

Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry

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We report the application of tandem mass spectrometry to prospective newborn screening for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. MCAD deficiency is diagnosed from dried blood spots on filter paper cards from newborns on the basis of the increase of medium chain length acylcarnitines identified by isotope dilution mass spectrometry methods. A robust and accurate semiautomated method for the analysis of medium chain length acylcarnitines as their butyl esters was developed and validated. Quantitative data from the analyses of 113 randomly collected filter paper blood spots from healthy newborns showed low concentrations of medium chain length acylcarnitines such as octanoylcarnitine. The maximum concentration of octanoylcarnitine was 0.22 $\mu\text{mol/L}$, with the majority being at or below the detection limit. In all 16 blood spots from newborns with confirmed MCAD deficiency, octanoylcarnitine was highly increased [median 8.4 $\mu\text{mol/L}$ (range 3.1–28.3 $\mu\text{mol/L}$)], allowing easy detection. The concentration of octanoylcarnitine was significantly higher in these 16 newborns (<3 days of age) than in 16 older patients (ages 8 days to 7 years) with MCAD deficiency (median 1.57 $\mu\text{mol/L}$, range 0.33–4.4). The combined experience of prospective newborn screening in Pennsylvania and North Carolina has shown a disease frequency for MCAD deficiency of 1 in 17 706. No false-positive and no known false-negative results have been found. A validated method now exists

for prospective newborn screening for MCAD deficiency.

Deficiency in the activity of medium-chain acyl-CoA dehydrogenase (MCAD) presents with a Reye-like syndrome, mild hypoglycemia, or sudden death [1–3].³ Approximately 20% of patients die before diagnosis, and a substantial proportion of the survivors has significant sequelae [1, 2]. With the exception of acute episodes, most patients are asymptomatic. After presymptomatic diagnosis, preventive treatment strategies including avoidance of fasting, a low-fat diet, and supplementation with L-carnitine have prevented mortality and largely reduced residual sequelae of subsequent metabolic decompensations [1, 2]. In Caucasian patients, a single mutation, A985G, accounts for 90% of the disease-causing alleles [4]. By screening for the frequency of this allele, the disease frequency in the Caucasian population of North Carolina has been estimated at 1:26 000 [5]. The frequency of the disease, the significant mortality and morbidity, and its prevention by relatively simple therapies make this disorder a good candidate for newborn screening [6].

The increase of medium chain acyl-CoA esters resulting from the enzymatic block leads to increased medium-chain fatty acids, dicarboxylic acids, acylglycines, and medium-chain acylcarnitines in plasma and urine. Strategies proposed for the identification of MCAD deficiency for newborn screening have included molecular screening for the common A985G mutation [7] or recognition of metabolites such as diagnostic increases of cis-4-decenoic acid [8], hexanoylglycine [9], or specific medium-chain

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³ Nonstandard abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; MS-MS, tandem mass spectrometry; MS1, first mass analyzer region; MS2, final mass analyzer region.

acylcarnitines in blood spots [10]. In a diagnostic clinical setting, analysis of acylcarnitines by tandem mass spectrometry (MS-MS) as their methyl esters allowed the diagnostic recognition of all patients with MCAD deficiency regardless of the underlying mutation, symptomatic state, or treatment [11]. Patients with MCAD deficiency have increased octanoylcarnitine ($>0.3 \mu\text{mol/L}$) and an increased octanoylcarnitine-to-decanoylcarnitine ratio (>5). Similar results were found in the analysis of newborn blood cards.

Analysis of acylcarnitines after derivatization as butyl esters was recently proposed [12]. Derivatization as butyl esters has the advantage to also allow amino acid analysis as the same sample preparation. The analysis of amino acids as their butyl esters has been validated for newborn screening of phenylketonuria, tyrosinemia, maple syrup urine disease, and homocystinuria [12–15]. In this study we validate the screening methodology for the analysis of acylcarnitines as their butyl esters from dried blood spots for the diagnosis of MCAD deficiency in newborns. Quantitative data obtained from prospective newborn screening are compared with data from newborns with MCAD deficiency. The evolution of the diagnostic acylcarnitines over the first days of life is investigated and compared with those in affected children. Finally, we report the first results of real-time newborn screening using this method in Pennsylvania and North Carolina.

Materials and Methods

SOLVENTS, REAGENTS, AND INTERNAL CALIBRATORS

High purity grade methanol was obtained from Burdick and Jackson. Glycerol, sodium octyl sulfate, acetylcarnitine, hexanoylcarnitine, octanoylcarnitine, and decanoylcarnitine were obtained from Sigma. Butanolic HCl (3 mol/L) was obtained from Regis. Stable isotopes were obtained from Cambridge Isotopes and include [$^2\text{H}_3$]acetylcarnitine, [$^2\text{H}_3$]octanoylcarnitine, and [$^2\text{H}_3$]palmitoylcarnitine. Sigma Tau Pharmaceuticals provided [$^2\text{H}_3$]propionylcarnitine.

BLOOD SPECIMEN COLLECTION

The specimens tested consisted of: (a) 16 500 dried blood spots from the North Carolina Division of Laboratory Services Newborn Screening Program obtained primarily from the Research Triangle region between 1993 and 1995; (b) a collection of blood spots from healthy newborns and from newborns confirmed with MCAD deficiency; these collections are part of a larger prospectively screened group of 267 303 samples from the supplemental newborn screening program at Neo Gen Screening in Pennsylvania that covered primarily Western and Central Pennsylvania between September 1992 and January 1997; and (c) a collection of blood spots of patients found to have MCAD deficiency from the medical genetics laboratory at the Mass Spectrometry Facility, Duke University Medical Center. All MCAD specimens were retrieved from storage of <3 years. All specimens from the North Carolina

Newborn Screening Program, Neo Gen Screening, and all the specimens received in the medical genetics laboratory were collected or prepared on S&S Grade 903 filter paper (Schleicher and Schuell).

SAMPLE PREPARATION

The semiautomated preparation of butyl ester derivatives of acylcarnitines and amino acids from blood spots consisted of a simple solvent extraction and derivatization procedure that takes ~ 2.5 h for 60 samples. This method has been described previously [13–15] with the following addition. The methanol stock solution that contained amino acid internal calibrators as described previously also contained the following additional internal calibrators: [$^2\text{H}_3$]acetylcarnitine (5 $\mu\text{mol/L}$), [$^2\text{H}_3$]propionylcarnitine (1 $\mu\text{mol/L}$), [$^2\text{H}_3$]octanoylcarnitine (1 $\mu\text{mol/L}$), and [$^2\text{H}_3$]palmitoylcarnitine (2 $\mu\text{mol/L}$). In brief, two $\frac{3}{16}$ -in. diameter punches from blood spots equivalent to 15.2 μL of whole blood [16] were extracted with 400 μL of this stock solution and evaporated to dryness at 50 $^\circ\text{C}$ under a gentle stream of nitrogen. Fifty microliters of butanolic HCl (3 mol/L) was added to each sample; the samples were then incubated at 65 $^\circ\text{C}$ for 15 min. After evaporation of excess butanolic HCl (3 mol/L), the derivatized samples were reconstituted with 35 μL of 1:1 (by vol) methanol:glycerol with 1 g/L sodium octyl sulfate for analysis by MS-MS.

METHOD VALIDATION

To estimate the linearity of this assay, four separate aliquots of whole blood from single donors were enriched with either acetyl-, hexanoyl-, octanoyl-, or decanoylcarnitine. In addition to unenriched whole blood, the following enrichments were prepared (five-point calibration curve): acetylcarnitine, 2.5–50 $\mu\text{mol/L}$; hexanoylcarnitine, 0.5–10 $\mu\text{mol/L}$; octanoylcarnitine, 0.5–10 $\mu\text{mol/L}$; decanoylcarnitine, 0.5–10 $\mu\text{mol/L}$. These samples were then spotted on filter paper and dried overnight. For recovery studies (extraction efficiency) of acylcarnitines from blood spots, aliquots of whole blood were prepared from a single donor pool and enriched with 0, 2.5, 10, and 80 $\mu\text{mol/L}$ of acetylcarnitine; 0, 0.5, 2, and 8 $\mu\text{mol/L}$ of octanoylcarnitine; and 0, 2, 10, and 20 $\mu\text{mol/L}$ each of hexanoylcarnitine and decanoylcarnitine. Before spotting, each set of aliquots was divided into two groups. One group of samples was spotted on filter paper, dried overnight, and prepared as described above. To the second group of whole-blood samples, [$^2\text{H}_3$]acetylcarnitine and [$^2\text{H}_3$]octanoylcarnitine were added to each aliquot (sample set) at the concentrations described above and mixed well. These whole-blood samples, containing internal calibrators, were spotted on filter paper, dried overnight, and prepared by extraction with pure methanol containing no internal calibrators. The remainder of the sample preparation procedure was followed as described above.

Instrument variability was measured by performing

10 replicate injections of one sample. Assay variability was measured by performing analyses of 10 control samples prepared from the same blood on one day (intraday variability) and over several weeks (interday variability).

MASS SPECTROMETRY

A VG Quattro quadrupole tandem mass spectrometer with a laboratory-based data system (Micromass) was used and operated in the static liquid secondary ionization mode. This mode incorporates an ion source containing a cesium ion gun operating at 10 keV and a manually operated insertion probe. Positively charged molecules are detected after separation in the first mass analyzer region (MS1) and in the final mass analyzer region (MS2). An intermediate hexapole located between the first quadrupole and the second quadrupole is used as the collision region into which argon gas is introduced. Tuning of the instrument was optimized with a solution containing deuterium-labeled calibrators prepared as butyl esters as described previously [12].

Product ion scans were produced by focusing MS1 on the molecular masses ($M+H$)⁺ of the butyl ester of octanoylcarnitine (m/z 344) and its internal calibrator [²H₃]octanoylcarnitine (m/z 347), while MS2 was used to scan fragment ions between m/z 50 and m/z 400. Mass spectra showing the fragmentation of octanoylcarnitine and its internal calibrator [²H₃]octanoylcarnitine were obtained. Parent ion scans of 85 Da were produced by scanning MS1 from m/z 255–550 while focusing MS2 on a single common product ion at m/z 85 produced by all acylcarnitine butyl esters. This resulted in a spectrum of parent ions (molecular ions) corresponding to ($M + H$)⁺.

Quantification of octanoylcarnitine was achieved by converting the ion abundance ratios of octanoylcarnitine to [²H₃]octanoylcarnitine (m/z 344:347) and then interpolating the concentration values for octanoylcarnitine by reference to a calibration curve. A calibration curve was generated from the analyses of blood spots containing serially added fixed concentrations of octanoylcarnitine. Quantification of acetylcarnitine (m/z 260) was achieved similarly with [²H₃]acetylcarnitine as the internal calibrator (m/z 260:263). No isotopically labeled internal calibrator was available for hexanoylcarnitine and decanoylcarnitine. Quantification of these compounds was achieved by calculating the ion abundance ratios of the pure unlabeled compound relative to [²H₃]octanoylcarnitine (i.e., hexanoylcarnitine, m/z 316:347, and decanoylcarnitine, m/z 372:347). A calibration curve was generated from the analyses of blood spots containing serially added fixed concentrations of either of these calibrators (hexanoyl- and decanoylcarnitine). No pure material was available for decenoylcarnitine. An approximation of the concentration of decenoylcarnitine was achieved by extrapolation from the calibration curve of the ion abundance ratios of decanoylcarnitine to [²H₃]octanoylcarnitine.

Results

ANALYSIS OF ACYLCARNITINES BY MS-MS

The fragmentation pattern of the protonated molecular ions, [$M+H$]⁺ of the butyl esters of octanoylcarnitine and [²H₃]octanoylcarnitine, are shown in Fig. 1. All other butyl

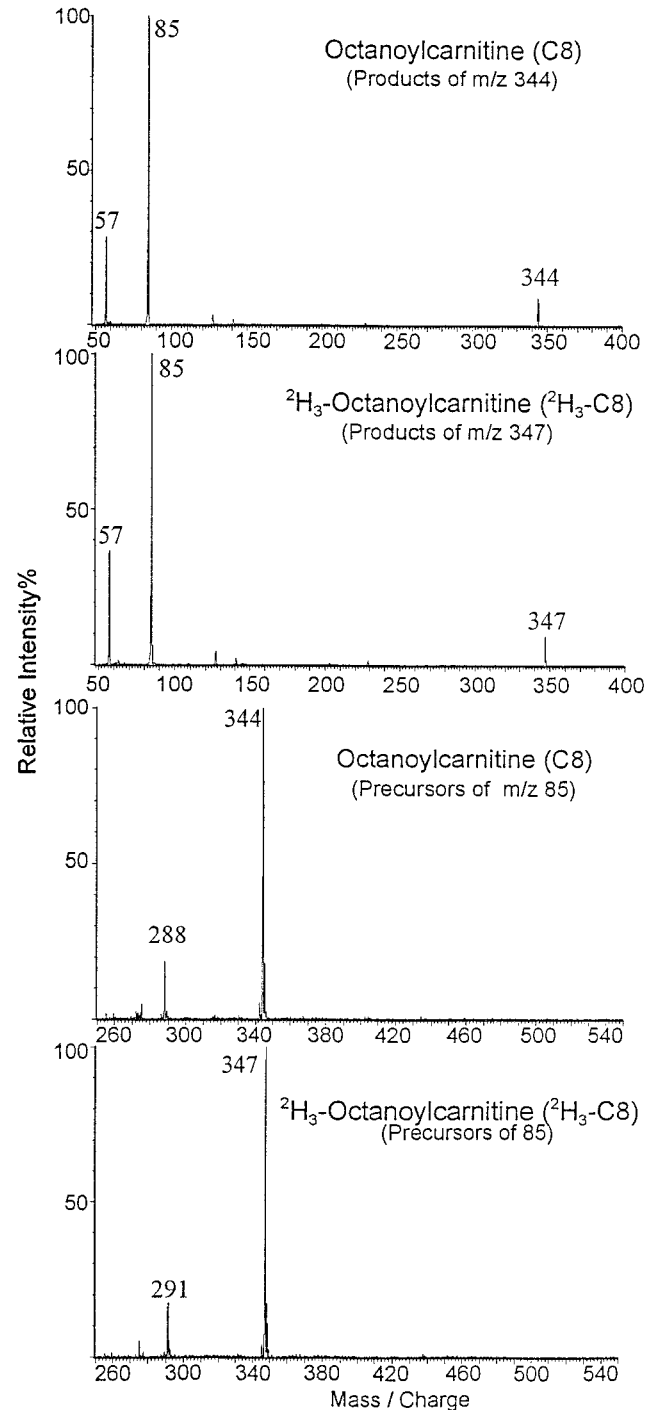


Fig. 1. Product ion mass spectra from the collision-induced dissociation of [$M+H$]⁺ ions of octanoylcarnitine (top) and [²H₃]octanoylcarnitine (2nd from top); precursor ion mass spectra of m/z 85 for octanoylcarnitine (3rd from top) and [²H₃]octanoylcarnitine (bottom).

esters of acylcarnitines (such as acetylcarnitine, hexanoylcarnitine, and decanoylcarnitine) share a similar fragmentation pattern with a common fragment ion at 85 Da. The fragmentation process, which is shown schematically in Fig. 2, involves loss of the fatty acid, trimethylamine, and butene, resulting in the generation of a stable fragment ion with m/z 85. Other acylcarnitine (acetyl-, hexanoyl-, and decanoylcarnitine) butyl esters exhibited similar fragmentation, resulting in a prominent fragment ion at m/z 85. Therefore, to analyze all acylcarnitines, the tandem mass spectrometer is set up such that MS1 will scan the entire mass range (m/z 225–550) while MS2 is set up to monitor m/z 85, the mass of the fragment ion common to acylcarnitine butyl esters. Product ions of 85 Da from acylcarnitine butyl esters are detected in MS2. Note that the mass of the parent ion is shown on the mass spectra although the product ion is actually detected. This method of MS-MS data acquisition is known as precursor ion scanning.

In biological samples, the parent ion scan detects short-chain acylcarnitines such as propionylcarnitine and butyrylcarnitine, long-chain acylcarnitines such as palmitoylcarnitine and linoleylcarnitine, dicarboxylic acid acylcarnitines such as glutarylcarnitine, or hydroxyacylcarnitines such as 3-hydroxyisovalerylcarnitine [12]. Fig. 1 shows the parent ion spectrum of the molecular ion (precursor ion) for pure octanoylcarnitine and [$^2\text{H}_3$]octanoylcarnitine. The parent ion spectrum of octanoylcarnitine and [$^2\text{H}_3$]octanoylcarnitine are not single peaks. In addition to the dominant molecular ion, there

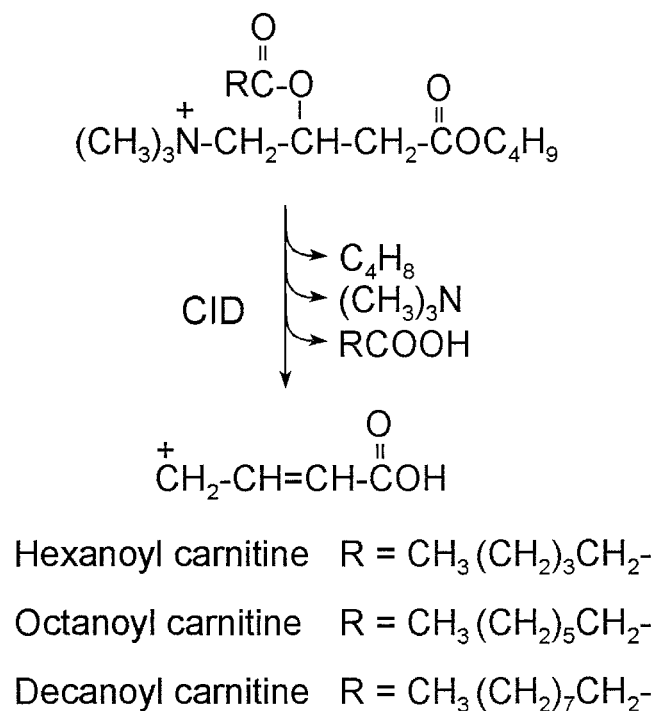


Fig. 2. Schematic representation of the specific fragmentation in the tandem mass spectrometer of acylcarnitine butyl ester derivatives.

are small peaks at m/z 288 and m/z 291 that may be the result of incomplete derivatization of acylcarnitines or of secondary fragmentation.

Figure 3 (top) shows a representative acylcarnitine profile of a blood spot from a fresh, normal newborn screening filter paper card obtained by using the parents of 85 Da scan function. Ion signals at representative masses of several acylcarnitines are m/z 260 (acetylcarnitine), m/z 274 (propionylcarnitine), m/z 456 (palmitoylcarnitine), and m/z 482 (linoleylcarnitine), and the internal calibrators at m/z 263 ($[\text{H}_3]$ acetylcarnitine), m/z 277

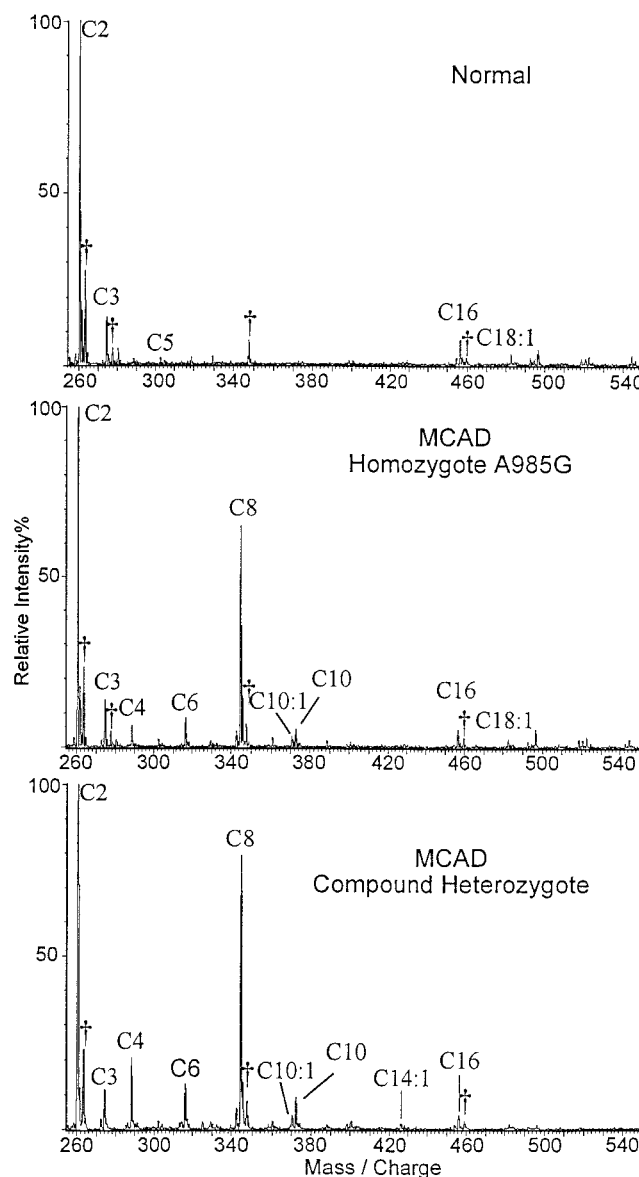


Fig. 3. MS-MS acylcarnitine profiles obtained with a precursor of m/z 85 Da scan function from a screening card of a healthy newborn (top), a screening card of a newborn with MCAD deficiency and subsequently found to be homozygous for the A985G mutation (middle), and a screening card of a newborn with MCAD deficiency and subsequently found to be compound heterozygous for the A985G mutation (bottom).

($[^2\text{H}_3]$ propionylcarnitine), m/z 347 ($[^2\text{H}_3]$ octanoylcarnitine), and m/z 459 ($[^2\text{H}_3]$ palmitoylcarnitine). The only significant signals detected are from acylcarnitines and their added internal calibrators. Octanoylcarnitine is generally present at very low concentrations in healthy newborn blood samples and the signal representative of this component is often at the limits of detection. Fig. 3 (middle) shows an acylcarnitine profile from a newborn screening blood card from an MCAD patient subsequently confirmed by DNA analysis as a homozygote for the common A985G mutation. The ion signal of octanoylcarnitine (m/z 344) is clearly increased higher than the internal calibrator ion signal at m/z 347. There is also an increase in the signal intensities of hexanoylcarnitine (m/z 316), decenoylcarnitine (m/z 370), and decanoylcarnitine (m/z 372). Fig. 3 (bottom) shows an acylcarnitine profile of a blood spot from a newborn screening filter paper card of an MCAD patient subsequently confirmed by DNA analysis to be a compound heterozygote with one copy of the gene for the A985G mutation present. Similar increases of diagnostically important acylcarnitines are seen in Fig. 3 (middle and bottom panels).

ASSAY CALIBRATION AND LIMITS OF DETECTION

Calibration curves for acetylcarnitine and octanoylcarnitine were generated with standard isotope dilution techniques. The ion signals corresponding to acetyl- or octanoylcarnitine and their respective deuterium-labeled internal calibrators were plotted as a function of the concentration of added acetyl- or octanoylcarnitine to blood. The calibration curves for acetyl- and octanoylcarnitine were linear over the concentration ranges of 0–50 and 0–10 $\mu\text{mol/L}$, respectively. The regression analyses and statistical data are presented in Table 1. Detection limits were based on a signal-to-noise ratio of 3:1.

Calibration curves for hexanoyl- and decanoylcarnitine were generated with slight variations of standard isotope dilution techniques whereby the internal calibrator is a

closely related isotope of the calibrator. The ion signal ratios of hexanoyl- or decanoylcarnitine to the internal calibrator, $[^2\text{H}_3]$ octanoylcarnitine, were plotted as a function of the concentration of added hexanoyl- or decanoylcarnitine to blood. The calibration curves for hexanoylcarnitine and decanoylcarnitine were each linear over the concentration range of 0–10 $\mu\text{mol/L}$ (Table 1). These results indicate excellent linearity of the assay within these ranges seen in most normal and all pathologic samples.

ANALYTICAL RECOVERY, PRECISION, AND ACCURACY

The analytical recoveries of acetyl-, hexanoyl-, octanoyl-, and decanoylcarnitine added to blood were determined in triplicate. These results show good recovery (Table 2). The analytical imprecision, CV, determined by 10 replicate analyses of the derivatized product of a single sample for acetyl-, hexanoyl-, octanoyl-, and decanoylcarnitine is described in Table 3. Overall precision of the assay calculated by replicate analyses was <8% of the same normal blood sample on the same day and on different days. The intraday and interday precision (CV) for acetyl-, hexanoyl-, octanoyl-, and decanoylcarnitine and the ratios of octanoylcarnitine to acetylcarnitine, hexanoylcarnitine to acetylcarnitine, and decanoylcarnitine to acetylcarnitine was <15% for all analytes. This is sufficient for the correct differentiation between normal and pathologic samples. The similar intra- and interday imprecision illustrates the stability of the method.

ANALYSIS OF BLOOD SPECIMEN COLLECTIONS

The range of normal values was determined in a sampling of 113 blood spots from the group of normal neonatal blood spots from Duke University Medical Center (Table 4). The concentration of acetylcarnitine, the most prominent physiologic metabolite, was 22.3 $\text{mmol/L} \pm 13.5$ (mean \pm SD). The minimum concentration observed was 3.5 mmol/L , and the maximum concentration 79.5

Table 1. Assay statistics.

Metabolite	Slope (SE)	Intercept (SE)	r^2 (root MSE)	Detection limit, $\mu\text{mol/L}$
Octanoylcarnitine	0.25 (0.006)	0.074 (0.027)	0.998 (0.05)	0.06
Acetylcarnitine	0.89 (0.002)	1.040 (0.043)	0.998 (0.08)	0.23
Hexanoylcarnitine	0.57 (0.014)	0.14 (0.07)	0.995 (0.15)	0.03
Decanoylcarnitine	0.52 (0.02)	0.15 (0.10)	0.998 (0.22)	0.04

Table 2. Analytical recovery data.

Octanoylcarnitine		Acetylcarnitine		Hexanoylcarnitine		Decanoylcarnitine	
Enr. ^a	% Recovery	Enr.	% Recovery	Enr.	% Recovery	Enr.	% Recovery
0	92 \pm 16	0	89 \pm 5	0	ND	0	ND
0.5	100 \pm 10	2.5	86 \pm 3	2	108 \pm 8	2	90 \pm 14
2.0	119 \pm 6	10	92 \pm 2	10	99 \pm 8	10	103 \pm 8
8.0	126 \pm 7	80	101 \pm 3	20	97 \pm 10	20	108 \pm 5

^a Enrichment, $\mu\text{mol/L}$.

ND, not detected.

Table 3. Imprecision.

Metabolite	Precision (CV, %) ^a		
	Single sample	Intraday	Interday
Acetylcarnitine	5.0	12.9	12.2
Octanoylcarnitine	7.3	8.8	8.9
Hexanoylcarnitine	4.5	8.0	11.8
Decanoylcarnitine	5.7	9.1	6.8

^a *df* = 9.

mmol/L. In these normal blood spots, the concentrations of the other pathologic acylcarnitines (hexanoyl-, octanoyl-, decanoyl-, and decenoylcarnitine) were very low, the median always being below the detection limit. For diagnostic distinction from the increased concentrations observed in blood spots from patients with MCAD deficiency, the upper range of normal must be determined. Table 4 describes the number of samples above the detection limit, and describes the upper ranges observed in normal blood spots as 95th percentile and maximum concentration observed. For hexanoyl-, octanoyl-, and decanoylcarnitine the upper quarter of the normal concentration range was within the sensitivity of the assay, and upper limits as quantified were well below 0.3 $\mu\text{mol/L}$. In no normal blood spot was decenoylcarnitine detected.

Sixteen original newborn blood spots collected <72 h after birth from neonates with MCAD deficiency verified by DNA mutation analysis were retrieved from both Neo Gen Screening and Duke University Medical Center. Ten patients were homozygous for the A985G mutation and six were compound heterozygotes. In these patient samples, all metabolites were well within the dynamic range of the assay, as shown in Table 4. All pathologic acylcarnitines were above the upper values of normal controls, allowing easy diagnostic distinction.

To evaluate the influence of patient age on the concentration of the acylcarnitines in MCAD deficiency, we

analyzed blood spots from 16 older patients outside the neonatal age. Ages ranged from 8 days to 11 years, and eight of these patients were homozygous for the A985G mutation. In these patients, the diagnostic acylcarnitines (hexanoyl-, octanoyl-, decenoyl-, and decanoylcarnitine) were increased but to a lesser degree than observed in the neonatal period ($P < 0.0001$, Mann-Whitney *U*-test) (Table 4). Octanoylcarnitine was still $>0.3 \mu\text{mol/L}$ but the diagnostic distinction more difficult. These samples were analyzed after storage in this laboratory and were not the results of the original analysis. Acetylcarnitine may degrade up to 50%, propionylcarnitine degrades $<50\%$, and palmitoylcarnitine $<10\%$ over 3 years [17].

Previous studies have demonstrated the diagnostic advantage of the ratio of metabolites in the same sample. In MCAD deficiency, previous studies have related the concentration of octanoylcarnitine to acetylcarnitine [11, 18] or to decanoylcarnitine [11]. Table 5 shows the ratios of the molar concentrations of the diagnostic metabolites to acetylcarnitine, and the molar ratio of octanoyl- to decanoylcarnitine. Both the ratios of octanoyl- to acetylcarnitine (C8/C2) and of octanoyl- to decanoylcarnitine (C8/C10) were clearly increased in all patients with MCAD deficiency, regardless of age, when compared with the neonatal control values (Table 5). This distinction was not so pronounced for the metabolite ratios C6/C2 and C10/C2.

Prospective newborn screening of 283 803 infants by Neo Gen Screening in Pennsylvania and Duke University Medical Center in North Carolina showed a disease frequency for MCAD of 1:17 706. Of these, nine of 16 MCAD patients were homozygous for the A985G mutation, the remaining being compound heterozygotes with one copy of this common mutation.

Discussion

MS-MS is an emerging analytical tool for neonatal screening for disorders of amino acid, fatty acid, and organic

Table 4. Acylcarnitines in blood spots collected <72 h after birth, $\mu\text{mol/L}$.

Acylcarnitine	Acetyl	Hexanoyl	Octanoyl	Decenoyl	Decanoyl
DL	0.23	0.03	0.06	0.04	0.04
<i>Normal (n = 113)</i>					
# >DL	113	29	31	0	45
75th Percentile	28.2	0.05	0.07	<DL	0.14
95th Percentile	48.8	0.13	0.14	<DL	0.25
Maximum	79.5	0.21	0.22	<DL	0.30
<i>Neonatal MCAD (n = 16)</i>					
Minimum	4.3	1.5	3.1	0.72	1.0
Median	12.7	1.2	8.4	0.56	0.26
Maximum	87.0	4.0	28.3	1.7	2.6
<i>Older MCAD (n = 16)</i>					
Minimum	0.79	0.13	0.33	0.13	0.11
Median	1.62	0.38	1.57	0.37	0.24
Maximum	6.3	1.2	4.4	1.1	0.49

DL, detection limit.

Table 5. Summary of the relative molar concentrations of acylcarnitines in blood spots.

Ratio	C6/C2	C8/C2	C10:1/C2	C10/C2	C8/C10
<i>Normal (n = 113)</i>					
# >DL	29	31	0	45	26
95th Percentile	0.010	0.013	<DL	0.018	0.95
Maximum	0.015	0.022	<DL	0.036	1.82
<i>Neonatal MCAD (n = 16)</i>					
Minimum	0.020	0.180	0.010	0.010	5.18
Median	0.111	0.599	0.042	0.073	10.1
Maximum	0.350	2.58	0.260	0.320	25.0
<i>Older MCAD (n = 16)</i>					
Minimum	0.04	0.27	0.03	0.04	2.32
Median	0.20	0.64	0.23	0.13	6.56
Maximum	0.36	1.35	0.61	0.27	8.82

acid metabolism. A semiautomated sample preparation, essential for the handling of high sample volumes for neonatal screening, has been developed. In this study, we used a simple semiautomated extraction and derivatization procedure applied in batches of 60. For accurate specific quantification, this method incorporates an isotope dilution technique. Because of its nonchromatographic nature, instrument time of the analysis of 12 amino acids and all acylcarnitines takes <3 min. Further automation can be expected with the use of alternative ionization methods such as electrospray [19], which may become the preferred ionization method for practical newborn screening by MS-MS.

The application of the stable isotope dilution techniques to the analysis of acylcarnitines as their butyl esters from newborn blood spots proved a highly accurate technique, with complete extraction from the filter paper, and an imprecision of <10%. The assay is robust, with intraday and interday imprecision of <15%. Interference due to overlapping components such as a small contribution of glutamate to the *m/z* 260 peak has a generally negligible effect on the accuracy of the quantification for its relevance to diagnostic application. Long-term sample storage leads primarily to the decline in the concentration of short-chain acylcarnitine due to sample degradation, but this is not relevant to the practice of newborn screening [17].

For accurate diagnosis the working range of an assay must cover the diagnostic region. The concentration of the pathologic metabolites hexanoyl-, octanoyl-, decenoyl-, and decanoylcarnitine in samples from healthy controls is very low, and is below the detection limit in approximately two-thirds of the population. However, the assay accurately detects and quantifies the upper third of the concentration range of the healthy population, and the whole range of concentrations found in patients with MCAD deficiency. This allows the development of diagnostic criteria, and the evaluation of their accuracy. In patients with MCAD deficiency all four metabolites are generally increased, with octanoylcarnitine always being well above the upper range of that seen in healthy

controls. The concentrations of the pathologic metabolites are higher in the newborn period than those observed in older patients in a routine diagnostic laboratory setting. This difference is more pronounced than can be explained by the degradation due to sample storage of the retrospectively analyzed samples of older MCAD patients. The progressive development of carnitine deficiency through renal loss represents one possible explanation for the lower values observed in older patients. These higher metabolite concentrations in the newborn period facilitate the detection of MCAD deficiency. Octanoylcarnitine concentrations >0.3 $\mu\text{mol/L}$ and a C8/C10 ratio >2 in patients of all age groups have to be considered as strongly indicative of MCAD deficiency, and is aided by C8/C2 >0.1. These criteria can easily be incorporated in a computer program for assistance in recognizing those patients who require further evaluation for a possible diagnosis of MCAD deficiency.

The higher concentrations of acylcarnitines in the neonatal period allow a higher cutoff. This can be advantageous since small increases can occur in normal heterozygote carriers, particularly during the neonatal period when diagnostic metabolites are more pronounced. Of the 267 303 samples screened at Neo Gen Screening, five samples that had small increases in octanoylcarnitine that were less than the diagnostic cutoff of 0.3 $\mu\text{mol/L}$ were found to have a single copy of A985G mutation for MCAD. No increase in octanoylcarnitine was found upon analysis of a second sample. So far no additional mutations have been recognized. Further study on the octanoylcarnitine concentration of obligate heterozygotes is under investigation. However, using the diagnostic criteria of 0.3 $\mu\text{mol/L}$ for octanoylcarnitine, the false-positive rate for MCAD deficiency is <0.01%.

Application of this technology to prospective newborn screening in a set of 283 803 samples from Pennsylvania and North Carolina revealed a disease frequency of 1:17 706. As previously reported [20], this incidence is considerably higher than estimates based on the gene frequency of the A985G mutation [5]. This is not attributable primarily to high-risk subpopulations such as the

Amish and Mennonites, although MCAD has been reported in these populations in Pennsylvania. This high frequency is also observed in the North Carolina samples. Prospective studies will have to establish to what extent these neonatally identified patients will become similarly symptomatic. If screening was DNA-based, the high heterozygote frequency of A985G mutation (1:84 Caucasians in North Carolina) would require frequent retesting by a second technique such as the analysis of acylcarnitines.

Some patients with MCAD deficiency (up to 7%) present acutely in the neonatal period [2, 21, 22]. These patients have typically presented on the third or the fourth day of life. Rapid diagnostic recognition will be required to timely ascertain these patients. The high concentration of the diagnostic acylcarnitines in the neonatal blood spots of affected patients indicates that early diagnostic recognition will be feasible, provided efficient sample collection and processing is available.

Because of the high incidence, the significant risk for death or serious sequelae, and the ease and efficacy of treatment, this disorder is considered a good candidate for newborn screening [6]. Real-time newborn screening for MCAD deficiency by analysis of acylcarnitines is now technically feasible. Prospective studies will have to establish how efficacious these screening programs will be in the ultimate prevention of mortality and morbidity. This obviously will require appropriate follow-up and treatment in addition to accurate and timely recognition.

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