Clinical Chemistry 53:7
1364–1369 (2007)

Pediatric Clinical Chemistry

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

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Background: **The expansion of newborn screening programs has increased the number of newborns diagnosed with inborn errors of metabolism in the presymptomatic phase, but it has also increased the number of costly, stress-producing false-positive results. Because propionylcarnitine (C3) is one of the analytes most frequently responsible for false-positive results, we aimed to develop a rapid liquid chromatography–tandem mass spectrometry (LC-MS/MS) method to identify free methylmalonic (MMA) and 3-OH propionic (3OH-PA) acids in blood spots.**

Methods: **We studied newborn screening spots from 250 healthy controls; 124 from infants with abnormal C3, of whom only 5 (4%) were truly affected; 124 from infants with altered isolated methylmalonylcarnitine; and 4 from clinically diagnosed patients. Whole blood was eluted from a 3.2-mm dried blood spot by a CH_3CN/H_2O 7:3 and 5 mL/L formic. This extract was injected into a LC-MS/MS equipped with pneumatically assisted electrospray without derivatization. Total analysis time was 5 min per sample.**

Results: **The assays were linear up to 3300 nmol/L for both metabolites. Intra- and interassay imprecision data were 3.6%–8% and 3.1%–6%, respectively, for MMA and 5.2%–20% and 3.6%–17% for 3OH-PA. Limit of detection and limit of quantitation were 1.95 and 4.2 μmol/L, respectively, for MMA and 8 and 10 μmol/L for 3OH-PA. The recoveries were 92.9%–106.1%. No deterioration was noted on the columns after 500 chromatographic runs. If the new method had been used as a 2nd-tier test for the 124 samples, only the 5 true positives would have been recalled for additional samples, and the positive predictive value would have been 100%.**

Conclusions: **This method has the potential to markedly reduce false-positive results and the associated costs and anxiety. It may also be suitable for diagnosing and routinely monitoring blood spots for methylmalonic aciduria and propionic acidemia.**

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The introduction of an expanded liquid chromatography–tandem mass spectrometry (LC-MS/MS)[3]–based newborn screening program has significantly increased early diagnoses of inborn errors of metabolism. False-positive screening results have also increased since the time when neonatal screening was limited to phenylketonuria and congenital hypothyroidism *(1, 2)*. This increase in retesting has had important consequences, including increased

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Received February 19, 2007; accepted April 24, 2007.

Previously published online at DOI: 10.1373/clinchem.2007.087775

[3] Nonstandard abbreviations: LC-MS/MS, liquid chromatography–tandem mass spectrometry; C3, propionylcarnitine; C4, isobutyryl/butyrylcarnitine; LA, lactic acid (2-hydroxypropionic acid); MMA, methylmalonic acid; SA, succinic acid; 3OH-PA, 3-OH-propionic acid; C4DC, methylmalonylcarnitine; PA, propionic acidemia; MRM, multiple reaction monitoring.

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Table 1. MMA, 3OH-PA, C3, and C4DC in samples from screening patients, false-positives samples, C4DC-positive spots, and controls.

Sample	Diagnosis year	MMA, μmol/L	3OH-PA, μmol/L	C3, μmol/L	C4DC, μmol/L
PA1	2002	ND[a]	76	7.89	0.03
PA2	2002	ND	69.4	8.79	0.03
PA3	2006	ND	104	10.76	0.14
PA4	2005	ND	106.7	11.83	0.41
MMA1	2006	37.4	11.3	7.25	0.25
MMA2	2006	109.2	31.9	9.84	0.32
MMA3	2005	111.1	29.9	6.88	0.54
MMA4	1999[b]	83.5	29.4	0.86[b]	0.09[b]
MMA5	2002[b]	190	27.9	4.4[b]	0.18[b]
False-positive samples (n = 124)		ND	ND	6.01–15.2	WR
C4DC-positive samples (n = 124)		ND	ND	WR[a]	0.55–1.1
Controls (n = 250)		ND	ND	WR	WR
Normal values		ND	ND	0.2–5.65	0.04–0.54

[a] ND, Not detectable; WR, within accepted interval.
[b] Newborn screening spot retrospectively tested in 2005.

laboratory analyses, personnel costs for repeat tests, and considerable anxiety for parents *(3–6)*. The impact of a screening recall on a family is substantial *(7, 8)*. Even after a normal retesting, a screening recall often results in increased anxiety over a child's health, an altered parent-child relationship, and increased hospitalizations for unrelated illnesses *(2, 6, 9)*. Many authors have tried to assess what levels of parental stress are acceptable *(10)*.

An expanded program for newborn screening by LC-MS/MS was established in Tuscany in 2001 (the first regional expanded newborn screening program in Italy). Our experience shows that some metabolites—propionylcarnitine (C3), isobutyryl/butyrylcarnitine (C4), and tyrosine—are more likely than others to cause false-positive results and increase recall rates. C3, in particular, causes a high number of false-positive results. In ~67 000 newborns screened by our center between November 2004 and November 2006, the recall rate for altered C3 was ≥20 percent of all recalls. Diagnosis of propionic acidemia (PA) was confirmed in 2 patients and methylmalonic aciduria in 3 patients. The positive predictive value of C3 was 4%.

These data encouraged us to develop a strategy to reduce false-positive rates and improve the positive predictive value of an initial abnormal result.

Materials and Methods

Chemical standard 2-hydroxypropionic acid [lactic acid (LA)], methylmalonic acid (MMA), and succinic acid (SA) were purchased from Sigma-Aldrich; 3-OH-propionic acid (3OH-PA) was from Tokyo Chemical Industries. A working calibrating solution containing 10 μmol/L of each was prepared in CH_3CN/H_2O 7:3 containing 5 mL formic acid per liter. MMA (methyl-D_3) was from Cambridge Isotopes Laboratories.

We tested 250 newborn screening spots from healthy controls, 119 spots with false-positive results due to abnormal values of C3, and 124 spots with isolated methylmalonylcarnitine (C4DC) outside the reference interval (Table 1), although we have not presupposed any recall for altered isolated C4DC. In addition, we studied newborn screening spots from 9 patients with confirmed diagnoses: 4 patients with PA and 5 patients with methylmalonic aciduria. Two patients with PA (PA3 and PA4) and 3 with methylmalonic aciduria (MMA1, MMA2, and MMA3) were identified by newborn screening; in 2 PA patients (PA1 and PA2) and 2 methylmalonic aciduria patients (MMA4 and MMA5), diagnosis was made clinically before the expanded newborn screening program was started. In the latter cases, stored dried blood spots from newborn screening were retrospectively analyzed. PA1 and PA2 had acute neonatal onset, whereas PA3 and PA4 were asymptomatic when newborn screening results were available. MMA1 was due to maternal cobalamin deficiency; in MMA2 no mutation was identified in the *MUT*[4] (methylmalonyl coenzyme A mutase) gene, and complementation studies are in progress. MMA3, MMA4, and MMA5 had mutase deficiencies; MMA2 and MMA3 had clinical symptoms when recalled for neonatal screening; MMA4 had acute neonatal onset; and MMA5 had acute late onset.

Specimens of dried blood spots used as controls were collected from neonates born in Tuscany. Our local ethics committee approved the procedure. Blood collection for newborn screening purposes is made between 48 and 72 h of life. Blood taken by heel stick is spotted on filter paper (903, Whatman), dried, and sent by courier to the screening center. Blood spot samples are stored at room temperature until analysis.

We punched a 3.2-mm filter paper disk containing ~3.4 μL whole blood from each dried blood spot and

[4] Human gene: *MUT*, methylmalonyl coenzyme A mutase.

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Table 2. MRM transitions, DP, CE, quantification strategies, and retention times.[a]

			MRM transitions				
Measured compound	**Chromatographic retention time, min**	**Polarity**	**Precursor ion**	**Fragment ion**	**DP, V**	**CE, eV**	**Quantification strategy**
Lactic acid	1.08	−	89	59	−50	−14	
3OH-PA	1.24	−	89	59	−50	−14	External calibration
SA	1.30	−	117.1	73	−40	−12	
$^2H_3$-MMA	2.37	−	120.1	76	−40	−12	
	2.39	−	117.1	73	−40	−12	Isotopic dilution

[a] DP, Declustering potential; CE, collision energy.

extracted it for 15 min with 200 μL of a solution containing CH_3CN/H_2O 7:3 and 5 mL/L formic acid, plus 330 nmol/L labeled MMA as internal standard. Calibrators, containing internal standard at 330 nmol/L, were at concentrations of 0, 33, 165, 330, and 3300 nmol/L. We injected 2 μL into the LC column. For enriching studies, we evaluated linearity by analyzing supplemented 3.2-mm dried blood spots prepared at 0, 33, 165, 330, and 3300 nmol/L. The resulting calibration values were 0, 1.947, 9.735, 19.47, and 194.7 μmol/L, respectively, compared with blood (3.4 μL diluted 59-fold). The solution was shaken on a vortex-mix system for 15 min at room temperature, and 2 μL solution was injected into the mass spectrometer.

The hardware configuration includes an Applied Biosystems/MDS Sciex API 4000™ Triple-Quad Mass Spectrometer equipped with the TurboV-Spray® source with the turbo gas temperature set at 425 °C. The source

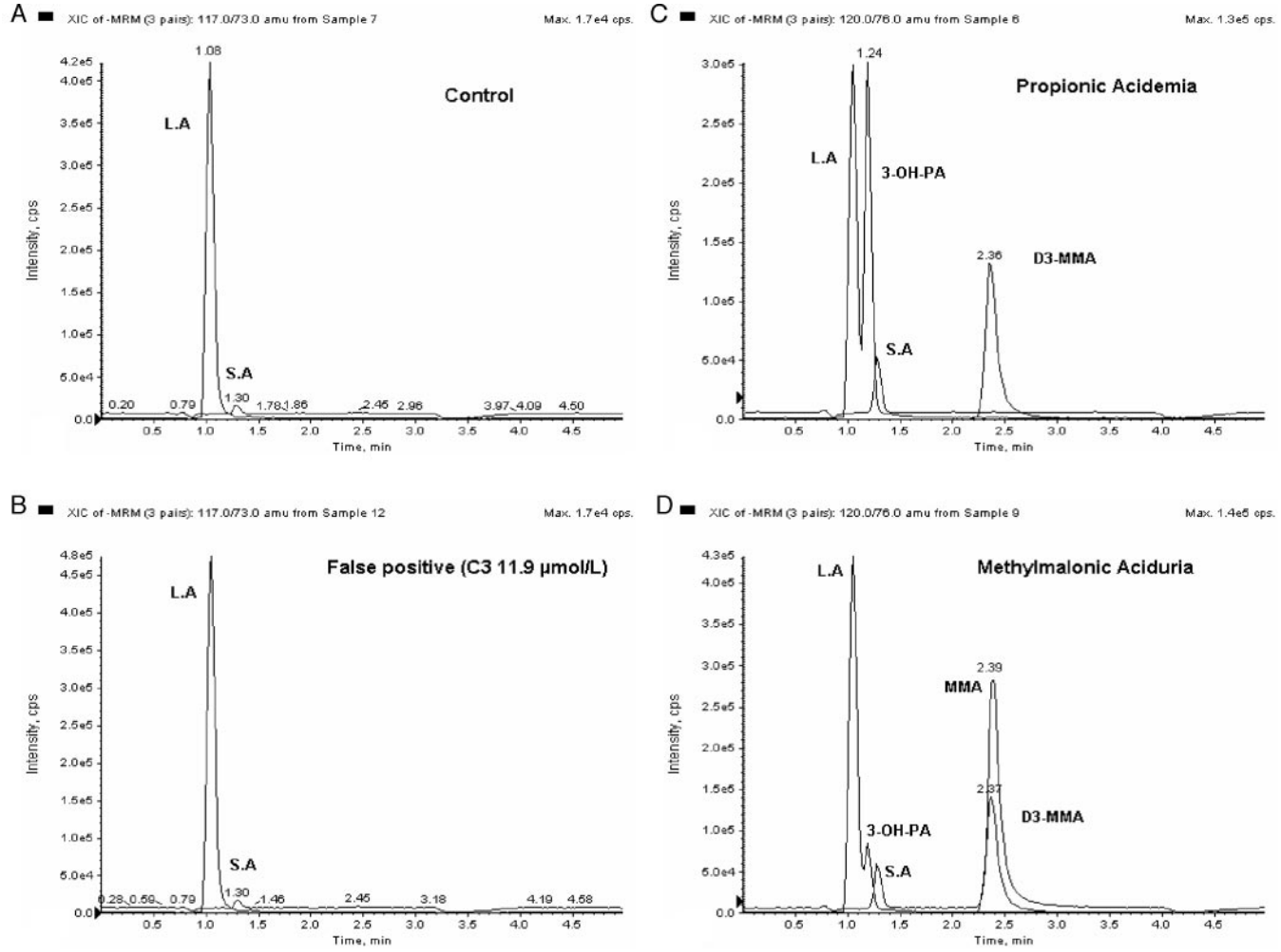

Fig. 1. Extract ion chromatograms of control (*A*), C3 false positive (*B*), PA (*C*), and methylmalonic aciduria (*D*).

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operates in negative ionization polarity at a potential of −4500 V. We performed multiple reaction monitoring (MRM) measurements using declustering potential and collision energy values as automatically optimized by the software functionality for each of the analytes. A list of exploited transitions, collision energies, and declustering potentials is reported in Table 2. The choice of ionization conditions for each analyte was made to give maximum sensitivity in the experimental conditions.

We used an Agilent 1100 Quaternary Capillary-Pump for chromatography and a Gemini C6-phenyl, 3-μm 100 × 2-mm (i.d.) column and a 4 × 2-mm precolumn cartridge (Phenomenex) for separation. The chromatographic run was performed at 200 μL/min with an isocratic profile of 40:60 between mobile phase of H_2O (eluent A) and CH_3CN (eluent B), each containing 5 mL/L formic acid. The next injection was performed after 5 min. Retention times are reported in Table 2.

Results

After extensive evaluation of several LC columns working either in normal phase or in reversed phase (data not shown), the best performance resulted from the C6-phenyl column: the eluent is identical to that used during newborn screening for acylcarnitines and amino acids. No substantial modifications were noted when 100% methanol was used both as extraction solvent and eluent B during a chromatographic run. In these conditions, the isobaric SA and MMA were completely resolved. The respective retention times were 1.30 and 2.39 min. In the case of LA and 3OH-PA, the retention times were 1.08 and 1.24 min, respectively (Fig. 1).

All analytes are acids and display poor sensitivity when ionized in positive mode. This implies that their measurement should be done in negative ion mode for better sensitivity. As already mentioned, where the isotopically labeled form of MMA was available, measurements were made by isotopic dilution strategy. The related labeled standard of 3OH-PA was not available, so we used the labeled MMA, which has chemical similarities, as internal standard.

The assays were linear up to 3300 nmol/L for both metabolites, but for the routine purpose of the protocol (screening), the MMA and 3OH-PA concentrations in samples from unaffected neonates were below detection limits. Intra- and interday imprecision data are reported in Table 3. For blood spots with added analyte, the limit of detection or limit of the blank (mean plus 3 SD of blank) for MMA was 1.95 μmol/L and the limit of quantification (mean plus 10 SD of blank) 4.2 μmol/L; for 3OH-PA the limit of detection was 8 μmol/L and the limit of quantitation 10 μmol/L. Intra- and interday imprecision (CV) was in the range of 3.5%–7.8% and 3.1%–6%, respectively, for MMA and 5.2%–19.6% and 3.6%–16.9% for 3OH-PA. Imprecision could not be determined for blood spots that had not been supplemented because the concentrations of MMA and 3OH-PA were lower than the limit of quantitation. The recoveries ranged from 92.9% to 106.1%. No deterioration was noted on the columns after 500 chromatographic runs.

Table 3. Intra- and interday imprecision.

Metabolite	Amount added, nmol/L	Intraday CV (n = 6), %	Interday CV (n = 6), %	Mean result, μmol/L	Recovery (n = 6)
Blood 1					
MMA	0	0	0.0	0.0	
MMA	33	3.5	3.1	34.0	103.1
MMA	165	4.9	3.7	158.0	95.8
MMA	330	7.8	6.0	336.2	101.9
MMA	3300	3.8	4.8	3299.7	100.0
PA	0	0	0.0	0.0	
PA	33	19.6	16.9	32.8	92.9
PA	165	6.6	4.8	169.6	100.3
PA	330	5.2	3.6	325.4	100.6
PA	3300	5.6	6.7	3300.2	100.3
Blood 2					
MMA	0	0	0.0	0.0	
MMA	33	7.8	6.1	42.6	123.0
MMA	165	9.4	8.0	175.0	106.1
MMA	330	1.4	1.8	308.9	93.6
MMA	3300	1.5	2.7	3301.5	100.0
PA	0	0	0.0	0.0	
PA	33	9.3	17.1	30.7	93.2
PA	165	4.4	10.6	168.3	102.0
PA	330	7.2	10.5	328.9	96.7
PA	3300	2.5	3.3	3300.0	100.0

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Between November 2004 and November 2006 (n = 67 586), the Newborn Screening Tuscany program, limited to LC-MS/MS testing, had a detection rate of 1:1950 and a false-positive rate of 0.83%. Of 564 total recalls, 124 (22%) were for abnormal values of isolated C3 (normal values 0.2 to 5.65 μmol/L) and 1 or more abnormal ratios (C3/free carnitine 0.03–0.13; C3/C4 1.1–12.5; C3/C16 0.11–1.16). Only 5 of the 124 were true positives: 3 methylmalonic acidurias and 2 PAs.

To assess whether 2nd-tier testing could be effective, we applied it to 250 blood spots reported as normal during newborn screening by LC-MS/MS, 124 spots recalled for abnormal values of C3, and 124 spots having C4DC outside the accepted intervals. In addition, we tested 9 truly positive newborn screening blood spots (5 methylmalonic acidurias and 4 PAs). No signal corresponding to free diagnostic acids was detected for controls, false positives, or C4DC-positive spots. Free 3OH-PA, free MMA, C3, and C4DC carnitine values from affected newborns are reported in Table 1.

This methodology succeeds in distinguishing true positives from false positives and controls, as shown in Fig. 1. In each panel, the extracted ion chromatograms referring to the implicated metabolites are highlighted for both the control/false positive and the affected patient.

Discussion

LC-MS/MS is increasingly gaining acceptance in clinical laboratories. In addition to its benefits in terms of sensitivity and specificity, it enables multiple compounds to be analyzed at one time. Routine analysis of multiple components in one fast step has been implemented in clinical laboratories for neonatal screening, steroid profiling, and so on. The method we describe allows qualification of the disorder and reduces the false-positive rate due to abnormal values of C3 and/or C4DC carnitines during newborn screening. To our knowledge, this is the first time free MMA and 3OH-PA involved in propionic and methylmalonic acidurias have been both monitored and quantified using a single blood spot sample.

Other methods that measure MMA and separate it from SA as *n*-butyl ester derivatives in plasma and urine by LC-MS/MS have been published by several authors *(11–13)*. There are several advantages of our method. It reveals contemporaneously free 3OH-PA and MMA, clearly separated from each other and differentiated from LA and SA by a fast chromatographic run in the same spot used in newborn screening. In addition, sample preparation is minimal and quick, without a derivatization step. Moreover, our method permits follow-up studies and a 1st rapid evaluation in suspected patients.

MS is a promising tool in clinical analysis, both for research (mainly through proteomic and metabolomic approaches) and in routine procedures (through the quantization of targeted metabolites and markers). In the past, mass spectrometry performance was noted for sensitivity, resolution, and selectivity factors. Today, the clinical laboratory demands those factors and also short- and long-term robustness. An analytical methodology cannot be exploited in the clinical domain if it is lacking robustness and requires labor-consuming sample preparation *(14, 15)*.

Our methodology is capable of monitoring and quantifying MMA and 3OH-PA during newborn screening as a 2nd-tier test. It can follow up on and diagnose PA and methylmalonic acidurias. The method is precise and robust and therefore suitable for implementation in routine clinical screening programs and quantification environments. In our opinion, the application of this method in newborn screening analysis will reduce or even eliminate false-positive results for C3 and prevent a great deal of unnecessary anxiety for parents and associated problems *(2)*. New and better strategies should be developed to provide testing options that, applied to newborn screening programs, reduce recall rate, overall expenses, and parental anxiety.

Grant/funding support: This work was partially supported by grants from the Family Association AMMEC.
Financial disclosures: None declared.

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