
<td>MTHFR deficiency (n = 3)</td>
<td>1.3 (1.1–2.5)</td>
<td>0.11 (0.05–0.13)</td>
</tr>
<tr>
<td>Maternal vitamin B12 deficiency (n = 8)</td>
<td>5.9 (2.6–8.1)</td>
<td>0.23 (0.20–0.29)</td>
</tr>
</tbody>
</table>

* C3, propionylcarnitine; C2, acetylcarnitine; C16, palmitoylcarnitine; ND, not detected.

b Medians and percents (1st to 99th) based on 469,279 newborn screening samples analyzed in our laboratory between June 2004 and December 2009.

c Medians and ranges based on 200 leftover NBS blood spots submitted to our laboratory for routine newborn screening and yielding normal results.
Fig. 2. Algorithms for newborn screening for inborn errors of propionic acid, Met, and Cbl metabolism using simultaneous determination of MMA, MCA, and tHCY in DBSs as a second-tier assay.

The cutoffs for C3-AC (A), high Met (B), low Met (C), and noted analyte ratios were established based on 469,279 newborn screening samples analyzed in our laboratory between June 2004 and December 2009 as well as disease ranges obtained from the Region 4 Collaborative Project (www.region4genetics.org).
Discussion

Newborn screening programs aim to identify patients with conditions that are clinically relevant and treatable. The analytical methods to achieve this goal for many conditions, however, do not allow for high diagnostic specificity without negatively affecting diagnostic sensitivity because they rely on biomarkers that are not intrinsically diagnostic. This has been true for phenylketonuria due to phenylalanine hydroxylase deficiency, the condition for which NBS was first implemented in the 1960s (14). The disease marker phenylalanine was deemed an excellent marker for phenylketonuria until it became apparent that it could also be transiently increased in healthy neonates and moderately increased in milder variants of phenylalanine hydroxylase deficiency as well as in cases affected with debilitating and poorly treatable defects in tetrahydrobipterin synthesis and recycling (15, 16). When amino acid and acylcarnitine profiling was introduced into NBS programs, the problem of differential diagnosis and the possibility of detecting conditions that may be deemed inappropriate for screening due to uncertain clinical relevance or absence of effective treatment options grew. In addition, the false-positive rate, particularly for relatively nonspecific markers, appeared to increase (17, 18). Overall, this led to a slow and unequal expansion of screening programs until the American College of Medical Genetics published the Uniform Panel of 29 conditions for which every baby should be screened and an additional 25 conditions that are mostly identified as part of the differential diagnosis in screening for primary conditions (1). Four of the primary conditions and two of the secondary targets are associated with abnormally increased C3-AC or Met concentrations. In addition, increases of C3-AC and abnormally low concentrations of Met are also observed in newborns with vitamin B12 deficiency (typically maternal in origin (19–21) and Cbl F deficiency [Table 1]). However, patients not affected by these genetic or nutritional conditions may also reveal C3-AC or Met concentrations beyond cutoffs that are typically determined based on mean values and standard deviations or percentile rankings of analytes observed in a population cohort. The overlap of concentrations for these analytes in affected and unaffected newborns may be caused by administration of parental nutrition to the newborn before NBS sample collection, other unrelated conditions causing liver disease and jaundice (personal observation), or unknown factors. Determination of analyte ratios, such as the C3/C2 ratio, and consideration of these ratios for the interpretation of abnormal C3-AC concentrations can help to reduce the number of false-positive results (6). However, only the measurement of pathognomonic analytes can markedly improve the balance between the diagnostic sensitivity and specificity of the test for nonspecific biomarkers (10, 22–24). Sometimes disease-specific markers such as succinylacetone can be included in routine screening (25, 26) or a condition is sufficiently prevalent in a defined population, justifying specific testing of every newborn (8). The assay we developed to overcome the problem of NBS for inborn errors of propionate, Met, and Cbl metabolism simultaneously quantifies MMA, MCA, and tHcy. The analytical time of the LC-MS/MS approach is 15.6 min between injections, which is one reason why this method cannot be applied as a primary screen of every sample without having to invest in additional equipment. The relatively long chromatographic separation, however, is essential for accurate identification and quantification of tHcy, MCA, and particularly of the structural isomers MMA and succinic acid (Fig. 1).

In our laboratory, this assay is applied to any NBS sample with abnormal C3-AC and/or Met concentrations in the primary NBS assay that measures amino acids and acylcarnitines (Fig. 2) (25). This approach has markedly improved screening performance. The false-positive rate for conditions associated with either increased C3-AC or Met was reduced fivefold from 0.049% to 0.009% when comparing the year before (2004) and after (2005) implementation of the second-tier assay. At the same time, the positive predictive value increased from 11% to 36%. With implementation of the second-tier assay, it was also possible to refine the cutoffs for increased C3-AC and Met, but also to introduce a new cutoff for low concentrations of Met (Fig. 2C). The latter enables identification of patients with remethylation defects, including Cbl D-Var1, Cbl E, Cbl G, and MTHFR deficiency, all of which are characterized by low concentrations of Met and increased tHcy (Table 1) (27, 28). Although treatable, these conditions were not included as either primary or secondary targets in the American College of Medical Genetics panel because low cutoffs are traditionally not applied to amino acids measured in NBS (1). Changing cutoffs to increase the diagnostic sensitivity occurs at the expense of diagnostic specificity and therefore is associated with an increase in samples that require follow-up. Our current cutoffs and decision algorithms (Fig. 2) are therefore only applicable because of the availability of the second-tier test, which is required for 2.3% of all NBS samples submitted. In 2009, with this approach in place, and based on the outcome of clinical and laboratory follow-up studies, the false-positive rate for increased C3-AC and either increased or low Met was only 0.008%, the positive predictive value was 60%, and the positive detection rate was 1:8141 newborns. Since 2005, we have prospectively identified two infants with β-cystathionine synthase
deficiency, one with MTHFR deficiency, one with Cbl G deficiency, two with methylmalonyl-CoA mutase deficiency, and seven with Cbl C deficiency, as well as eight mothers with vitamin B_{12} deficiency. The overall false-positive rate and positive predictive value from 2005 through 2009 was 0.009% and 44%, respectively. This indicates a substantial benefit to the affected and unaffected population screened. To optimize the utilization of resources, the assay is performed in batches, twice per week, because tHCY, MMA, and MCA are sufficiently stable for at least 7 days after sample collection, even when stored at room temperature. Only a markedly increased concentration of C3-AC triggers immediate second-tier testing to clarify the possibility of propionic acidemia or severe MMA (Fig. 2A). The other targeted conditions typically do not present acutely in the first week of life. Further cost containment could be achieved if screening programs shared the burden by regionalization of this and other second-tier tests, which has been shown to be effective and efficient for NBS for congenital adrenal hyperplasia (29). Through the MS/MS laboratory quality improvement project of the Region 4 Genetics Collaborative (www.region4genetics.org), our laboratory is already providing this service to several NBS programs in the US and elsewhere and is supporting other programs in the implementation of second-tier assays in their own laboratories. Introduction and performance of second-tier tests in NBS programs is also supported by the Centers for Disease Control and Prevention’s Newborn Screening Quality Assurance program, which is likely to provide quality-control DBS materials for tHCY, MMA, and MCA in the future, as it already does for second-tier testing for congenital adrenal hyperplasia and maple syrup disease (30). With these analytical improvements available, addition of remethylation defects to the NBS core conditions should be strongly considered to prevent serious complications, including death, in patients affected with these treatable conditions.

**References**


14. Guthrie R, Susi A. A simple phenylalanineline method for detecting phenylketonuria in large popula-