Analysis of Methylcitrate in Dried Blood Spots by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract Accumulation of propionylcarnitine (C3) in neonatal dried blood spots (DBS) is indicative of inborn errors of propionate metabolism including propionic acidemia (PA), methylmalonic aciduria (MMA), and cobalamin (Cbl) metabolic defects. Concentrations of C3 in affected newborns overlap with healthy individuals rendering this marker neither specific nor sensitive. While a conservative C3 cutoff together with relevant acylcarnitines ratios improve screening sensitivity, existing mass spectrometric methods in newborn screening laboratories are inadequate at improving testing specificity. Therefore, using the original screening DBS, we sought to measure 2-methylcitric acid (MCA), a pathognomonic hallmark of C3 disorders to decrease the false positive rate and improve the positive predictive value of C3 disorders. MCA was derivatized with 4-[2-(*N,N*-dimethylamino)ethylamino-sulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE). No separate extraction step was required

and derivatization was performed directly using a 3.2-mm disc of DBS as a sample (65°C for 45 min). The reaction mixture was analyzed by liquid chromatography tandem mass spectrometry. MCA was well separated and eluted at 2.3 min with a total run time of 7 min. The median and (range) of MCA of 0.06 $\mu\text{mol/L}$ (0–0.63) were in excellent agreement with the literature. The method was applied retrospectively on DBS samples from established patients with PA, MMA, Cbl C, Cbl F, maternal vitamin B12 deficiency ($n = 20$) and controls ($n = 337$). Comparison with results obtained by another method was satisfactory ($n = 252$). This method will be applied as a second tier test for samples which trigger positive PA or MMA results by the primary newborn screening method.

Introduction

Accumulation of propionate metabolites in body fluids can be caused by defects in propionyl coenzyme A or cobalamin (Cbl) metabolism (Fenton et al. 2001). These metabolites are chiefly derived from the catabolism of certain amino acids and odd chain fatty acids. Propionate accumulation results in increased propionylcarnitine (C3) concentration in biological samples. C3 is a marker of a number of inborn errors of metabolism including methylmalonyl CoA mutase deficiency (OMIM ID: 251000) and propionic acidemia (PA, OMIM ID: 606054) (Zytkovicz et al. 2001; Rashed et al. 1997). Elevated C3 can also be associated with defects in several other genes involved in Cbl metabolism (Fowler et al. 2008; Watkins and Rosenblatt 2011). A subset of C3 related metabolic disorders have been recommended as screening targets and are widely adopted by newborn screening programs around the world

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(Watson et al. 2006). Clinically, these are often accompanied by episodic metabolic acidosis, ketosis, hyperammonemia, and may result in severe sequelae including neurological symptoms or death in infancy. Early diagnosis is key to effective management and favorable outcome is expected should treatment start before the appearance of symptoms (Hori et al. 2005; Schulze et al. 2009).

In neonatal dried blood spot (DBS) specimens, concentration of C3 in affected newborns overlaps with healthy individuals rendering screening for relevant disorders using this metabolic intermediate as a sole marker neither specific nor sensitive. In Ontario, screening for PA and MMA started in 2006 based on C3 and one baby with a false negative screen (methylmalonyl-CoA mutase deficiency) was identified among 507,428 infants screened in the first 4 years. By adjusting C3 cutoff and introducing the ratio of C3 to acetylcarnitine (C3/C2), the sensitivity and specificity can be improved (Wilcken et al. 2003; Chace et al. 2003; Lindner et al. 2008), however, the positive predictive value remains generally poor (La Marca et al. 2007).

In our program, screen positive newborns are recalled for further diagnostic workup. Simultaneous testing of mothers to eliminate a maternal condition is also not uncommon and most of these babies turn out to be unaffected. The high false positive rate negatively impacts the cost-benefit ratio of newborn screening, leads to parental anxiety, and increases risk for parent-child dysfunction (Gurian et al. 2006). Unfortunately, routine MS/MS-based newborn screening methodology is inadequate to reduce the false positive rate and new analytical strategies are needed to improve the screening performance.

Second tier testing whereby the initial screening sample is tested for a different marker or using alternative technology is efficient in improving specificity (Minutti et al. 2004; Janzen et al. 2007). 2-Methylcitric acid (MCA) is generally described as a pathognomonic hallmark for disorders involving propionyl CoA metabolism. MCA can be detected in DBS and has been shown to reduce the false positive rate and improve the positive predictive value for PA and MMA (Turgeon et al. 2010).

In this paper, we describe a novel method to analyze MCA in DBS as a derivative of 4-[2-(*N,N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE). Similar to other carboxylic acids, the poor ionization and fragmentation of MCA in electrospray ionization (ESI)-MS/MS can be improved by this approach (Al-Dirbashi et al. 2007, 2008). The superior sensitivity attained allows for MCA detection using a single 3.2 mm disc. In a newborn screening laboratory setting, this method can be used for quantifying MCA in the original screening DBS to provide additional analytical information and reduce the number of false positive results.

Materials and Methods

Chemicals and Standard Solutions

MCA and d3-MCA used as internal standard (IS) were obtained from CDN Isotopes (Pointe-Claire, QC, Canada). DAABD-AE was synthesized according to the published method (Tsukamoto et al. 2005) but can also be purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 4-(dimethylamino)pyridine (DMAP), and perfluorooctanoic acid (PFOA) were obtained from Sigma-Aldrich. HPLC grade acetonitrile was from Burdick's and Jackson (Muskegon, MI, USA). Water used throughout this study was generated by a Milli-QUV Plus Ultra pure water system (Millipore SA, Molsheim, France).

Stock solutions of MCA and IS were prepared by dissolving the proper amounts in 50% methanol to give a concentration of 10 mmol/L. These solutions were stable for a minimum of 6 months when stored at -20°C . A working IS solution at 2.5 $\mu\text{mol/L}$ was prepared in 50% acetonitrile.

Controls and Patients Samples

Our Institutional Research Ethics Board granted approval to this study. DBS specimens received at Newborn Screening Ontario laboratory and producing normal profiles for all screened conditions were used to determine MCA reference range ($n = 337$). In general, samples were collected at 24–72 h on Whatman 903TM Specimen Collection Paper. Archived DBS samples from confirmed patients with PA ($n = 2$), MMA ($n = 8$), Cbl C ($n = 1$), Cbl F deficiency ($n = 1$), and maternal vitamin B12 deficiency ($n = 8$) were also analyzed. These samples were stored at -80°C from the time of the initial positive screening result.

Sample Preparation

A single 3.2 mm disc was punched from the DBS into a 2.0 mL polypropylene tube. After adding 20 μL of IS working solution, the following were added successively: 25 μL of EDC (25 mmol/L in water), 25 μL of DMAP (25 mmol/L in acetonitrile), and 50 μL of DAABD-AE (2 mmol/L in acetonitrile). The tubes were tightly capped and heated at 65°C for 45 min. The reaction was stopped by adding 120 μL of 10% methanol containing PFOA at a concentration of 0.5 g/L. The sample tubes were centrifuged at 13,000 rpm for 1 min and a 10 μL portion of the resultant supernatant was injected onto the LC-MS/MS. The derivative stability was evaluated by analyzing the reaction mixture stored at 8°C in a tightly sealed vial at 0, 2, 4, 8, 24, and 48 h after reaction.

LC-MS/MS system

The LC-MS/MS consisted of a Waters ACQUITY Ultra Performance LC system (Waters, Milford, MA, USA) for solvent delivery and sample introduction interfaced with a Xevo XE tandem mass spectrometer (Micromass, Manchester, UK). The ESI source was operated in the positive ion mode at a capillary voltage of 3.5 kV. Cone voltage and collision energy were 35 V and 22 eV, respectively, using argon as collision gas. The ion source and desolvation temperatures were maintained at 120 and 350°C, respectively. MCA and IS were detected by selected reaction monitoring (SRM) using transitions of mass to charge (m/z) of 499 to 151 and 502 to 151, respectively, with a dwell time of 0.07 s. Separation was performed on an ACQUITY UPLC BEH C₈ column (2.1 × 50 mm, 1.7 μm, Waters). Mobile phase A was 10% methanol and mobile phase B was 90% methanol, and both contained 0.5 g/L PFOA. The following gradient program was used: 0–1.3 min 98% of A, 1.3–2.6 min from 98% to 50% of A, and 2.6–2.7 min 50% A at a flow rate of 0.4 mL/min. The column was re-equilibrated with 98% of mobile phase A for 4.3 min at a flow rate of 0.65 mL/min. The injection-to-injection time was 7 min.

Method Validation

To determine the linear range, DBS calibrators were prepared by adding MCA stock solution to whole blood from healthy volunteers to yield 0.5, 1, 2, 4, 8, and 16 μmol/L. Non-enriched blood was used as a blank. Quality control (QC) samples at 2.5 and 10 μmol/L were also prepared. Calibrators and QCs were applied manually onto Whatman 903™ Specimen Collection Paper and allowed to dry at ambient temperature overnight. DBS calibrators were stored at –20°C in sealed plastic bags with a desiccant.

The limit of quantification of MCA was calculated using DBS samples prepared from spiked blood that was serially diluted to give a final concentration of 0.1 μmol/L.

Within-day ($n = 19$) and between-day ($n = 14$) variations were evaluated by repeatedly analyzing QC samples. Coefficient of variation (CV%) was calculated according to the following equation [$CV\% = 100 \times \text{standard deviation}/\text{mean}$]. Analytical recovery was calculated using data obtained from QC samples as follows [$\text{Recovery}\% = 100 \times (\text{concentration measured} - \text{concentration in non-enriched sample})/\text{concentration added}$].

Stability of MCA in DBS was assessed by storing samples spiked at 1.5 and 3.5 μmol/L at various temperatures (ambient, –20°C and 32°C). Analysis was carried out as described over a period of 3 weeks.

For method comparison, samples with normal and abnormal MCA levels ($n = 252$) were analyzed in parallel by the current methods and a published method after minor modification (Turgeon et al. 2010). The modification involved extending the derivatization reaction time from 15 min to 4 h.

Results

Sample Preparation

Extraction and derivatization of MCA was accomplished in a single step. IS and reagents required for derivatization were added directly onto a 3.2 mm DBS disc and incubated at 65°C. The derivatization yield which represents the extraction of MCA from DBS and the formation of DAABD-MCA derivative reached its maximum at 45 min or more. All the following experiments hence were performed at 65°C for 45 min. DAABD-MCA derivatives were stable for at least 48 h when stored in a tightly sealed vial at 8°C.

MS/MS and LC-MS/MS Experiments

The reaction mixture was infused into the first quadrupole of the MS/MS. Scanning in the positive ion ESI-MS revealed ions at m/z of 517 and 520 corresponding to $[\text{MH}]^+$ of DAABD-AE derivatives of MCA and d3-MCA, respectively. We also observed another set of more intense ions at m/z of 499 and 502. These were attributed to intramolecular condensation of MCA and d3-MCA and loss of water. The transmission of these ions into the collision cell and subsequent scanning by the second resolving quadrupole for fragments revealed a simple fragmentation pattern with an intense fragment at m/z of 151 common to all studied ions. This was assigned to the *N,N*-dimethylaminoethylaminosulfonyl moiety originating from DAABD-AE. Figure 1 shows the MS (Fig. 1a) and MS/MS (Fig. 1b) spectra of DAABD-MCA. It is noteworthy to mention that water loss may vary between different ion source designs, therefore, the use of appropriate stable isotope labeled IS is essential to compensate for this potential cause of variation.

Various chromatographic columns were evaluated aiming at the retention of DAABD-AE derivatives while allowing other compounds which may cause ion suppression including excess reagents to elute. This was achieved using a reversed phase C8 column in combination with a gradient program that increases methanol concentration from 2% to 50% (v/v) over the course of the run. The use of PFOA as a mobile phase additive improved the ionization process and enhanced the peak shape. Figure 2 shows

Fig. 1 ESI-MS spectrum of DAABD-MCA derivative (**a**) and ESI-MS/MS spectrum of m/z 499 (**b**). Experimental details are as described in the text

extracted mass chromatograms obtained with a 3.2 mm DBS disc from a healthy control (Fig. 2a) and that from a patient with an MMA (Fig. 2b). As shown, MCA was well separated and eluted at 2.3 min. After each injection, the column was reconditioned for 4.3 min to remove ion suppression effects of late eluting compounds, therefore the analytical time between successive injections was 7 min. It is worth mentioning that the automatic switching valve was programmed to divert column effluent to waste for the first 1.8 min and the last 4 min of each run to avoid loading the mass spectrometer with contaminating material from samples or reagents.

Assay Validation

Linearity was established by using DBS enriched with MCA at 0.5, 1, 2, 4, 8, and 16 $\mu\text{mol/L}$. Non-enriched DBS was also analyzed to correct for endogenous MCA. Regression analysis over the studied range revealed a linear relationship ($y = 0.041x - 0.002$, $r = 0.9999$), with y as the peak area ratio (MCA/IS) and x as the concentrations in DBS ($\mu\text{mol/L}$). The limit of quantification defined as MCA concentration that gives a signal to noise ratio (S/N) of ≥ 10 was found to be 0.1 $\mu\text{mol/L}$ whereas the limit of detection (S/N = 3) was calculated to be 0.03 $\mu\text{mol/L}$.

Fig. 2 Extracted mass chromatograms obtained with a DBS from a healthy individual (**a**) and from an MMA patient (**b**). Solid lines represent MCA and dotted lines represent the IS. The arrow points at MCA in the healthy individual's sample

Analysis of DBS specimens containing MCA at 1.5 and 3.5 $\mu\text{mol/L}$ stored for a period of 3 weeks at -20°C , 23°C (ambient) and 32°C revealed that this compound is reasonably stable at the conditions described (Fig. 3).

Within-day ($n = 19$) and between-day ($n = 14$) imprecision were evaluated by repeated analysis of QC samples at 2.5 and 10 $\mu\text{mol/L}$. Table 1 summarizes the imprecision expressed as coefficient of variation (%) and analytical recovery.

Figure 4 illustrates the correlation between concentrations of MCA in DBS samples ($n = 252$) measured by the current method and in parallel by a published method (Turgeon et al. 2010).

Analysis of Controls and Patients Samples

MCA concentrations obtained by the current method using DBS samples from healthy individuals ($n = 337$) and from patients ($n = 20$) with confirmed PA, MMA, Cbl C, Cbl F, and maternal vitamin B12 deficiency are summarized in Table 2 which also shows the corresponding C3 and C3/C2 levels obtained by the primary newborn screening method.

Discussion

As proposed by the American College of Medical Genetics, PA, MMA, Cbl A, and Cbl B defects are included in the newborn screening core panel whereas Cbl C and Cbl D are considered secondary targets (Watson et al. 2006). These disorders are screened for by MS/MS using C3 as a primary marker together with appropriate ratios. Due to the overlap in C3 concentration between affected and unaffected newborns these disorders present a significant challenge to newborn screening laboratories. In affected patients, a gradient of C3 concentration is observed including subtle elevations that may be overlooked. To maximize screening sensitivity, laboratories tend to apply conservative cutoffs but this decreases test specificity resulting in a high rate of false screen positives, and potentially reveals incidental findings of non-targeted disorders. To mitigate this, second tier tests were devised to improve the screening process for this group of disorders aiming at measuring MMA, 3-hydroxypropionic acid, and MCA in DBS samples (Turgeon et al. 2010; Matern et al. 2007; La Marca et al. 2007). Among these, MCA, which is traditionally detected by gas chromatography mass spectrometry as part of the

Fig. 3 Stability of MCA in DBS samples stored at different temperatures

Table 1 Recovery, intra- and inter-day reproducibility of MCA analysis

Concentration added (μmol/L)	Intra-day (<i>n</i> = 19)				Inter-day (<i>n</i> = 14)			
	Mean (μmol/L)	SD ^a	CV ^b (%)	Recovery ^c (%)	Mean (μmol/L)	SD	CV (%)	Recovery (%)
2.5	2.2	0.1	4.5	88.0	2.3	0.2	8.7	92.0
10.0	11.4	0.7	6.1	114.0	11.5	0.7	6.1	115.0

^aSD = standard deviation

^bCV = coefficient of variation

^cRecovery (%) = 100 × found concentration /added concentration

Fig. 4 Comparison of MCA concentrations in DBS (*n* = 252) obtained by the current method versus a reference method after modification (Turgeon et al. 2010). Experimental details are as described in the text

organic acid profile (Rinaldo 2008) is known to accumulate in patients with defects in propionate metabolism. The MCA method is appealing because it is also capable of measuring MMA and homocysteine simultaneously. We attempted to adopt the MCA method of Turgeon et al. (2010) as described, but we encountered inadequate sensitivity and large fluctuations in reproducibility. We decided to extend the butylation reaction time beyond 15 min and monitor the formation of the tributyl MCA derivative and found a 10-fold increase in peak intensity at 4 h of incubation or more. Under the modified conditions, the reproducibility was also improved, but this modification together with a lengthy chromatographic run of 15.6 min did not meet our screening objective which necessitates that samples not be batched and that reflexive testing is completed within the same working day as the initial screen positive result.

We sought to develop a more efficient method for MCA in DBS with sufficient sensitivity, still allowing for early referral of screen positive results. MCA, a tricarboxylic acid is a hydrophilic compound with disappointing chromatographic

Table 2 Median and range of MCA, C3, and C3/C2 levels in healthy individuals and confirmed patients studied in this work

Sample type	MCA ($\mu\text{mol/L}$) Median (range)	C3 ($\mu\text{mol/L}$) Median (range)	C3/C2 Median (range)
Control ($n = 337$)	0.06 (0–0.63)	1.87 (0.46–7.15)	0.08 (0.03–0.21)
Propionic acidemia ($n = 2$)	10.4 (7.3–13.4)	20.8 (16.5–25.1)	2.3 (1.97–2.55)
Methylmalonyl CoA mutase deficiency ($n = 8$)	13.3 (5.2–19.4)	17.6 (8.1–49.0)	0.79 (0.38–2.06)
Cbl C deficiency ($n = 1$)	6.7	18.5	0.77
Cbl F deficiency ($n = 1$)	0.83	5.73	0.27
Maternal vitamin B12 deficiency ($n = 8$)	0.28 (0–2.76)	8.4 (6.4–14.7)	0.26 (0.14–0.40)

and mass spectrometric behavior when analyzed by LC-MS/MS. To overcome this, we used chemical derivatization with DAABD-AE to generate a highly ionizable hydrophobic derivative. DAABD-AE forms stable amides upon reaction with carboxylic acids and introduces a chargeable moiety suitable for detection by ESI-MS/MS in the positive ion mode (Al-Dirbashi et al. 2007; Al-Dirbashi et al. 2008). Using reversed-phase chromatography, the hydrophobic DAABD-MCA derivative can be well separated from the early eluting ion-suppressing compounds. The high organic content coinciding with DAABD-MCA peak elution enhanced the ionization process and contributed to the increased overall sensitivity. DAABD-AE offers outstanding reactivity and allows for derivatization using DBS specimens without the need for a dedicated extraction step. The simple sample preparation which consisted of a single 45-min step for both extraction and derivatization resulted in excellent recovery and reproducibility. With a 7-min chromatographic time, we are able to meet our required turn around time and easily integrate this second tier method into our routine screening process. Compared with the published MCA method (Turgeon et al. 2010), our sample preparation does not require extraction and subsequent evaporation and our injection-to-injection time is more than halved conserving more than 50% of the instrument time.

MCA is present in DBS from healthy individuals at low, yet detectable levels ($<0.7 \mu\text{mol/L}$) (Turgeon et al. 2010). On the contrary, this marker is detected at significantly higher concentrations in PA or MMA patients. To obtain the maximum diagnostic value, we designed our calibrators to cover a wide concentration range encompassing physiological and pathological MCA levels (0.5–16 $\mu\text{mol/L}$). The use of stable isotope IS to generate the calibration curve enhanced the quality of quantitative data obtained.

MCA levels in DBS achieved in this work were compared to those obtained by a modified version of a published LC-MS/MS method (Turgeon et al. 2010). The two methods performed adequately and showed satisfactory agreement as demonstrated by linear regression analysis shown in Fig. 4.

The potential usefulness of the proposed method as a second tier test was assessed by a retrospective study using archived DBS samples from known patients ($n = 12$) with MMA, PA, Cbl C, and Cbl F defects randomized with DBS samples from babies of maternal vitamin B12 deficiency ($n = 8$) and healthy newborns ($n = 337$). Median MCA concentration was $0.06 \mu\text{mol/L}$ (range 0–0.63 $\mu\text{mol/L}$) in healthy newborns. As shown in Table 2, elevated MCA was detected in all known patients regardless of the underlying genetic defect. In DBS samples from babies born to mothers with confirmed vitamin B12 deficiency ($n = 8$), MCA was elevated in only two out of the eight samples in contrast to C3 which was elevated in all samples. With the implementation of MCA analysis as second tier test, we anticipate reduction of the false positive rate while maintaining excellent sensitivity. As previously reported (Turgeon et al. 2010), some patients with certain Cbl metabolic defects may not be detected by MCA analysis, hence, complementary analysis of other relevant markers such as MMA may be considered.

Many enzymes and small molecule markers are known to be stable in blood collected on filter paper as the dry nature of this matrix provides a favorable environment that decreases degradation. In this work, we found MCA in DBS to be invariably stable for at least 3 weeks at temperatures ranging between -20°C and 32°C as shown in Fig. 3. This finding is significant as stability during transport of DBS samples is essential to guarantee sample integrity and result validity.

In this work we developed and validated a novel, simple, and robust method to determine MCA in DBS using a single 3.2 mm disc. The excellent reactivity of the commercially available DAABD-AE reagent allowed for derivatization within 45 min, completely eliminating the extraction step. Injection-to-injection time was 7 min. The short sample preparation and chromatography time permits integration of this lean assay as a second tier method into routine newborn screening work flow without prolonging the turn around time. Reference intervals obtained are in agreement with the literature, and the method was able to

detect confirmed cases of MMA, PA, Cbl C, and Cbl F defects with 100% sensitivity. Prospective application of this method as a second tier test to improve screening for C3 disorders is in progress.

Take-Home Message

Measuring methylcitric acid in dried blood spots improves newborn screening for disorders in which propionate metabolism is impaired.

Compliance with Ethics Guidelines

Conflict of Interest

Osama Y. Al-Dirbashi, Nathan McIntosh, Christine McRoberts, Larry Fisher, Mohamed S. Rashed, Nawal Makhseed, Michael T. Geraghty, Tomofumi Santa, and Pranesh Chakraborty declare that they have no conflict of interest.

Informed Consent

Not applicable.

Details of the Contribution of Individual Authors

OYA and PC designed and coordinated the study. NM, CM, and LF carried out the analysis of samples. TS synthesized the derivatization reagent. OYA, MSR, NM, MTG, and PC provided subspecialist consultation services, clinical examination, and diagnostic testing. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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