Clinical Chemistry 56:11
1686–1695 (2010)

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

Coleman T. Turgeon,[1] Mark J. Magera,[1] Carla D. Cuthbert,[2] Perry R. Loken,[1] Dimitar K. Gavrilov,[1] Silvia Tortorelli,[1] Kimiyo M. Raymond,[1] Devin Oglesbee,[1] Piero Rinaldo,[1] and Dietrich Matern[1*]

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 4.8-mm DBS punch with acetonitrile:water:formic acid (59:41:0.42) containing dithiothreitol and isotopically labeled standards (d_3-MMA, d_3-MCA, d_8-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.

Several inborn errors of propionic acid, methionine (Met)[3], and cobalamin (Cbl) metabolism are included in the panel of conditions for which every newborn should be screened, as recommended by the US Health and Services Administration and the American College of Medical Genetics, and now adopted by all newborn screening (NBS) programs in the US (Table 1; http://genes-r-us.uthscsa.edu/nbsdisorders.htm) *(1)*. These conditions are indicated by abnormal concentrations of either propionylcarnitine (C3-AC) and/or Met. Unfortunately, these markers lack disease specificity and have poor diagnostic sensitivity due to the overlap of their concentrations in affected and unaffected newborns *(2, 3)*. Adjustment of cutoffs to increase the diagnostic sensitivity will therefore lead to a higher number of false-positive results, which causes unnecessary anxiety and follow-up costs. Reverse adjustment of cutoffs to increase specificity will decrease the false-positive rate, but at the expense of diagnostic sensitivity. The determination of analyte ratios, which became possible with the introduction of acylcarnitine and amino acid profiling by tandem mass spectrometry (MS/MS), has been shown to improve but not eliminate this predicament *(4–6)*. Measurement of more specific disease markers such as total homocysteine (tHCY), methylmalonic acid (MMA), 3-hydroxy propionic acid, and 2-methylcitric acid (MCA) as part of the primary NBS test would be desirable but not possible without a major investment in additional equipment and effort. However, as was recently shown for NBS in Qatar (ca. 15000 births per year), where

[1] Biochemical Genetics Laboratory, Mayo Clinic College of Medicine, Rochester, MN; the [2] Division of Laboratory Sciences, NCEH, Centers for Disease Control and Prevention, Atlanta, GA.
* Address correspondence to this author at: Biochemical Genetics Laboratory, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905. Fax 507-266-2888; e-mail matern@mayo.edu.
Received April 19, 2010; accepted July 15, 2010.
Previously published online at DOI: 10.1373/clinchem.2010.148957

[3] Nonstandard abbreviations: Met, methionine; Cbl, cobalamin; NBS, newborn screening; C3-AC, propionylcarnitine; MS/MS, tandem mass spectrometry; tHCY, total homocysteine; MMA, methylmalonic acid; MCA, methylcitric acid; LC-MS/MS, liquid chromatography–tandem mass spectrometry; DBS, dried blood spot; MTHFR, methylenetetrahydrofolate reductase.

Table 1. Expected metabolite abnormalities in disorders of propionic acid, Met, and Cbl metabolism as determined by routine NBS and second-tier test results for MMA, MCA, and tHCY.

	Initial NBS result		Second-tier test result		
	C_3-AC	Met	MMA	MCA	tHCY
Propionyl-CoA carboxylase deficiency (propionic acidemia)[a]	↑ ↑	N	N	↑ ↑	N
Methylmalonyl-CoA mutase deficiency (Mut^0 or Mut^-)[a]	↑ to ↑ ↑	N	↑ ↑	↑	N
Cystathionine β-synthase deficiency (homocystinuria)[a]	N	↑ to ↑ ↑	N	N	↑ ↑
Cbl metabolism					
Adenosyl-Cbl–specific deficiencies					
Cbl A,[a] Cbl B,[a] and Cbl D-Var2[b] deficiencies	↑	N	↑	N	N
Combined methyl-Cbl and adenosyl-Cbl deficiencies					
Cbl C,[c] Cbl D,[c] and Cbl F[b] deficiencies	↑	↓	↑	N	↑
Methyl-Cbl–specific deficiencies					
Cbl D-Var1, Cbl E, and Cbl G deficiencies[b]	N	↓	N	N	↑
Met adenosyltransferase I/III deficiency[c]	N	↑	N	N	N (to ↑)
Adenosylhomocysteine hydrolase deficiency[c]	N	↑	N	N	N (to ↑)
Glycine N-methyltransferase deficiency[c]	N	↑	N	N	N
Methylene tetrahydrofolate reductase deficiency[b]	N	N to ↓	N	N	↑
Nutritional Cbl deficiency in mother or other Cbl metabolism defect[b] (e.g., transcobalamin II deficiency)	N to ↑	↓ to N	N to ↑	N	N to ↑
Vitamin B_6 deficiency[b]	N	N to ↑	N	N	N to ↑

[a] Primary target conditions.
[b] Conditions that have not been considered for inclusion in the universal NBS panel *(1)*.
[c] Secondary target conditions.

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 homogenous 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 to achieve final concentrations of 0, 5, 10, 50, 100, and 200 μmol/L and were then spotted on filter paper (Whatman ProteinSaver 903) and dried overnight at room temperature. The spotted cards were then transferred to a sealed bag with desiccator and stored at −20 °C.

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 were negative for all screened conditions, including inborn errors of propionic acid, Met, or Cbl metabolism, and not sugges-

tive of nutritional vitamin B_{12} deficiency. We used these samples to determine reference ranges for tHCY, MMA, and MCA. Samples obtained from the original NBS specimens of 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 [methylenetetrahydrofolate reductase (MTHFR), n = 3; Cbl G, n = 3; Cbl D variant 1, n = 1], and maternal vitamin B_{12} 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 4.8-mm 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 d_3-MMA, 1 μmol/L d_3-MCA, 0.5 μmol/L d_8-homocystine) and dithiotreitol (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 laboratory grade water. The vials were centrifuged at 1570*g* for 3 min, and the supernatants were transferred to LC autosampler vials and capped for LC-MS/MS analysis.

MS/MS PROCEDURE

We used a triple-quadrupole MS/MS system (Applied Biosystems/MDS Sciex API 5000) operated in positive ion mode (source voltage, 5500 V). Mass calibration and resolution of both resolving quadrupoles were automatically optimized with a poly(propylene)glycol solution introduced by an infusion pump. Method optimization for the detection of MMA, MCA, and tHCY by selected reaction monitoring was performed by infusing a 0.5 μmol/L solution of MMA, MCA, HCY, and their corresponding isotopically labeled standards as butylesters at 0.6 mL/h. The instrument was optimized automatically by an internal algorithm to monitor the transitions *m/z* 231.1 to *m/z* 119.0 and *m/z* 234.1 to *m/z* 122.0 for unlabeled and labeled MMA, *m/z* 375.2 to *m/z* 199.1 and *m/z* 378.2 to *m/z* 202.1 for unlabeled and labeled MCA, and *m/z* 192.1 to *m/z* 90.1 and *m/z* 196.1 to *m/z* 94.1 for unlabeled and labeled HCY (see Supplemental Figure in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue11). These selected reaction monitoring experiments (300-ms dwell for each experiment) were then added to the MS/MS method. Sample introduction into the atmospheric pressure ionization source was achieved by a Perkin-Elmer 200 Series Autosampler and Perkin-Elmer 200 Series LC pumps. Autosampler injections of 10 μL per sample were made into the LC mobile phase flow of 0.95 mL/min. Gradient elution of the analytes was achieved using a program with 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 T_3 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 6 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 $R^2 = 0.9994$; MCA $R^2 = 0.9995$; HCY $R^2 = 0.9972$). Intraassay imprecision for MMA, MCA, and tHCY was determined at 3 concentrations (Table 2), and interassay imprecision was determined at 2 concentrations by the analysis of quality control samples that were analyzed by 5 different technologists over a period of 2 months.

STABILITY

The stability of extracted and prepared specimens was assessed by analysis of 2 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.

Table 2. Imprecision for MMA, MCA, and tHCY.[a]

	Intraassay imprecision (n = 10)			Interassay imprecision (n = 20)	
MMA	6.6% (6.1)	5.7% (10.8)	6.9% (100.0)	7.8% (6.6)	9.6% (27.5)
MCA	3.9% (9.3)	4.8% (13.6)	6.7% (101.2)	14.3% (9.6)	11.4% (29.9)
tHCY	6.6% (17.9)	6.0% (22.3)	7.5% (112.9)	14.8% (18.4)	14.1% (44.4)

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

The percent change in concentration for MMA, MCA, and tHCY 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 2 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 tHCY 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 HCY at 2 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

Table 3. Recovery of MMA, MCA, and tHCY from spiked DBS specimens.[a]

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

[a] n = 5 interassay analyses at each concentration.

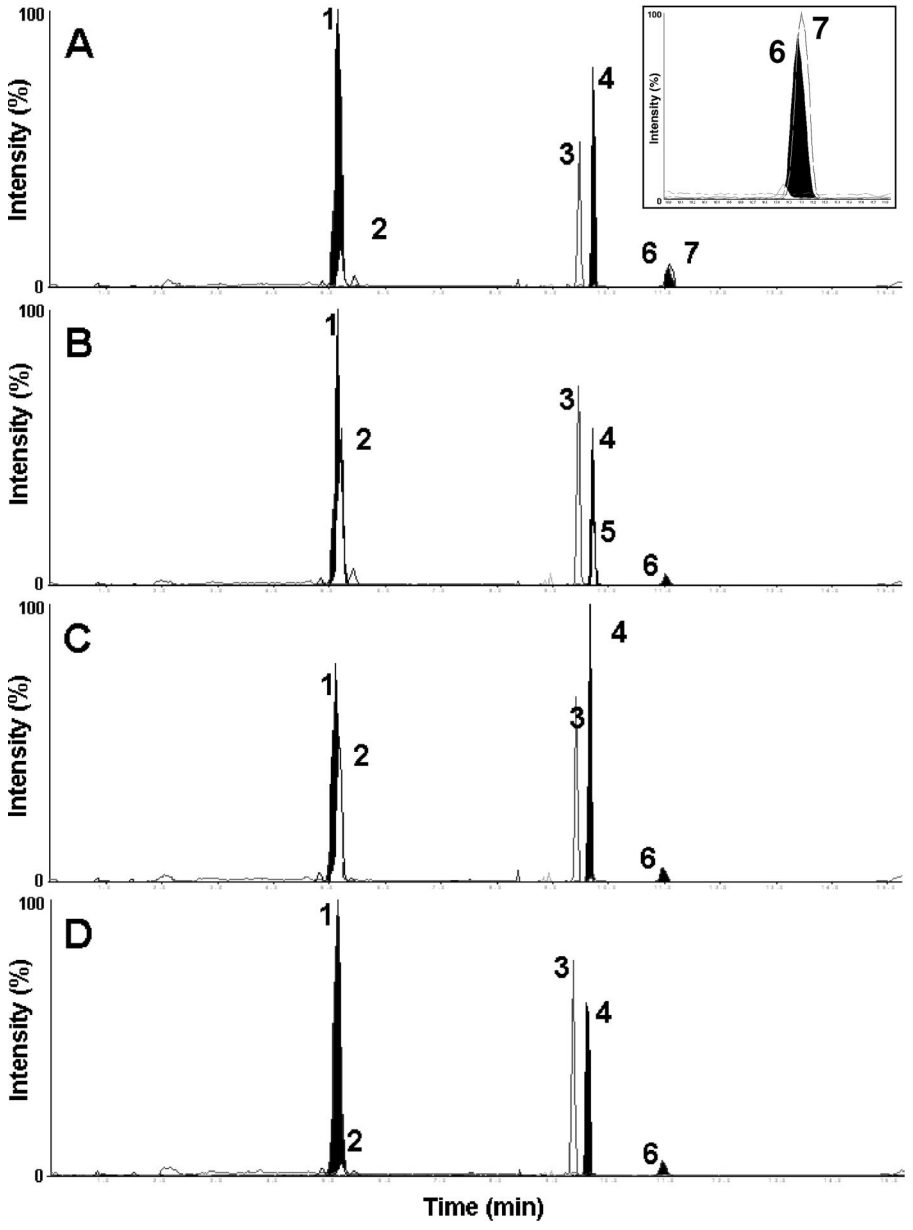

Fig. 1. Multiple reaction monitoring chromatograms of eluents from DBS samples of a newborn with propionic acidemia (A), Cbl C deficiency (B), homocystinuria (C), and a healthy control (D).

1, d_4-Homocysteine (internal standard); 2, tHCY; 3, succinic acid; 4, d_3-MMA (internal standard); 5, MMA; 6, DL-2-methyl-d_3-citric acid (internal standard); 7, MCA.

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 and online Supplemental Table).

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.

	Primary screening result					Second-tier test results		
	C3[a] (μmol/L)	C3/C2	C3/C16	Met (μmol/L)	Met/Phe	MMA (μmol/L)	MCA (μmol/L)	tHCY (μmol/L)
Controls	1.9 (0.6–5.1)[b]	0.08 (0.04–0.19)[b]	0.8 (0.3–2.7)[b]	21.5 (11.3–55.5)[b]	0.41 (0.23–0.80)[b]	0.6 (0.2–2.0)[c]	0.2 (ND to 0.7)[c]	5.2 (0.8–10.9)[c]
Propionyl-CoA carboxylase deficiency (n = 2)	25.8 (17.3–34.4)	0.96 (0.81–1.10)	8.3 (6.1–10.7)	18.1 (15.8–20.4)	0.37 (0.37–0.37)	0.3 (0.3–0.3)	12.30 (12.0–12.6)	3.8 (3.4–4.3)
Methylmalonyl-CoA mutase deficiency (n = 4)	8.9 (6.0–119.0)	0.43 (0.29–5.40)	7.4 (2.0–165.5)	22.3 (16.2–23.0)	0.40 (0.31–0.42)	144.0 (34.9–358)	4.8 (0.7–9.8)	1.68 (1.3–3.7)
Cystathionine β-synthase deficiency (n = 4)	0.9 (0.5–2.1)	0.10 (0.06–0.12)	1.4 (0.7–2.4)	308.4 (47.8–683)	5.95 (0.95–24.7)	0.8 (0.6–1.7)	0.05 (ND to 0.6)	49.5 (34.1–189)
Cbl C deficiency (n = 7)	5.5 (0.3–7.7)	0.22 (0.17–0.52)	2.1 (1.1–3.7)	19.0 (14.5–42.3)	0.41 (0.21–0.61)	29.2 (12.3–108)	0.9 (0.4–5.9)	51.8 (12.6–121)
Cbl G deficiency (n = 3)	1.6 (1.4–1.9)	0.08 (0.05–0.09)	0.8 (0.5–0.9)	8.1 (4.7–9.7)	0.14 (0.10–0.16)	1.0 (0.4–2.4)	0.2 (0.1–0.2)	62.6 (45.0–74.9)
Cbl D variant 1 deficiency (n = 1)	1.7	0.05	0.7	5.5	0.09	1.0	ND	64.4
MTHFR deficiency (n = 3)	1.3 (1.1–2.5)	0.11 (0.05–0.13)	0.7 (0.4–0.7)	5.9 (4.8–6.4)	0.10 (0.10–0.12)	1.0 (0.8–1.1)	0.1 (0.1–0.2)	97.8 (73.7–157)
Maternal vitamin B12 deficiency (n = 8)	5.9 (2.6–8.1)	0.23 (0.20–0.29)	2.2 (1.5–3.4)	20.9 (15.1–27.6)	0.48 (0.31–0.52)	12.3 (5.1–31.9)	0.6 (0.5–0.8)	24.9 (5.7–52.7)

[a] C3, propionylcarnitine; C2, acetylcarnitine; C16, palmitoylcarnitine; Phe, phenylalanine; ND, not detected.
[b] Medians and percentiles (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.

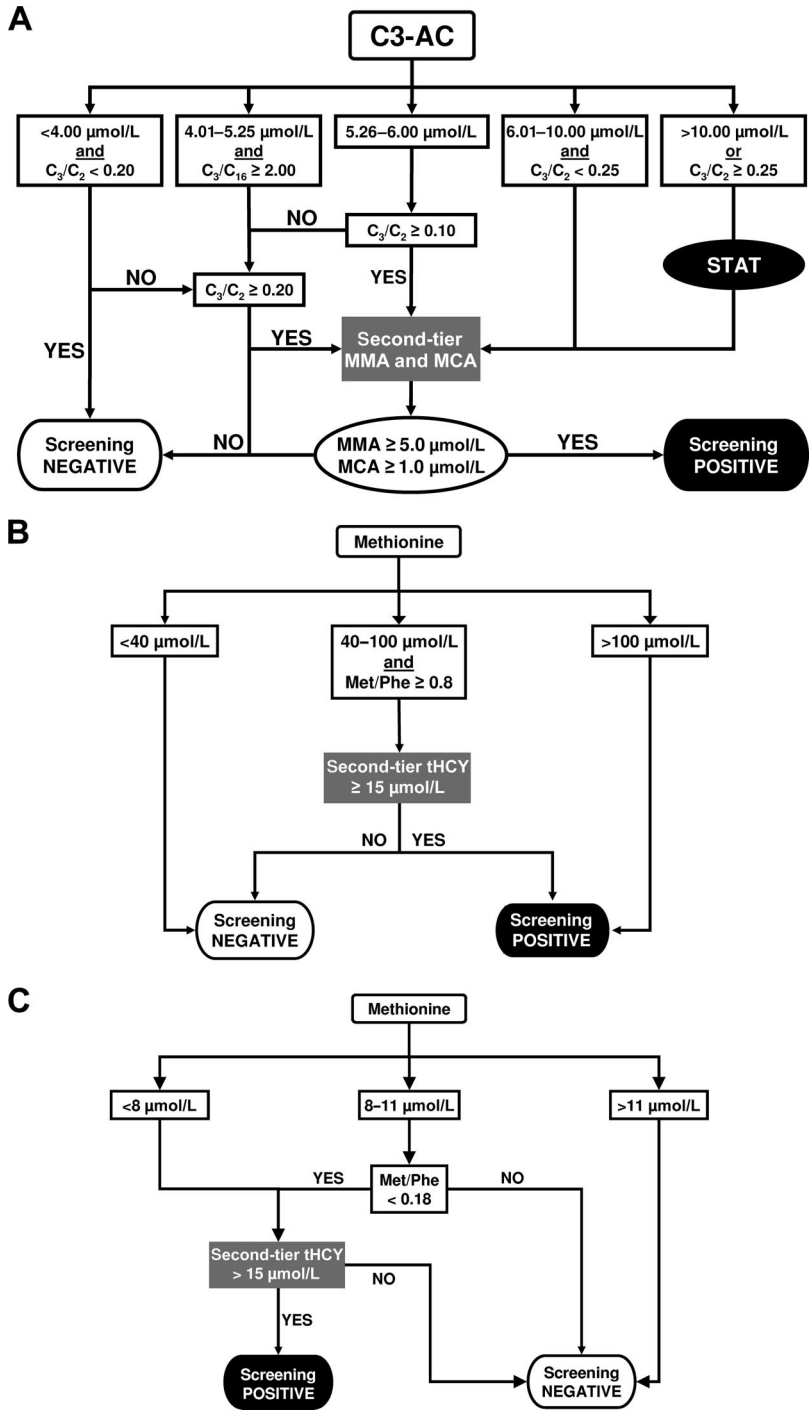

Fig. 2. Algorithms for newborn screening for inborn errors of propionic acid, Met, and Cbl metabolism using simultaneous determination of MMA, MCA, and tHCY in DBSs as a second-tier assay.

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). Phe, phenylalanine.

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 tetrahydrobiopterin 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 2 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 B_{12} 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 parenteral 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 5-fold 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 2 infants with β-cystathionine synthase

deficiency, 1 with MTHFR deficiency, 1 with Cbl G deficiency, 2 with methylmalonyl-CoA mutase deficiency, and 7 with Cbl C deficiency, as well as 8 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 urine 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.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures of Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: The authors would like to thank Mark McCann (Minnesota Department of Health, St. Paul, MN), Kyriakie Sarafoglou (Amplatz Children's Hospital, University of Minnesota, Minneapolis, MN), James S. Lim (Kentucky Newborn Screening Program, Frankfort, KY), Steve Baumgart and Uta Lichter-Konecki (Children's National Medical Center, Washington, DC), Allen Bergum (New Jersey Department of Health, Trenton, NJ), Debra-Lynn Day-Salvatore (St. Peter's University Hospital, NJ), Jose Abdenur (Children's Hospital of Orange County, Orange, CA), Jonathan A. Bernstein (Stanford University School of Medicine, Palo Alto, CA), Fred Lorey (California Department of Public Health, Richmond, CA), Klaas Wierenga (The University of Oklahoma Health Sciences Center, Oklahoma City, OK), and Sharon Vaz (Oklahoma State Department of Health, Oklahoma City, OK) for assisting in the retrieval of original newborn screening samples of patients with confirmed diagnoses.

References

1. Watson MS, Lloyd-Puryear MA, Mann MY, Rinaldo P, Howell RR. Newborn screening: toward a uniform screening panel and system [Executive Summary]. Genet Med 2006;8:1S–12S.
2. Peterschmitt MJ, Simmons JR, Levy HL. Reduction of false negative results in screening of newborns for homocystinuria. N Engl J Med 1999;341:1572–6.
3. ten Hoedt AE, van Kempen AA, Boelen A, Duran M, Kemper-Proper EA, Oey-Spauwen MJ, et al. High incidence of hypermethioninaemia in a single neonatal intensive care unit detected by a newly introduced neonatal screening programme. J Inherit Metab Dis 2007;30:978.
4. Chace DH, Hillman SL, Millington DS, Kahler SG, Adam BW, Levy HL. Rapid diagnosis of homocystinuria and other hypermethioninemias from newborns' blood spots by tandem mass spectrometry. Clin Chem 1996;42:349–55.
5. Chace DH, DiPerna JC, Kalas TA, Johnson RW, Naylor EW. Rapid diagnosis of methylmalonic and propionic acidemias: quantitative tandem mass spectrometric analysis of propionylcarnitine in filter-paper blood specimens obtained from newborns. Clin Chem 2001;47:2040–4.
6. Weisfeld-Adams JD, Morrissey MA, Kirmse BM, Salveson BR, Wasserstein MP, McGuire PJ, et al. Newborn screening and early biochemical follow-up in combined methylmalonic aciduria and homocystinuria, cblC type, and utility of methionine as a secondary screening analyte. Mol Genet Metab 2010;99:116–23.
7. Zschocke J, Kebbewar M, Gan-Schreier H, Fischer C, Fang-Hoffmann J, Wilrich J, et al. Molecular neonatal screening for homocystinuria in the Qatari population. Hum Mutat 2009;30:1021–2.
8. Gan-Schreier H, Kebbewar M, Fang-Hoffmann J, Wilrich J, Abdoh G, Ben-Omran T, et al. Newborn population screening for classic homocystinuria by determination of total homocysteine from Guthrie cards. J Pediatr 2010;156:427–32.
9. la Marca G, Malvagia S, Pasquini E, Innocenti M, Donati MA, Zammarchi E. Rapid 2nd-tier test for measurement of 3-OH-propionic and methylmalonic acids on dried blood spots: reducing the false-positive rate for propionylcarnitine during expanded newborn screening by liquid chromatography-tandem mass spectrometry. Clin Chem 2007;53:1364–9.
10. Matern D, Tortorelli S, Oglesbee D, Gavrilov D, Rinaldo P. Reduction of the false-positive rate in newborn screening by implementation of MS/MS-based second-tier tests: the Mayo Clinic experience (2004–2007). J Inherit Metab Dis 2007;30:585–92.
11. Magera MJ, Helgeson JK, Matern D, Rinaldo P. Methylmalonic acid measured in plasma and urine by stable-isotope dilution and electrospray tandem mass spectrometry. Clin Chem 2000;46:1804–10.
12. Magera MJ, Lacey JM, Casetta B, Rinaldo P. Method for the determination of total homocysteine in plasma and urine by stable isotope dilution and electrospray tandem mass spectrometry. Clin Chem 1999;45:1517–22.
13. Annesley TM. Ion suppression in mass spectrometry. Clin Chem 2003;49:1041–4.
14. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large popula-

tions of newborn infants. Pediatrics 1963;32: 338–43.
15. Kaufman S, Holtzman NA, Milstien S, Butler LJ, Krumholz A. Phenylketonuria due to a deficiency of dihydropteridine reductase. N Engl J Med 1975;293:785–90.
16. Danks DM, Cotton RG, Schlesinger P. Variant forms of phenylketonuria [Letter]. Lancet 1976;1: 1236–7.
17. Tarini BA, Christakis DA, Welch HG. State newborn screening in the tandem mass spectrometry era: more tests, more false-positive results. Pediatrics 2006;118:448–56.
18. Rinaldo P, Zafari S, Tortorelli S, Matern D. Making the case for objective performance metrics in newborn screening by tandem mass spectrometry. Ment Retard Dev Disabil Res Rev 2006;12: 255–61.
19. Campbell CD, Ganesh J, Ficicioglu C. Two newborns with nutritional vitamin B12 deficiency: challenges in newborn screening for vitamin B12 deficiency. Haematologica 2005;90:ECR45.
20. Marble M, Copeland S, Khanfar N, Rosenblatt DS. Neonatal vitamin B12 deficiency secondary to maternal subclinical pernicious anemia: identification by expanded newborn screening. J Pediatr 2008;152:731–3.
21. Chapman KA, Bennett MJ, Sondheimer N. Increased C3-Carnitine in a Healthy Premature Infant. Clin Chem 2008;54:1914–7.
22. Magera MJ, Gunawardena ND, Hahn SH, Tortorelli S, Mitchell GA, Goodman SI, et al. Quantitative determination of succinylacetone in dried blood spots for newborn screening of tyrosinemia type I. Mol Genet Metab 2006;88:16–21.
23. la Marca G, Malvagia S, Casetta B, Pasquini E, Donati MA, Zammarchi E. Progress in expanded newborn screening for metabolic conditions by LC-MS/MS in Tuscany: update on methods to reduce false tests. J Inherit Metab Dis 2008 Oct 27. DOI: 10.1007/s10545-008-0965-z. JIMD short report #127 (2008) online.
24. Oglesbee D, Sanders KA, Lacey JM, Magera MJ, Casetta B, Strauss KA, et al. Second-tier test for quantification of alloisoleucine and branched-chain amino acids in dried blood spots to improve newborn screening for maple syrup urine disease (MSUD). Clin Chem 2008;54:542–9.
25. Turgeon C, Magera MJ, Allard P, Tortorelli S, Gavrilov D, Oglesbee D, et al. Combined newborn screening for succinylacetone, amino acids, and acylcarnitines in dried blood spots. Clin Chem 2008;54:657–64.
26. la Marca G, Malvagia S, Pasquini E, Innocenti M, Fernandez MR, Donati MA, Zammarchi E. The inclusion of succinylacetone as marker for tyrosinemia type I in expanded newborn screening programs. Rapid Commun Mass Spectrom 2008; 22:812–8.
27. Tortorelli S, Turgeon CT, Lim JS, Baumgart S, Day-Salvatore DL, Abdenur J, et al. A two-tier approach to the newborn screening of methylenetetrahydrofolate reductase deficiency and other remethylation disorders by tandem mass spectrometry. J Pediatr 2010:157;271–5.
28. Shinawi M. Hyperhomocysteinemia and cobalamin disorders. Mol Genet Metab 2007;90: 113–21.
29. Kirk K, Steckel T, King P, Kline D, Rhoades E, Copeland K. A second-tier screening approach for CAH: Oklahoma's experience. 2007 Newborn Screening and Genetic Testing Symposium; 2007 May 7–10; Minneapolis, Minn. http://www.aphl.org/profdev/conferences/proceedings/Pages/2007APHLNBSandGeneticsTestingSymposium.aspx (Accessed September 2010).
30. De Jesús VR, Mei JV, Bell CJ, Hannon WH. Improving and assuring newborn screening laboratory quality worldwide: 30-year experience at the centers for disease control and prevention. Semin Perinatol 2010;34:125–33.