

Electrospray Tandem Mass Spectrometry for Analysis of Acylcarnitines in Dried Postmortem Blood Specimens Collected at Autopsy from Infants with Unexplained Cause of Death

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Background: Deaths from inherited metabolic disorders may remain undiagnosed after postmortem examination and may be classified as sudden infant death syndrome. Tandem mass spectrometry (MS/MS) may reveal disorders of fatty acid oxidation in deaths of previously unknown cause.

Methods: We obtained filter-paper blood from 7058 infants from United States and Canadian Medical Examiners. Acylcarnitine and amino acid profiles were obtained by MS/MS. Specialized interpretation was used to evaluate profiles for disorders of fatty acid, organic acid, and amino acid metabolism. The analyses of postmortem blood specimens were compared with the analyses of bile specimens, newborn blood specimens, and specimens obtained from older infants at risk for metabolic disorders.

Results: Results on 66 specimens suggested diagnoses of metabolic disorders. The most frequently detected disorders were medium-chain and very-long-chain acyl-CoA dehydrogenase deficiencies (23 and 9 cases, respectively), glutaric acidemia type I and II deficiencies (3 and 8 cases, respectively), carnitine palmitoyl transferase type II/translocase deficiencies (6 cases), severe carnitine deficiency (4 cases), isovaleric acidemia/2-methylbutyryl-CoA dehydrogenase deficiencies (4 cases), and long-chain hydroxyacyl-CoA dehydrogenase/trifunctional protein deficiencies (4 cases).

Conclusions: Postmortem metabolic screening can explain deaths in infants and children and provide estimates of the number of infant deaths attributable to

inborn errors of metabolism. MS/MS is cost-effective for analysis of postmortem specimens and should be considered for routine use by Medical Examiners and pathologists in unexpected/unknown infant and child death.

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It is estimated that up to 3–6% of sudden unexpected infant deaths are attributed to inherited disorders of fatty acid oxidation (FAO)¹ (1–9). A partial list of some of the most common hereditary disorders includes medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (10–15), very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (2), long-chain hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency (16), and systemic carnitine deficiency (2, 3, 17, 18). Currently, deaths attributable to metabolic disorders often are undiagnosed because autopsy findings are inconclusive with only nonspecific findings. Typical autopsy or postmortem investigations of unexplained sudden deaths in infants include clinical and

¹ Nonstandard abbreviations: FAO, fatty acid oxidation; MCAD, medium-chain acyl-CoA dehydrogenase; VLCAD, very-long-chain acyl-CoA dehydrogenase; LCHAD, long-chain hydroxyacyl-CoA dehydrogenase; MS/MS, tandem mass spectrometry; MADD, multiple acyl-CoA dehydrogenase deficiency; SIDS, sudden infant death syndrome; mrm, multiple reaction monitoring mode; FC, free carnitine; TC, total carnitine; SC, short chain; MC, medium chain; LC, long chain; MSUD, maple syrup urine disease; CPT II, carnitine palmitoyl transferase type II; TFP, trifunctional protein; GA-I, glutaric acidemia type I; IVA, isovaleric acidemia; PA, propionic acidemia; MMA, methylmalonic acidemia; C2, acetylcarnitine; C3, propionylcarnitine; C4, butyrylcarnitine; C5, isovalerylcarnitine; C6, hexanoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C12, dodecanoylcarnitine; C14, myristoylcarnitine; C16, palmitoylcarnitine; C18, octadecanoylcarnitine; C10:1, decenoylcarnitine; C14:1, tetradecenoylcarnitine; C16:1, hexadecenoylcarnitine; C18:1, octadecenoylcarnitine; C4OH, 3-OH-butyrylcarnitine; C16OH, 3-OH-hexadecenoylcarnitine; C18:1OH, 3-OH-octadecenoylcarnitine; C18OH, 3-OH-octadecenoylcarnitine; and C5DC, glutaryl carnitine.

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forensic information, such as reports from pathology, toxicology, microbiology, site investigations as to manner of death, and family history. Before 1996, only a small number of infant and child deaths were investigated for a metabolic disorder. This was in part a result of poor access to postmortem screening for inborn errors of metabolism, the high cost of these services from highly specialized laboratories, and limited awareness of new technology such as tandem mass spectrometry (MS/MS) for diagnosing potential disorders in FAO.

Pathological findings that reveal a fatty liver or cardiomyopathy (5) suggest the possibility that death was caused by an underlying metabolic disturbance. Biochemical analysis is required, however, to provide additional information in support of this hypothesis. Biochemical tests will often indicate an atypical blood chemistry or metabolic profile for a variety of inherited metabolic disorders. Negative biochemical findings rule out only those disorders that are detected by specific biochemical tests or metabolic disorders that present with only mild aberrations in the metabolic pattern of the specimen tested. Often, extensive biochemical testing is not performed if pathological findings are negative, essentially ruling out the possibility of finding an underlying metabolic disease. In other cases, the presence of factors such as infection may mask the true cause of death. Hence, deaths that appear to be attributable to pneumonia, flu, or other infection may, in fact, have been attributable to an underlying metabolic disease. In the same manner that toxicology is ordered in most suspicious deaths (because no pathological evidence is available in many poisonings), biochemical tests can be considered in cases of infant death of unknown or uncertain cause.

Emerging biochemical tests that can be performed on postmortem specimens primarily use MS/MS to quantify acylcarnitines and acylglycines in blood, urine, and bile (1–3, 9, 12, 19–27). For each of these studies, the diagnosis of a metabolic disorder requires complex interpretation and specialized protocols (3, 28–30). Until recently, these biochemical tests were not ordered as routinely as toxicology tests are ordered after an infant death of unknown cause. Advances in technology, such as electrospray MS/MS analysis, could facilitate routine postmortem screening for inborn errors of metabolism that may be the underlying cause of death in some infants and children.

Studies have suggested that infants born with MCAD deficiency have a 33% statistical chance of premature death (14). If we assume that many other disorders, such as VLCAD, multiple acyl-CoA dehydrogenase deficiency (MADD), or LCHAD, share these similar statistics (some disorders may be more lethal, whereas others are less so), we can estimate the number of deaths per year attributable to metabolic disorders detectable by MS/MS methods. The known frequency of these metabolic disorders, based on a screening program at Neo Gen Screening involving 1 million infants, is 1:4500. Using statistics from the CDC (31) that 3 880 894 infants were born in the US in

1997, we can approximate that 465 infants will be born per year with a metabolic disorder detectable by MS/MS. This produces an estimate that one-third of these infants, or more specifically, 155 infants, will die from one of these metabolic disorders. Additional statistics provided by the CDC in 1997 (31) showed an infant mortality rate of 7.2 deaths per 1000 live births. Furthermore, ~0.77 births per 1000 (2996 deaths) were categorized as sudden infant death syndrome (SIDS). Therefore, the percentage of unknown deaths that were a result of a FAO defect or an organic acidemia (detectable by MS/MS) can be estimated at 5% among the SIDS population or 0.55% within the population of total infant deaths. These numbers support findings provided by other investigators (2, 3).

We have developed a method to screen for inborn errors of fatty acid and organic acid metabolism that uses small amounts of blood routinely obtained at autopsy, applied to filter paper, and sent to our laboratory, where MS/MS is used to obtain a metabolic profile similar to that obtained routinely for neonatal blood specimens. The technique is inexpensive when coupled with high-volume automated analyses for newborn or high-risk screening. It can be easily adapted to analyze other biological fluids, including bile, vitreous fluid, and urine. We provide details of our method as well as results from the analysis of >7000 postmortem blood specimens from infants who died predominantly of unknown causes. In addition, we compare the postmortem analysis of dried blood specimens with bile specimens and provide reference data for newborn and high-risk blood specimens.

Materials and Methods

SOLVENTS, REAGENTS, AND INTERNAL STANDARDS

HPLC-grade solvents, methanol, and acetonitrile were obtained from Fisher Scientific. Butanolic HCl (3 mol/L) was obtained from Bodman Industries. Two sets of dried mixtures of stable isotopes were obtained from Cambridge Isotopes. Set A contained the following L-amino acids: [¹⁵N,²⁻¹³C]glycine, [²H₄]alanine, [²H₈]valine, [²H₃]leucine, [²H₃]methionine, [²H₅]phenylalanine, [²H₄]tyrosine or [¹³C₅]tyrosine, [²H₃]aspartate, [²H₃]glutamate, [²H₂]ornithine-2 HCl, [²H₂]citrulline, and [²H₅,¹³C]arginine-HCl. Set B contained the following L-carnitines and L-acylcarnitines: [²H₉]carnitine, [²H₃]acetylcarnitine (²H₃-C2), [²H₃]propionylcarnitine (²H₃-C3), [²H₃]butyrylcarnitine (²H₃-C4),² [²H₉]isovalerylcarnitine (²H₉-C5),² [²H₃]octanoylcarnitine (²H₃-C8), [²H₉]myristoylcarnitine (²H₉-C14), and [²H₃]palmitoylcarnitine (²H₃-C16).

² C4 and C5 are generalized notations for a 4- or 5-carbon fatty acylcarnitine. Although identified as the common forms butyrylcarnitine and isovalerylcarnitine, these notations may also represent isomers such as methylbutyrylcarnitine (also C5). Other acylcarnitines may also have isomeric forms not unlike the amino acids Leu and Ile, where many authors represent that mass simply as Leu.

BLOOD AND BILE SPECIMENS

All specimens used in this study were received by this laboratory between May 1, 1996, and November 30, 2000. They are characterized as follows: 7058 dried postmortem blood specimens prepared by applying 25 μL of blood, collected at autopsy, to S&S Grade 903 filter paper (Schleicher and Schuell); 1195 dried blood specimens from healthy newborns obtained from routine newborn screening at Neo Gen Screening; 263 dried blood spots from older infants (age >30 days) with negative findings for metabolic disorders; and 30 dried postmortem bile specimens prepared by applying 25 μL of bile in the same manner as blood specimens. Postmortem blood specimens were received from Medical Examiners and coroners from throughout the United States and Canada. Blood specimens from healthy newborns obtained from Neo Gen Screening's supplemental and comprehensive screening programs primarily included babies born in Pennsylvania (~75%).

SAMPLE PREPARATION

The following method of specimen preparation contained substantial modifications from original articles published previously (23, 32). A single 0.48-cm (3/16-inch) disk was punched from dried blood and dried bile specimens into polystyrene flat-bottomed 96-well microtiter plates (Evergreen Scientific). The volume of blood contained in a dot was ~7.6 μL , whereas the volume of a dried bile specimen was estimated at 4.2 μL (33). Stock solutions of internal standards were prepared by dilution of amino acid Set A and acylcarnitine Set B with 1 mL of 1:1 (by volume) methanol-water or absolute methanol, respectively. These solutions were mixed 1:1 and further diluted with methanol to produce the working calibration solution of amino acids and acylcarnitines in the following concentrations: 8.33 $\mu\text{mol/L}$ [^{15}N , ^{13}C]glycine; 1.67 $\mu\text{mol/L}$ each of [$^2\text{H}_4$]alanine, [$^2\text{H}_8$]valine, [$^2\text{H}_3$]leucine, [$^2\text{H}_3$]methionine, [$^2\text{H}_5$]phenylalanine, [$^2\text{H}_4$]tyrosine or [$^{13}\text{C}_5$]tyrosine, [$^2\text{H}_3$]aspartate, [$^2\text{H}_3$]glutamate, [$^2\text{H}_2$]ornithine-2 HCl, [$^2\text{H}_2$]citrulline, and [$^2\text{H}_5$, ^{13}C]arginine-HCl; 0.506 $\mu\text{mol/L}$ [$^2\text{H}_9$]carnitine; 0.126 $\mu\text{mol/L}$ $^2\text{H}_3$ -C2; 0.025 $\mu\text{mol/L}$ each of $^2\text{H}_3$ -C3, $^2\text{H}_3$ -C4, $^2\text{H}_9$ -C5, $^2\text{H}_3$ -C8, and $^2\text{H}_9$ -C14; and 0.05 $\mu\text{mol/L}$ [$^2\text{H}_3$]hexanoylcarnitine ($^2\text{H}_3$ -C6). These stock solutions were placed in 10 \times 75-mm test tube reservoirs positioned in a no. 217 rack as part of the Gilson Model 215 Liquid Handler with an eight-syringe configuration. Microtiter plates were placed on a no. 505H rack, which accommodates 10 shallow-well microtiter plates.

We added 300 μL of internal working solution to each well, using the Liquid Handler. Plates were placed on an orbital shaker for 30 min and returned to the Liquid Handler, where the extract was transferred to a clean round-bottomed microtiter plate (Evergreen Scientific). Solvent was removed by a gentle stream of nitrogen in a Speed Dry 96 or a EvapArray nitrogen evaporator (Jones Chromatography). Derivatization reagent (3 mol/L HCl in *n*-butanol; 50 μL) was added to each well with an

Eppendorf pipette rather than a stainless steel syringe to prevent corrosion. Specimens were covered with a polystyrene plate cover and heated to 65 $^\circ\text{C}$ for exactly 15 min. Specimens were promptly dried in the nitrogen evaporators to remove the volatile derivatization reagent. One hundred microliters of a 1:1 (by volume) solution of acetonitrile-deionized water containing 0.2 mL/L formic acid was added to each microtiter well. Microtiter plates were sealed with a 96-well Micromat plate cover (Sun Brokers) before MS/MS analysis.

MS

The tandem mass spectrometers (Applied Biosystems/MDS Sciex Models API 300, 365, and 3000) were equipped with standard ion spray sources. A Gilson Model 215 (single syringe) or a Perkin-Elmer Series 200 LC autosampler was equipped with a Model 7010 or a Model 7725 Rheodyne injector fitted with 10- μL loops. Sample were injected at 2-min intervals in a flowing stream of 1:1 (by volume) acetonitrile-water containing 0.2 mL/L formic acid. Perkin-Elmer Series 200 LC or Shimadzu Model LC10AD liquid pumps were used to deliver a constant 18 or 20 $\mu\text{L}/\text{min}$ of mobile phase into the electrospray ion sources. Nitrogen was used as the collision gas, and compressed zero-grade air was used as the nebulization gas. MS/MS analysis was optimized by infusion of a tuning solution containing isotopically labeled internal standards using a Harvard Syringe pump Model 11 (Harvard Apparatus).

Each MS/MS analysis comprised five separate methods for data acquisition, called scan functions. Scan functions defined the mass range of the analysis, type of MS/MS analysis, and tuning parameters or state files. Each function acquired data for a defined period followed by the next scan function in series. One complete scan was defined as the sum of the acquisition periods for each sequential scan function. With the API 300 or 365 tandem mass spectrometer, 6 complete scans were acquired during data acquisition, with data acquisition lasting 1.33 min, whereas 14 complete scans were acquired with the API 3000, with data acquisition lasting 1.5 min. The scan functions used in series were as follows: (a) multiple reaction monitoring mode (mrm) scan of free carnitine (FC) using precursors of 103 Da (Pre 103), (b) mrm scan of C2 using precursors of 85 Da (Pre 85); (c) full scan of the remaining acylcarnitines using Pre 85; (d) full scan of amino acids using neutral loss of 102 (NL 102); and (e) mrm scan of basic amino acids using neutral loss of 119 (NL 119) for citrulline, homocitrulline, and ornithine or neutral loss of 161 (NL 161) for arginine.

Acquired data were processed on a power MAC G3 system using Neonatal Script (Ver. 1.0G; Applied Biosystems/MDS-Sciex). The desired masses for quantification were entered in this program as well as important ion ratios and conversion factors used to calculate concentrations. A mass spectrum produced from each abnormal scan function was printed together with concentration

data. In addition, all data from a complete series of analyses were converted to a format compatible with PC-based software, such as Microsoft Excel, where additional detailed data analysis was obtained. Methods for quantification of acylcarnitines and amino acids have been described previously (23, 32).

RESULT PROCESSING

Isomeric acylcarnitines. Several isomeric forms of acylcarnitines cannot be differentiated on the basis of mass alone, as is the case with Leu and Ile described previously (34). Isomeric forms exist for both C4 and C5 acylcarnitines. For example, methylbutyryl and isovaleryl carnitines, both 5-carbon fatty acylcarnitines, have the same mass and are conveniently labeled C5. Therefore, an increase in a particular metabolite, e.g., C5, may be indicative of 2-methylbutyryl-CoA dehydrogenase deficiency or isovaleric acidemia, depending on whether the metabolite measured is primarily methylbutyrylcarnitine or isovaleryl carnitine. Additional investigation requires the use of chromatographic techniques (such as gas chromatography-mass spectrometry of organic acids) to differentiate these metabolites and hence the likely metabolic disorders present. However, for simplicity, the notation C4 and C5 will be used throughout this report to identify these 4- and 5-carbon fatty acylcarnitines isomers, noting of course that they represent isomeric mixtures.

Concentration calculations. Concentrations were obtained from the ratio of ion intensity (cps) at the mass that represented a specific acylcarnitine, e.g., m/z 344 for C8, to that for its isotopically labeled internal standard, e.g., m/z 347 for $^2\text{H}_3$ -C8. Subsequent corrections were applied to adjust for blood volume, extraction efficiency, hydrolysis, and other interferences as described previously (23, 35). For acylcarnitines that did not have an isomeric internal standard, the closest internal standard of similar mass and structure was used. For compounds that had no structurally similar standards, e.g., dicarboxylic acid acylcarnitines or hydroxyacylcarnitines, the internal standard of nearest mass was used. In addition, an assumption was made that equimolar mixtures of these metabolites and the internal standards have a response factor of 1:1. These procedures have been described previously (23).

Metabolite ratios. Molar ratios were obtained by calculating the concentration of one metabolite relative to another, e.g., the ratio of the concentration of octanoylcarnitine to decanoylcarnitine is denoted C8/C10. Other, more complex molar relationships are defined below. The acyl/free ratio was defined as the sum of the concentration of all measured acylcarnitines divided by the concentration of FC, whereas total carnitine (TC) was defined as the concentration of all acylcarnitines measured plus FC. The short chain (SC) index is the sum of the concentrations of C2, C3, and C4 divided by TC. Similarly, the medium

chain (MC) index is the sum of the concentrations of C6, C8, decanoylcarnitine (C10:1), and C10 divided by TC, whereas the long chain (LC) index is the sum of the concentrations of tetradecenoylcarnitine (C14:1), C14, hexadecenoylcarnitine (C16:1), C16, octadecenoylcarnitine (C18:1), and octadecenoylcarnitine (C18) divided by TC. The sum of the long-chain hydroxyacylcarnitines is denoted as total hydroxy (total OH) and includes 3-OH-myristoylcarnitine, 3-OH-hexadecenoylcarnitine (C16OH), 3-OH-octadecenoylcarnitine (C18:1OH), and 3-OH-octadecenoylcarnitine (C18OH).

Results

ANALYSIS OF CONTROL SPECIMENS

Tandem mass spectra of acylcarnitines obtained during typical analyses of dried postmortem blood and bile specimens are shown in Fig. 1, A and B, respectively. The complete profile for these specimens included four additional spectra, free carnitine, C2, neutral and acidic amino acids, and basic amino acids. Tandem mass spectra from postmortem blood specimens are predominantly characterized by increased free carnitine and short-chain acylcarnitines, e.g., C2, C3, C4, and C6, as well as hydroxybutyryl- (C4OH) carnitines. By comparison, the tandem mass spectra of control postmortem bile specimens are characterized by high medium- and long-chain acylcarnitines, i.e., C10:1, C10, dodecenoylcarnitine (C12:1), dodecanoylcarnitine (C12), C14:1, and C14 and relatively low short-chain acylcarnitines. A quantitative summary of the mean concentrations of carnitine and selected acylcarnitines are provided in Table 1.

In nearly all postmortem blood specimens, amino acid profiles were characterized by nonspecific, generalized large increases of most amino acids as observed in the control profile shown in Fig. 2A. It was thought that these profiles would be generally nondiagnostic for amino acidopathies, but as observed in one postmortem specimen, an extremely high Leu concentration relative to other amino acids was observed, as shown in Fig. 2B. This specimen was diagnosed as maple syrup urine disease (MSUD).

Acquisition of the amino acid profiles is, on the other hand, extremely helpful in the investigation of sample integrity. In addition to the potential to diagnose some amino acid disorders, the amino acid profile provides important information with regard to specimen integrity. For example, an MS/MS analysis of a postmortem specimen that contains amino acid concentrations at concentrations characteristic of healthy newborns may indicate that the specimen is not from an autopsy, that insufficient blood or bile sample was applied to the filter paper, or that there were other problems with the specimen. Information provided by Medical Examiners is not always complete, and therefore, information derived from specimen characteristics (amino acid concentrations near the expected values) is invaluable in the interpretation.

Separate acquisition of FC enables a more sensitive and

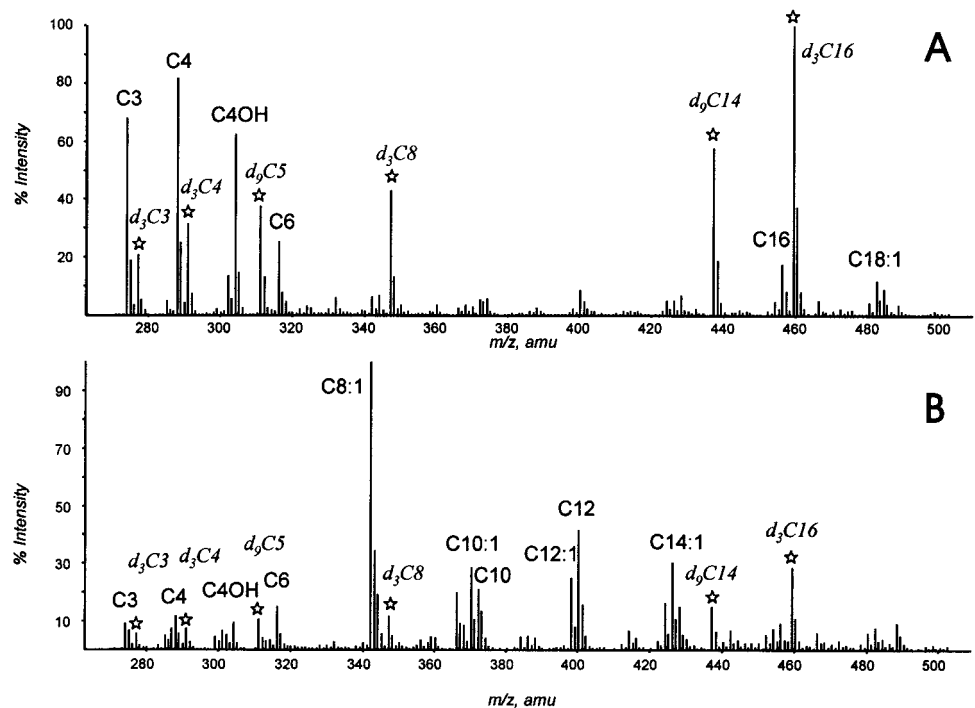


Fig. 1. Acylcarnitine profiles of a dried blood specimen (A) and a bile specimen (B) obtained at autopsy from a deceased infant for whom no cause of death was determined.

These MS/MS spectra were obtained using the Pre 85 scan functions appropriate for acylcarnitine butyl ester analyses. The stars and *italic font* denote stable-isotope internal standards.

reliable scan function, e.g., Pre 103 rather than Pre 85 as has been described previously (23). In a similar manner, separate acquisition of C2 enables optimal tuning (the tuning of C2 is quite different from other acylcarnitines) of this short-chain acylcarnitine. Furthermore, visualization of the acylcarnitine profiles is facilitated by acquiring C2 in a separate spectrum because with all MS/MS spectra, the largest peak serves to normalize the remaining spectra. Quantitatively, however, there is no advantage to separating acylcarnitines into C2 and C3 to C18 ranges.

Analyses of filter-paper bile profiles often show a markedly increased peak at m/z 342. This mass would be

representative of octenoylcarnitine (C8:1). However, this identification (m/z 342 is C8:1) is questionable in part because its relative increase is not observed in a dried blood specimen from the same patient and the increase of C8:1 acylcarnitine by itself has not been linked to a specific metabolic disorder. It is possible that this peak is produced by a component in bile that is present at very high concentrations and has a minor fragment ion at m/z 85. Additional research is required for identification of the possible components that are detectable at this mass. It has been observed that C8:1 often is mildly increased in cases of MADD. Additional research in this area is indicated. It is worth noting, however, that identification of

Table 1. Comparison of postmortem blood and bile specimens with newborn and high-risk blood specimens.

	Median (range)			
	Postmortem blood (n = 855)	Postmortem bile (n = 30)	Newborn blood (n = 1195)	High-risk blood (n = 263)
FC, $\mu\text{mol/L}$	162 (23–729)	249 (42–1728)	53 (18–271)	29 (8.6–70)
TC, $\mu\text{mol/L}$	216 (39–895)	370 (72–1916)	79 (29–312)	44 (15–108)
Acyl/Free	0.29 (0.07–1.6)	0.32 (0.11–1.1)	0.47 (0.12–1)	0.51 (0.21–1.74)
SC index	21 (6.1–78)	16.8 (5.5–44)	24 (6.6–40)	28 (12–56)
MC index	0.52 (0.07–2.1)	2.4 (0.7–7.8)	0.47 (0.1–1.8)	0.57 (0.21–2)
Very-long-chain index	0.96 (0.13–9.2)	2.3 (0.63–23)	7.6 (1.7–15)	5.1 (1.4–17)
C2, $\mu\text{mol/L}$	28 (3.5–134)	29 (12–104)	14 (3–42)	8.9 (2.8–33)
C3, $\mu\text{mol/L}$	2.9 (0.11–12)	3.4 (1–9.8)	1.5 (0.21–4.7)	1 (0.2–4.3)
C4, $\mu\text{mol/L}$	4 (0.14–33)	4.1 (1.5–7.6)	0.23 (0.05–1)	0.18 (0.06–0.72)
C4OH, $\mu\text{mol/L}$	2.4 (0.07–43)	2.2 (0.65–5.3)	0.2 (0.04–0.9)	0.11 (0.02–0.5)
C5, $\mu\text{mol/L}$	0.52 (0.03–5.5)	1.5 (0.5–6.8)	0.17 (0.04–0.61)	0.12 (0.03–0.67)
C8, $\mu\text{mol/L}$	0.21 (0.02–1.03)	2.7 (0.47–24)	0.08 (0.01–0.36)	0.06 (0.01–0.2)
C14, $\mu\text{mol/L}$	0.16 (0.02–1.72)	0.96 (0.16–32)	0.41 (0.08–1.1)	0.1 (0.01–0.39)
C16, $\mu\text{mol/L}$	0.67 (0.05–8.5)	1.3 (0.47–10)	3.4 (0.25–9.7)	0.84 (0.23–6.1)

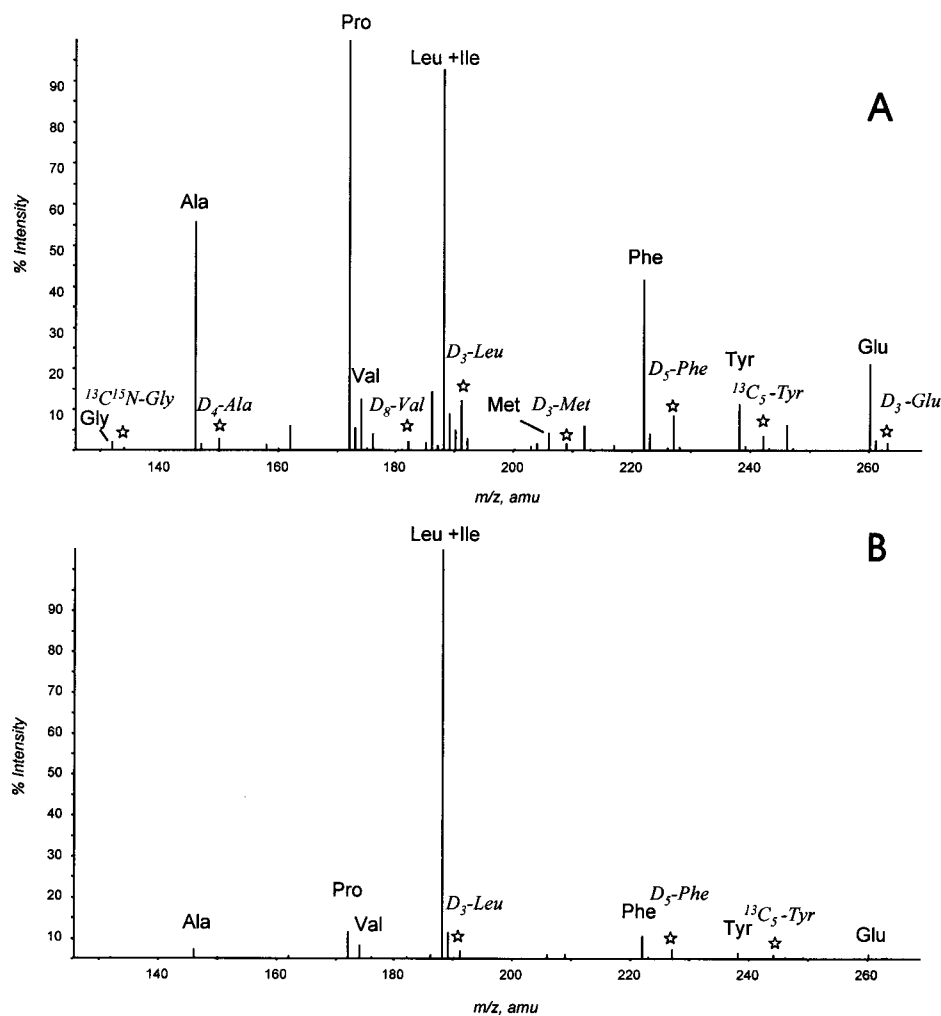


Fig. 2. Amino acid profiles of dried blood specimens from a control post-mortem specimen (A) and a specimen of a deceased infant presumptive for MSUD (B).

These MS/MS spectra were obtained using the NL 85 scan functions appropriate for amino acid butyl ester analyses. The stars and *italic font* denote stable-isotope internal standards.

very large increases in C8:1 is useful as a reference point in bile acylcarnitine profile interpretation.

A comparison of blood and bile postmortem specimens demonstrates substantially higher concentrations of FC and TC in bile compared with blood. This is demonstrated by the quantitative data in Table 1. Medium- and long-chain acylcarnitines are routinely 3- to 20-fold higher in postmortem bile compared with blood. However, some acylcarnitines, such as short-chain acylcarnitines, are comparable in blood and bile. These results compare well with data from other investigators reporting on MS/MS acylcarnitine analyses of bile specimens (3, 28). Proportionately, the relative concentration of short-chain acylcarnitines to medium- and long-chain acylcarnitines is higher in blood than in bile. This may be attributable to the role of bile in the elimination of longer chain acylcarnitines.

COMPARISON WITH NEWBORN AND OTHER SCREENING SPECIMENS

Important information regarding interpretation of a post-mortem specimen can be obtained by comparison and

reference to control data for newborn and clinical (high-risk) blood specimens. This is a simplified way to age match reference ranges into just two groups: newborn specimens (age, birth through 7 days) and high-risk individuals (age, 7 days through adult). The data presented in Table 1 demonstrate the characteristic differences between these two age groups. These differences may assist in the interpretation of postmortem specimens in many cases.

Acylcarnitine profiles of dried blood specimens from a healthy newborn and a healthy older infant are shown in Fig. 3, A and B, respectively. The newborn acylcarnitine profile (Fig. 3A) is characterized by the presence of relatively moderate concentrations of long- and short-chain acylcarnitines, whereas the medium-chain acylcarnitines are at low concentrations. In older infants (the high-risk group), a representative acylcarnitine profile from a blood specimen (Fig. 3B) shows substantially reduced long-chain acylcarnitines, moderate short-chain acylcarnitines, and very low medium-chain acylcarnitines. Clearly, the medium- and long-chain acylcarnitines, relative to other acylcarnitines, are reduced in the

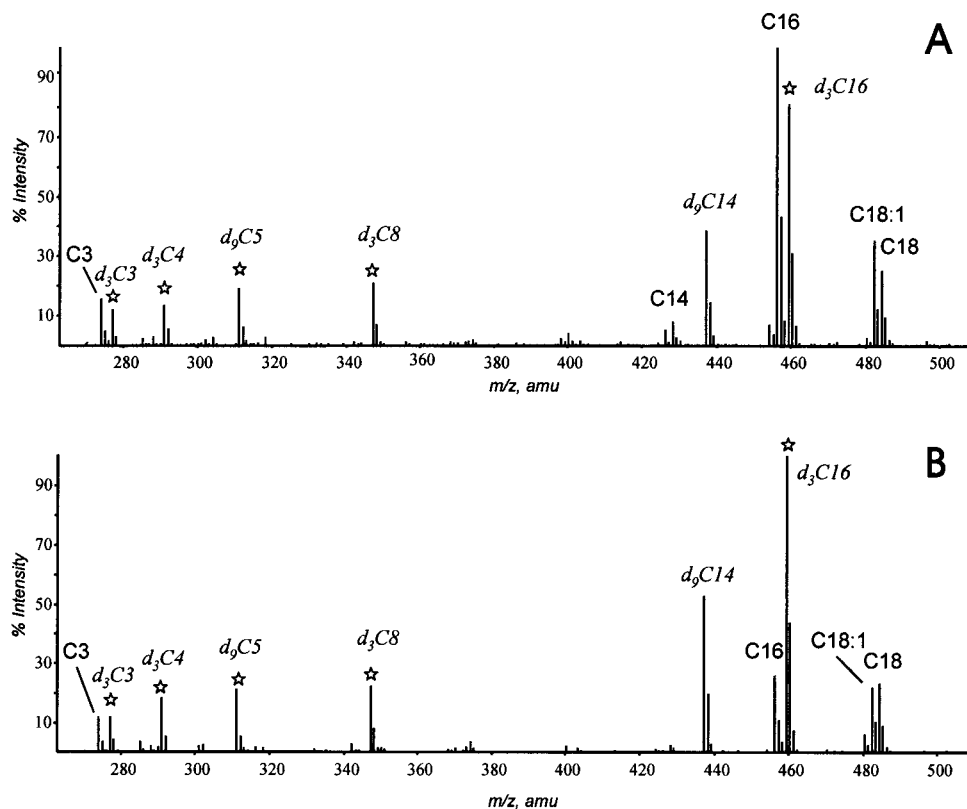


Fig. 3. Acylcarnitine profiles of dried blood specimens from a healthy newborn (A) and an older healthy infant (B).

These MS/MS spectra were obtained using the Pre 85 scan functions appropriate for acylcarnitine butyl ester analyses. The stars and *italic font* denote stable-isotope internal standards.

specimen obtained from older children compared with newborns.

A quantitative summary of the mean concentration of carnitine and several acylcarnitines for newborn and high-risk screening groups is presented in Table 1. As seen in Table 1, the mean concentrations of FC and TC in the high-risk population were reduced in comparison with the newborn population. Relative to postmortem specimens, both newborn and high-risk specimens had substantially lower FC and TC. With regard to the SC and MC indices, it is interesting to find that they were comparatively similar among newborn, high-risk, and postmortem blood specimens (Table 1), although individual acylcarnitine concentrations were higher in postmortem blood. For the LC index, postmortem blood specimens showed a reduced value compared with high-risk and newborn blood specimens. The greatest acyl/free ratio was found in newborn and high-risk blood specimens compared with postmortem blood specimens.

ANALYSIS OF PRESUMPTIVE METABOLIC DISORDERS OF FAO

MCAD deficiency. The MS/MS acylcarnitine profile of a postmortem blood specimen for an infant who died from MCAD deficiency at 4 days of age is shown in Fig. 4A. This infant's original newborn blood specimen, collected on the 2nd day of life, is shown in Fig. 4B. The classical group of abnormal metabolites (C6, C8, C10, and C10:1) for MCAD deficiency were observed in both the newborn

and postmortem blood profiles. Molecular analysis revealed one copy of the common A985G mutation. Additional studies found another mutation (currently being characterized), indicating that this infant was a compound heterozygote for MCAD deficiency. An MCAD profile from an infant who died at 8 months of age and was homozygous for the A985G mutation is shown in Fig. 4C for comparison. In this older infant, the characteristic medium-chain acylcarnitines were observed.

A total of 23 infant deaths were attributed to MCAD deficiency in our postmortem screening program. The primary cause of death was marked as SIDS in most cases for which this information was available. All MCAD infant deaths were of Caucasian race and equally divided among males ($n = 10$) and females ($n = 9$). Sixteen of these specimens were found to be homozygous for the A985G mutation, 5 were compound heterozygotes, and 2 were inconclusive. Three infants died within the newborn period at <7 days of age. Infants who died of MCAD deficiency did not show a preponderance in any one state. Infant deaths were found in Oregon, Pennsylvania, Texas, Minnesota, Virginia, Missouri, Georgia, Ohio, Illinois, Alabama, Michigan, Maryland, New York, and Kentucky.

The median concentration for the important diagnostic acylcarnitines in MCAD deficiency is provided in Table 2. A comparison of results for MCAD-affected deceased infants with controls (Table 1) showed that the median concentrations of C8 and the MC index were clearly increased. The C8/C10 ratio was useful in differentiating

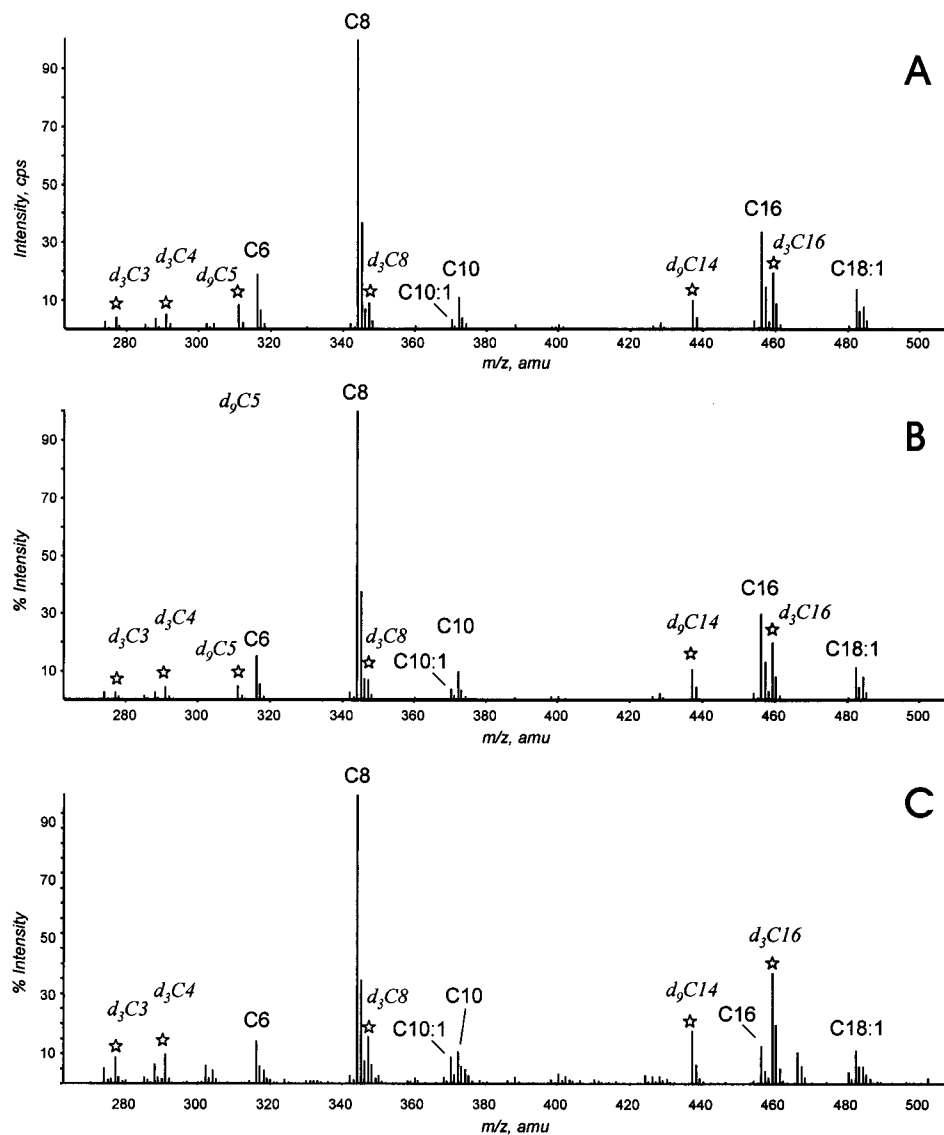


Fig. 4. Acylcarnitine profiles of dried blood specimens from an infant diagnosed with MCAD deficiency.

(A), MS/MS spectrum (Pre 85) obtained from postmortem blood profile. (B), MS/MS spectrum obtained from the original newborn screening specimen. (C), MS/MS spectrum obtained from another infant who died at age 8 months. The stars and *italic font* denote stable-isotope internal standards.

postmortem specimens from infants with MCAD deficiency from specimens from infants with other metabolic disorders. For example, the median C8/C10 ratio was 11 for MCAD-confirmed postmortem specimens. This value was greater than or equal to median values reported previously for plasma (20) and newborn blood specimens (23). Both the C8/C10 ratio and molecular analyses helped to limit false-positive results for MCAD because the median concentration of C8 was approximately three times higher in postmortem MCAD specimens than in controls. The median FC and TC concentrations were lower in MCAD-deficient specimens relative to control postmortem specimens. Further investigation of unknown mutations is currently underway. It is also worth noting that specimen demographics may reflect more active Medical Examiner screening programs rather than frequencies of disorders in those states for which an MCAD deficiency case was found or not found.

VLCAD deficiency. An acylcarnitine profile of a postmortem blood specimen obtained from an infant who died of VLCAD deficiency is shown in Fig. 5, and the profile of a postmortem bile specimen collected from this infant is shown in Fig. 5B. In both acylcarnitine profiles, a substantial increase in long-chain acylcarnitines (C14:1, C14, C16:1, C16, C18:1, and C18) was observed. These metabolites are those traditionally found to be increased in identified high-risk and newborn screening specimens from patients with VLCAD deficiency. Comparison of blood and bile acylcarnitines showed a similar metabolic profile but a substantially higher concentration of these acylcarnitines in bile (nearly 10-fold higher). The observation of high concentrations of long-chain acylcarnitines in bile has been reported previously (3, 28, 36).

Nine infant deaths were presumptive for VLCAD deficiency. All VLCAD infant deaths were of Caucasian race with five cases occurring in males. Mutation analysis

Table 2. Comparison of presumptive FAO and organic acid disorders in postmortem blood specimens collected on filter paper.

	Median (range)									
	MCAD (n = 13)	VLCAD (n = 7)	CPT II/Translocase (n = 4)	MADD (n = 6)	GA-1 (n = 3)	CN Def ^a (n = 4)	LCHAD/TFP (n = 3)	IVA (n = 3)		
FC, $\mu\text{mol/L}$	63 (26-249)	61 (35-250)	76 (57-152)	150 (59-283)	150 (97-270)	5.7 (3-9.7)	71 (6.1-116)	128 (12-140)		
TC, $\mu\text{mol/L}$	89 (38-295)	95 (46-295)	117 (76-204)	235 (76-441)	187 (112-317)	9.1 (3.9-13)	95 (9.2-136)	165 (41-170)		
Acyl/Free	0.24 (0.11-0.64)	0.34 (0.16-1.6)	0.4 (0.34-0.62)	0.55 (0.22-0.58)	0.18 (0.15-0.24)	0.43 (0.27-0.7)	0.33 (0.18-0.5)	0.29 (0.21-2.34)		
SC index	18 (7.7-27)	9 (6.1-19)	13 (9.5-29)	28 (13-35)	15 (12-19)	35 (16-118)	18 (3.7-3)	24 (12-30)		
MC index	4.6 (1.4-15)	0.35 (0.14-0.9)	0.39 (0.27-0.71)	0.92 (0.57-1.2)	0.32 (0.13-0.35)	2.2 (1.4-2.4)	0.87 (0.24-0.99)	0.37 (0.29-1.5)		
LC index	2.6 (0.98-6.2)	1.1 (5.3-43)	13 (9.2-16)	4 (1.7-6)	0.99 (0.59-1)	2.7 (2-3.6)	8 (4.2-11)	1.8 (0.97-9.4)		
C8, $\mu\text{mol/L}$	2.9 (1.1-12)	0.08 (0.02-0.14)	0.14 (0.04-0.24)	0.43 (0.13-1.1)	0.08 (0.05-0.12)	0.06 (0.01-0.07)	0.07 (0.01-0.47)	0.14 (0.12-0.2)		
C8/C10	11 (5.4-29)	0.67 (0.18-0.88)	0.59 (0.4-0.77)	0.63 (0.41-1.2)	1.6 (0.83-2.4)	1.1 (0.47-1.4)	0.58 (0.33-6.71)	1.1 (0.87-1.7)		
C14:1, $\mu\text{mol/L}$	0.06 (0.03-0.49)	1.1 (0.4-10)	0.27 (0.12-0.44)	0.51 (0.12-2.5)	0.1 (0.04-0.2)	0.02 (0-0.02)	0.26 (0.01-1.39)	0.12 (0.03-0.18)		
C14, $\mu\text{mol/L}$	0.1 (0.07-0.79)	2.4 (0.38-12.6)	1 (0.6-1.7)	0.83 (0.48-2.5)	0.1 (0.09-0.23)	0.03 (0.01-0.06)	0.41 (0.14-1.33)	0.13 (0.12-0.3)		
C16, $\mu\text{mol/L}$	0.49 (0.23-5.8)	7.7 (1.3-13)	9.6 (6.4-13.6)	1.6 (1.2-4.4)	0.79 (0.17-1.2)	0.07 (0.04-0.14)	1.4 (0.35-4.8)	1.4 (0.54-2.2)		
C16/C14:1	10 (3.8-77)	5.7 (1-27)	46 (17-79)	2.9 (1.3-14)	5.9 (1.7-20)	6 (2-38)	5.2 (3.4-35)	12 (4.5-47)		
C14:1/C12:1	1.8 (1-4)	16 (7.8-24)	2.2 (2-3.4)	3.2 (1.3-4.4)	4 (2-5)	1.5 (0.33-5)	3.7 (1-6.6)	3.6 (1.5-4)		
C5DC, $\mu\text{mol/L}$	0.09 (0.02-0.26)	0.07 (0-0.19)	0.17 (0.03-0.21)	0.15 (0.09-0.28)	0.24 (0.23-1.7)	0.02 (0.01-0.03)	0.04 (0.02-0.1)	0.08 (0.07-0.16)		
Total OH, $\mu\text{mol/L}$	0.11 (0.03-0.33)	0.31 (0.07-0.76)	0.31 (0.1-0.34)	0.26 (0.12-0.94)	0.07 (0.06-0.13)	0.02 (0.01-0.02)	1.7 (0.69-1.8)	0.07 (0.07-0.19)		

^a CN Def, carnitine deficiency.

was not available within our laboratory. Four infants died within the newborn period at <7 days of age. In several cases, a postmortem bile specimen, an original newborn specimen, or both were obtained for additional confirmation of this disease. Results from these analyses provided additional support that our presumptive diagnosis for VLCAD deficiency was correct. Most cases were categorized as SIDS. In two cases, significant fatty changes in the liver were noted in two siblings, one of whom died at 3 days of age, whereas the other died at 3 months. Information on other cases was sparse and was not accessible. We therefore developed and relied on key indices and substantially increased metabolite concentrations. In Table 2, the median concentrations of C14 and C16, and the LC index were clearly increased relative to control values (Table 1). Traditionally, the most reliable indicator of VLCAD deficiency, the acylcarnitine C14:1, had a median concentration of 1.1 $\mu\text{mol/L}$, 10-fold higher than that of control postmortem blood specimens (0.1 $\mu\text{mol/L}$). To differentiate VLCAD deficiency from other long-chain metabolic disorders, a ratio of C14:1 to C12:1 was applied in the interpretation. The median for the ratio of C14:1 to C12:1 in VLCAD deficiency was 16, which is substantially higher than in infants suspected of other disorders [e.g., carnitine palmitoyl transferase type II deficiency (CPT II)] and postmortem control values (median C14:1/C12:1 = 2.5). The median concentrations of FC and TC were lower in VLCAD patients compared with control values.

CPT II/Translocase deficiencies. CPT II/Translocase deficiencies are inherited metabolic disorders characterized by increased very-long-chain fatty acylcarnitines, primarily those containing fatty acids of 16 carbons or more (C16, C18, and C18:1). A characteristic postmortem acylcarnitine profile for CPT II/translocase deficiencies is shown Fig. 6A. Six infant deaths were attributed to either CPT II or translocase deficiencies (these two metabolic disorders cannot be differentiated on the basis of an acylcarnitine profile alone). In at least one case, the newborn blood specimen was received in less than 24 h because of serious illness. A stat analysis revealed the presumption of CPT II/translocase deficiency. This child died on day 4 regardless of the clinical intervention given. Molecular analyses for CPT II deficiency were not available in our laboratory. We therefore relied primarily on information provided by the Medical Examiner, including clinical history if available.

The median concentrations of C16 and other long-chain acylcarnitines (C18 and C18:1) are clearly increased in postmortem specimens from infants with CPT II/translocase deficiencies relative to control autopsy specimens (10-fold excess of these metabolites) as shown in Table 2. The median concentration of the LC index was markedly higher than in controls. Because VLCAD and CPT II/translocase deficiencies share similar increases in long-chain acylcarnitine concentrations and the LC index, a ratio of C16 to C14:1 was used to distinguish these two

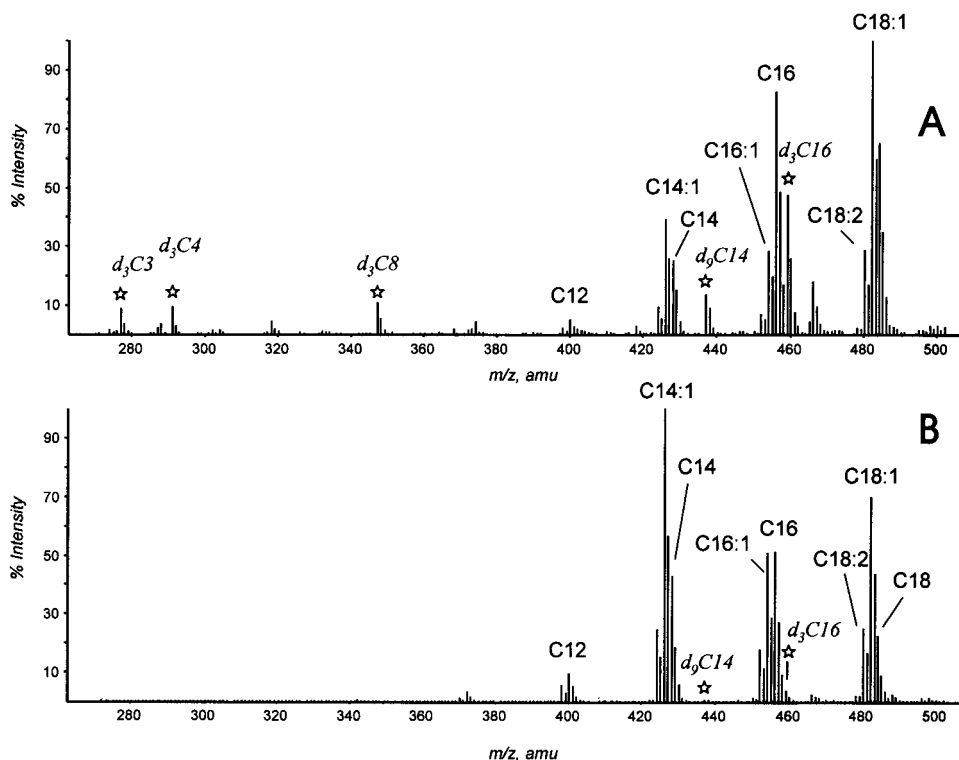


Fig. 5. Acylcarnitine profiles of a post-mortem dried blood specimen (A) and bile specimen (B) from an infant diagnosed with VLCAD deficiency.

MS/MS spectra were obtained using the Pre 85 scan functions appropriate for acylcarnitine butyl ester analyses. The stars and *italic font* denote stable-isotope internal standards.

different classes of metabolic disorders. The ratio was based on the finding that C14:1 and C14 were characteristically increased in cases of VLCAD deficiency but not typically in CPT II/translocase deficiencies. The median value for C16/C14:1 for CPT II/translocase deficiencies was 46, compared with 5.7 for VLCAD deficiency. As observed in both MCAD and VLCAD deficiencies, the median FC and TC concentrations were lower in CPT II/translocase deficiencies compared with control values. The median acyl/free index appeared to be marginally increased in CPT II/translocase deficiencies compared with other metabolic disorders, such as MCAD and VLCAD deficiencies, and with control specimens.

LCHAD/Trifunctional protein (TFP) deficiency. Four infant deaths were attributed to LCHAD/TFP deficiency through screening of postmortem blood specimens. The acylcarnitine profile for an infant diagnosed with LCHAD/TFP deficiency is shown in Fig. 6B. This post-mortem acylcarnitine profile is characterized by a mild increase in the long-chain hydroxyacylcarnitines (C16OH, C18:1OH, and C18OH). Few other acylcarnitine metabolites were substantially increased, although mild increases in C16, C18, and C18:1 may be observed. Molecular analysis of the most common LCHAD mutation was performed for each presumptive specimen. Two specimens were homozygous for the G1528C mutation, whereas one other specimen was heterozygous for the G1528C mutation. In one case, an infant with LCHAD/TFP deficiency was identified through newborn screening

but subsequently died at 5 days of age. However, another child identified through clinical screening as LCHAD was doing well clinically.

The median concentration of long-chain hydroxyacylcarnitines (Table 2) in infants with LCHAD/TFP deficiency was mildly increased compared with control post-mortem specimens. However, the sum of all major long-chain hydroxyacylcarnitines (total OH) showed a more significant combined concentration of 1.7 vs 0.13 $\mu\text{mol/L}$ for control postmortem specimens. The median concentrations of C14 and C16 were only mildly increased relative to the controls in a few specimens. Examination of the LC index, however, showed a clear increase in this value compared with controls (nearly eightfold increase in the LC index for LCHAD specimens). Often it is helpful to express the ratio of C16OH to C16 to detect increases in hydroxyacylcarnitines. We found that this ratio was clearly increased in LCHAD specimens (0.46) compared with control specimens (0.08). As with other disorders of FAO described earlier in this report, the FC and TC were each decreased relative to control specimens.

MADD. The recognition of MADD, also known as glutaric acidemia type II, is the most challenging of the inherited metabolic disorders to identify in the postmortem specimen. Eight cases of MADD were presumptively identified. This disorder is characterized by a highly variable acylcarnitine profile whose distribution may show higher concentrations of medium- or long-chain acylcarnitines or organic acids such as glutarylcarnitine (C5DC) or C5. The

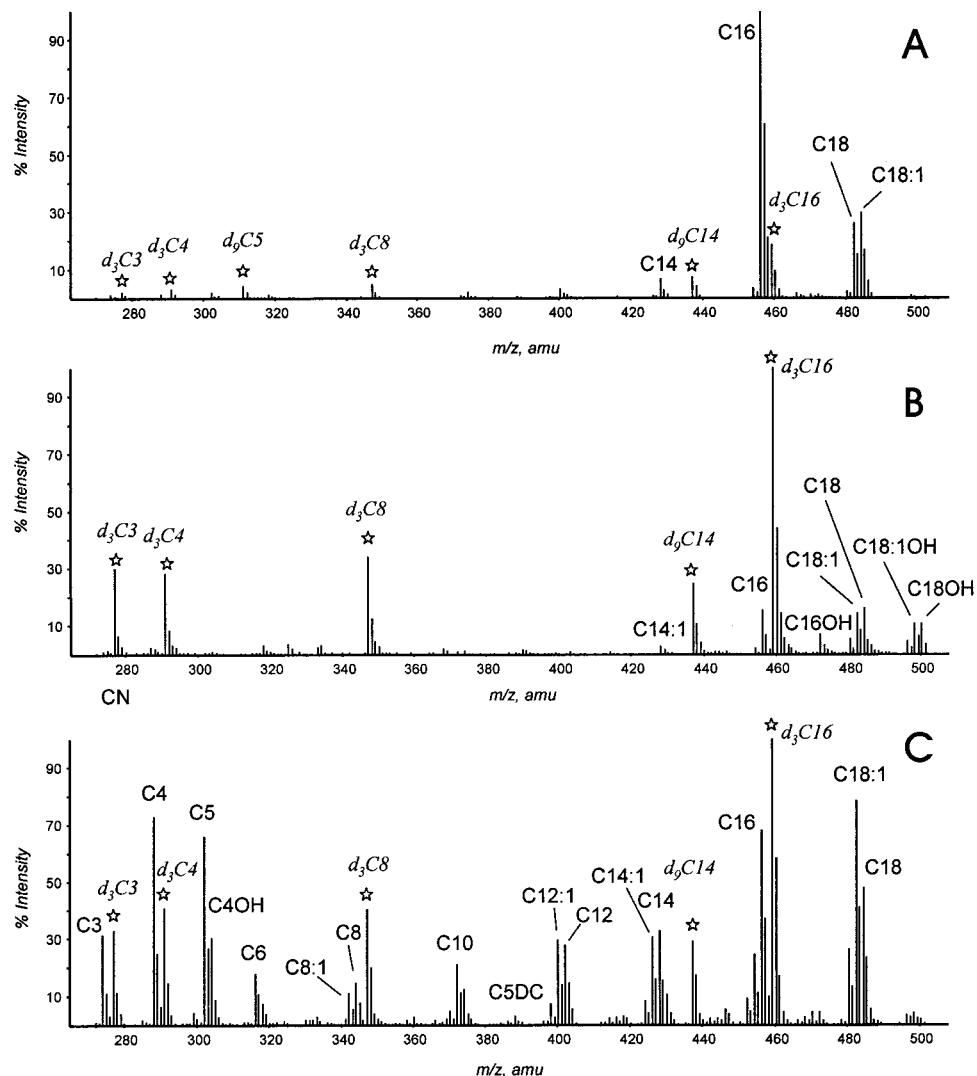


Fig. 6. Postmortem acylcarnitine profiles of dried blood specimens from infants diagnosed with CPT II/translocase deficiencies (A), LCHAD deficiency (B), or MADD (C).

MS/MS spectra were obtained using the Pre 85 scan functions appropriate for acylcarnitine butyl ester analyses. The stars and *italic font* denote stable-isotope internal standards.

most common postmortem acylcarnitine profile indicative of MADD as identified in this study is shown in the example presented in Fig. 6C. This profile reveals generalized mild increases of most fatty acylcarnitines and some organic acylcarnitines. However, mild increases in some acylcarnitines, although quite variable, are observed frequently in presumably nonpathologic postmortem specimens. How, therefore, is MADD presumptively detected in postmortem specimens? (a) Examination of the medium-chain acylcarnitines provides the initial evidence of the possibility of MADD deficiency as is the case in the interpretation of both newborn and high-risk specimens. Mild to moderate increases of C8 and, to a lesser extent, C8:1 are observed. (b) Long-chain acylcarnitines almost always appear increased compared with control as observed in the quantitative values for the median concentrations of C14 and C16 in Tables 1 and 2. Furthermore, other important acylcarnitines are mildly to moderately increased, including the medium-chain acylcarnitines C10:1, C10, C12:1, and C12 and the organic acid acylcar-

nitines C5DC and C5. Increases in C4 that are often characteristic of MADD in newborns and high-risk specimens are masked by the generalized increase characteristic of most postmortem specimens.

Quantitative analysis appears to be important in the diagnosis of multiple mild increases. The median concentration of C8 is ~2.5-fold higher than that for control postmortem specimens as demonstrated by the data in Tables 1 and 2. In several cases of MADD, C5DC may be increased. The median concentration of C5DC is 0.15 $\mu\text{mol/L}$ for the MADD group compared with 0.09 $\mu\text{mol/L}$ for the control postmortem group. In one case, a substantial increase of C5DC was found that suggested glutaric acidemia type I (GA-I). However, retrieval of the original newborn blood specimen showed a pattern (increased medium- and long-chain acylcarnitines) consistent with MADD rather than GA-I. Other important diagnostic metabolites include C14 and other long-chain acylcarnitines. At least a threefold increase in the median concentration of long-chain acylcarnitines (Table 2) was

noted compared with controls (Table 1). More characteristic of MADD was the increased concentrations of intermediate-chain length acylcarnitines C10 and C12. Median concentrations generally showed a three- to fourfold increase over control values. Examination of other data demonstrated mild increases in the SC and MC indices, whereas the LC index demonstrated significant increases compared with the control. The acyl/free ratio was found to be an important diagnostic indicator for diagnosis of MADD. This ratio was highest in MADD compared with other disorders and nearly double the control values. The median FC and TC concentrations were not significantly lower in this disease group vs the control. Clearly, the difficulty in confirming MADD requires the development of methods or standard protocols to confirm MADD through either molecular or enzyme-based analyses.

Severe carnitine deficiency. It was observed that several postmortem profiles showed very low concentrations of both free carnitine and acylcarnitines. This can be indicative of either a metabolic disorder or an insufficient or poor blood specimen. Rinaldo and co-workers (19, 37) reported findings of carnitine deficiency in postmortem bile samples and characterized them as a carnitine uptake disorder. We have observed four cases of significantly low FC and low C2 and other acylcarnitines. The acylcarnitine profile (data not shown) was characterized by the absence of acyl and free carnitine, whereas the amino acid profile remained consistent (increased amino acids) with an autopsy blood specimen. Quantification of free and total carnitine in these specimens (Table 2) clearly showed a substantial reduction in FC and TC: the median concentrations for each were nearly 20-fold lower than those for control postmortem specimens.

To prevent a false-positive result because of poor-quality specimens, the amino acid profiles were referenced. If a generalized increase of amino acids was observed as is the case with most postmortem specimens, then it was concluded that the specimen contained sufficient blood to determine the relative concentration of acylcarnitines. In addition, as with all specimens, their quality was physically examined. If the specimen was pale or spotty and did not meet the general requirements for proper blood specimens, another postmortem specimen (whole blood is often stored by the Medical Examiner and is reapplied to the filter paper) was requested or the newborn specimen was requested. In either case, however, an attempt was made to interpret the profile based on our experience with plasma specimens (data not shown). Presumptive positive results were reported as carnitine deficiency attributable to an uptake disorder, as primary carnitine deficiency, or as secondary carnitine deficiency attributable to a metabolic disorder that causes depletion of carnitine and affects our ability to find abnormal increases of acylcarnitines.

ANALYSIS OF PRESUMPTIVE METABOLIC DISORDERS OF ORGANIC ACID METABOLISM

GA-I. Increased glutaric acid was observed in several autopsy blood specimens. Significant increases in this metabolite are associated with the presumptive diagnosis of GA-I. The acylcarnitine profile of a postmortem specimen identified from a patient with GA-I in which the metabolite C5DC is clearly increased is shown in Fig. 7A. We have presumptively identified 3 cases of GA-I in our 7058 analyses. The criteria for presumption of GA-I are based on the observation of an increased C5DC together with the lack of other increased acylcarnitines that may indicate other metabolic disorders, such as glutaric acidemia type II. As observed in Fig. 7A, no other relevant acylcarnitine metabolite was increased that differed substantially from control. The median concentration of C5DC was 0.24 $\mu\text{mol/L}$ (Table 2) compared with control values of 0.09 $\mu\text{mol/L}$. Other indices (LC, MC, SC), and both FC and TC were essentially the same as for controls. Information for these cases with regard to the clinical phenotype was not made available to us.

Isovaleric acidemia (IVA)/2-methylbutyryl-CoA dehydrogenase deficiency. Four presumed cases of IVA/2-methylbutyryl-CoA dehydrogenase deficiency were found. It is important to note that without additional confirmation, we cannot ensure which disorder is present because, as described previously, C5 may be either isovalerylcarnitine or 2-methylbutyrylcarnitine. The postmortem acylcarnitine profile for one of these cases is shown in Fig. 7B. As observed, C5 was significantly increased. The median concentration of C5 was 5.8 $\mu\text{mol/L}$, whereas control specimens (Table 1) had a C5 concentration of 0.52 $\mu\text{mol/L}$. In two postmortem cases, a metabolic disorder was confirmed by retrieval of the original newborn blood specimen in which significantly increased C5 was noted. No deficiency of FC or TC was observed in these presumptive specimens. All indices were within control limits.

Propionic and methylmalonic acidemia (PA and MMA). A substantial increase of C3 was observed in three postmortem blood specimens. An acylcarnitine profile from one presumptive case of either PA or MMA is presented in Fig. 7C. Note the relatively high C3 concentration as well as the relative increase of C3 to C2 as shown in the inset of Fig. 7C. An increase of C3 does not provide direct evidence for PA, MMA, or cobalamin deficiency. Analysis of urine, if available, would be required for elucidation of the exact metabolic disorder. Numerous postmortem specimens revealed increased concentrations of both C3 and C2 and a normal C3/C2 ratio, and thus a normal result. This ratio is critical for reducing false-positive postmortem results. The median concentration of C3 was 2.9 $\mu\text{mol/L}$ and the C3/C2 ratio was 0.1 for the control group, whereas the PA/MMA group had a median C3 concentration of 16 $\mu\text{mol/L}$ and a C3/C2 ratio of 0.85.

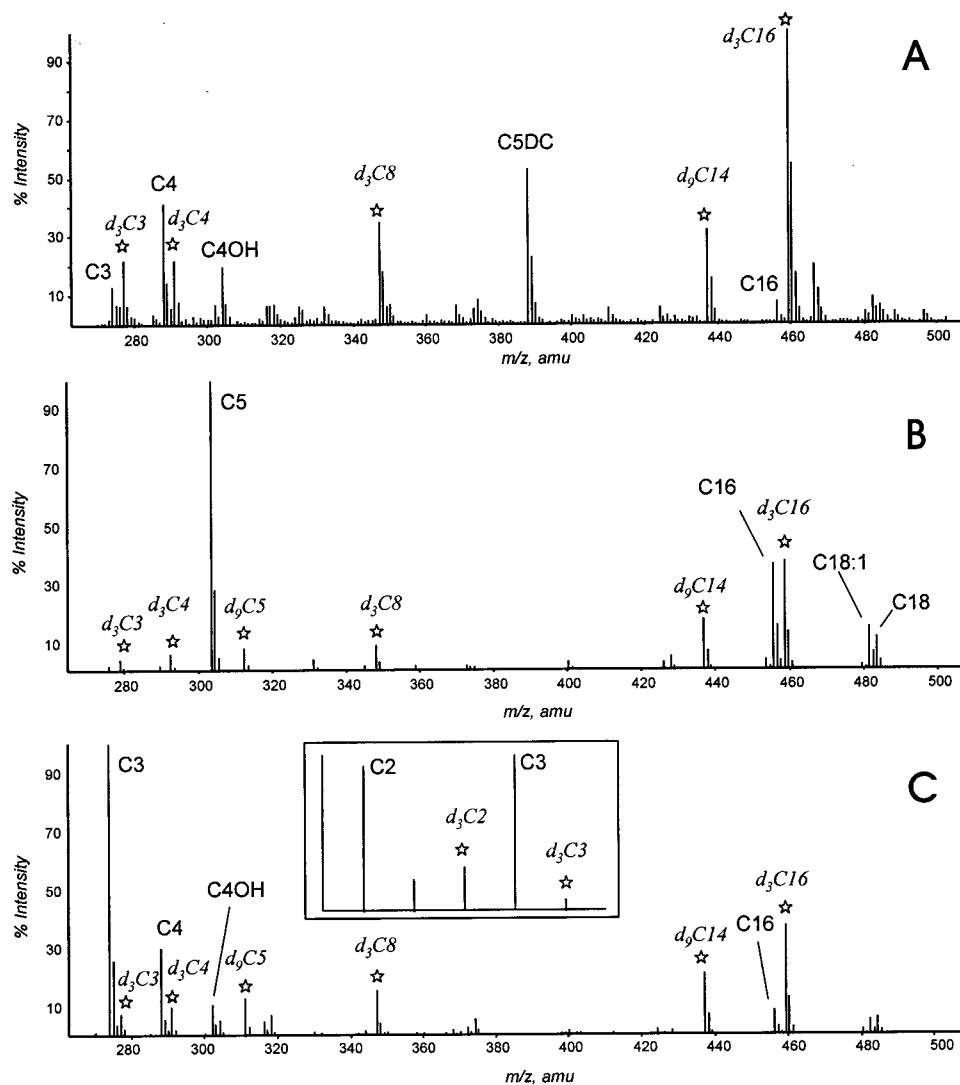


Fig. 7. Postmortem acylcarnitine profiles of dried blood specimens from infants with GA-I (A), IVA/2-methylbutyryl-CoA dehydrogenase deficiency (B), and PA/MMA (C).

MS/MS spectra were obtained using the Pre 85 scan functions appropriate for acylcarnitine butyl ester analyses. (*Inset*), MS/MS spectrum (Pre 85) of C2 and C3, showing only the concentration of C3 relative to C2. The stars and *italic font* denote stable-isotope internal standards.

Interestingly, FC was depressed in the affected group, whereas TC was within the range of values obtained for controls. This low value for FC affects the acyl/free ratio, which was 1.3 for the presumptive positive group vs 0.29 for control postmortem specimens. Not all specimens received by our screening program come from infants and children; one case that showed a particularly high C3 concentration was later found to be from a 16-year-old male.

Other disorders. Several other abnormal profiles were observed. One autopsy specimen revealed a very high concentration of 3-OH-isovalerylcarnitine ($2.5 \mu\text{mol/L}$) compared with a median concentration of $0.37 \mu\text{mol/L}$ for control specimens. In another case, both C3 and C5 were substantially increased but no specific metabolic disorder was defined, although these two metabolites have been observed to be increased in cases of biotinidase deficiency or multiple CoA carboxylase deficiency. In another notable case, the concentration of C4OH was 48

$\mu\text{mol/L}$, which was clearly greater than any increase of C4OH observed in control specimens. This profile suggests the presumption of short-chain hydroxyacyl-CoA dehydrogenase deficiency. Finally, one case of an infant death from MSUD was found in an international specimen submitted to our postmortem screening program as described earlier and shown in Fig. 2B.

DEMOGRAPHICS

The collection of >7000 specimens received by our laboratory included specimens from all 50 US states and from most Canadian provinces as well as a few specimens from other countries. The patients were of all races and were primarily infants <1 year of age; the numbers of males and females were approximately equal. More than one-half of the specimens were from patients with deaths of unknown cause. The trend of samples from the early screening specimens (the first 1000) compared with the latest specimens shows an increasing number of infants who died of any cause including SIDS. Some medical

examiners are sending us specimens from every infant who dies in their state at <2 years of age. With regard to causes of death that we believe are highly presumptive or confirmed by our screening program, we have found 66 cases (Table 3). Of 57 cases for which we have information, 30 were male, 27 were female, 12 were African American, 21 were Caucasian, 1 was Asian, and 2 were of mixed race. Of 49 cases for which we have date of death, in 7 cases the infants died at ages <3 days, whereas in another 6 cases, infants died at <7 days of age.

Discussion

The preparation and MS/MS analyses of postmortem dried filter paper blood specimens are easily performed by clinical laboratories proficient in clinical, "high-risk" screening by MS/MS. This method, currently used to analyze an average of 10 postmortem specimens per day, can easily be accommodated in batch analyses alone or combined with other blood specimens. This enables the analytical portion of the testing to be highly cost-effective. In fact, it is likely that some toxicology screens can be achieved from the same postmortem blood specimen used for inborn error metabolic screens. Preliminary results were presented for a combined metabolic and benzoylecognine (a cocaine metabolite) screen at the 2000 American Society of Mass Spectrometry Conference (www.ASMS.org) in Long Beach, CA. Specimen collection is relatively simple with 25 μ L of blood collected at autopsy (preferably with tubes containing heparin as the anticoagulant) and allowing a free-flowing drop produced during ejection of the syringe to fall onto the filter paper. After drying for 3 h, the specimen is mailed via regular post to the metabolic screening laboratory for analysis. Furthermore, the filter paper "carrier" is amenable to other biological specimens, such as bile, urine, plasma, cerebrospinal fluid, and vitreous humor. Blood specimens are additionally advantageous because DNA can be extracted from them for identification (chain of custody) or for additional genetic testing.

MS/MS spectra from postmortem blood specimens are generally characterized by increased carnitine, acylcarnitines, and amino acids. By newborn screening or high-risk screening interpretation standards, postmortem screening results are almost always abnormal. In fact, postmortem acylcarnitine and amino acid profiles together often enable identification of the origin of the blood specimen, if unknown. Identification of a postmortem specimen can be based on its characteristic increases in the short-chain aliphatic and hydroxyacylcarnitines C4 and C4OH together with increased FC and amino acids. To identify an abnormal postmortem result requires many new parameters that are not used in the interpretation of newborn screening or high-risk results. Applying newborn screening or high-risk parameters to postmortem interpretation would produce a very high false-positive rate. Therefore, several new indices were used to investigate the relative concentrations of important diagnostic metabolites. As the data show, these indices were extremely important in the interpretation process and the identification of several metabolic disorders.

As shown in Table 3, based on the interpretation scheme and results described, we found 66 of 7058 postmortem specimens to be highly presumptive for a metabolic disorder. Statistically, this represents a <1% positive finding for the specimens we have screened. However, these numbers do not predict the rate of occurrence of metabolic disorders because the population and characterization of screening specimens is less defined. For example, Medical Examiners determine their own criteria for death attributable to SIDS (some being broadly defined, whereas others are more narrowly defined). Many Medical Examiners send specimens only when evidence is sufficient to suspect a metabolic disorder, whereas more recently, Medical Examiners in some states and counties are sending specimens from all infant deaths for screening regardless of suspected or known cause. These cases include deaths from any one of numerous apparent causes, including accidental, criminal, unknown but suspected (suffocation attributable to cosleeping or overlay), and stillbirths. With such a mixed population of specimens, it is difficult to estimate a reliable frequency of metabolic disorders among any specific classification, such as SIDS and sudden unexplained death syndrome (SUDS).

As the volume of specimens increases, our statistics will more closely resemble the percentage of deaths from a metabolic disorder relative to the infant mortality rate. Our current statistics lie somewhere between the predicted infant deaths attributable to metabolic disorders among the unknown death population vs the 2–5% of deaths attributable to highly suspicious sudden infant death. Furthermore, we have found deaths attributable to underlying metabolic disease in infants and children that should have been identified by illness, i.e., several organic acidemias. Our findings include organic acidemias and one case of MSUD, an amino acid disorder. These diseases

Table 3. Highly presumptive or confirmed diagnosis of metabolic disorders in 7058 postmortem blood specimens.

Disorder	Total cases found
MCAD deficiency	23
VLCAD deficiency	9
Glutaric acidemia type II (MADD deficiency)	8
CPT II/Translocase deficiencies	6
Carnitine deficiency/Carnitine uptake	4
LCHAD deficiency/TFP deficiency	4
SCHAD ^a deficiency	1
GA-I	3
IVA/2-Methylbutyryl-CoA dehydrogenase deficiency	4
Other organic acid disorders	3
MSUD	1
Total	66

^a SCHAD, short-chain hydroxyacyl-CoA dehydrogenase.

and others have not often been considered traditionally in postmortem screening for SIDS, as have the FAO disorders. In addition to the 66 metabolic disorders detected by MS/MS, we have also identified 6 cases of congenital adrenal hyperplasia within the same postmortem population using a non-MS/MS enzyme immunoassay for 17-OH-progesterone.

The age at death may affect interpretation. Our experience has shown that the postmortem blood specimen obtained from an infant who died in the newborn period (1–7 days) more closely resembles the profile of a newborn, especially with regard to abnormal specimens. The concentration of carnitine is critically important to the recognition and diagnosis of abnormal acylcarnitine profiles. As the child ages, lower carnitine concentration may alter the metabolic patterns; hence, this fact must be noted during the interpretation process. In cases where specimens reveal a clear carnitine deficiency, a metabolic disorder is highly presumptive although it cannot pinpoint which FAO disorder is present. The challenge to interpretation is the borderline low carnitine concentrations that present a difficulty in diagnosing a carnitine deficiency or a specific metabolic disorder because expected metabolites for various disorders may not be present at concentrations high enough to identify an abnormality, whereas the concentration of carnitine is not low enough to diagnose carnitine deficiency. It is in these cases that the ratio of a metabolite to total carnitine is important. All of the indices described incorporate the ratio of individual or summed metabolites to the concentration of total carnitine and are critical in reducing false-negative and -positive screening results.

The choice of specimen for postmortem screening is important. Postmortem bile and blood revealed few differences in the profiles for medium- and long-chain acylcarnitine, but TC and long-chain acylcarnitines were substantially higher in bile than in blood. However, the relative ratios of metabolites were similar. One report suggested that bile was superior to plasma and blood for postmortem analyses (36). Our experience with plasma, although limited, would support this assumption, in part because our experience shows that long-chain acylcarnitines are not found in plasma to the extent that they are found in whole blood. However, we would disagree, based on the findings presented here, that blood is unsuitable. As shown in the example of VLCAD deficiency (Fig. 5), both specimens demonstrated clearly abnormal metabolic profiles. Additional investigations and specimen comparisons are needed to better understand the differences of each profile, although the data provided here provide a preliminary basis for that assessment. It is also important to remember that blood contains extractable DNA that can be used in screening or in confirmation of metabolic disease. In addition, based on our experience, it may be easier to diagnose organic acidemias and short-chain acylcarnitine disorders in blood rather than in bile because, relatively speaking, these metabolites are

apparently excreted at lower concentrations in bile than in blood (based on the data presented here).

As described above, the analytical phase of testing is very similar to approaches used in newborn and high-risk testing for metabolic disorders. However, this analytical similarity ends at this phase. The results produced by MS/MS analysis of postmortem blood specimens are quite different from those obtained in newborn screening. These results require both experience in the visual interpretation of results together with the assistance of automated computer interpretation using many of the parameters developed in this report. Our high-volume screening program has demonstrated both the need for an extensive interpretation scheme to recognize abnormal results while minimizing false positives as well as an extensive list of procedures for follow-up and confirmatory testing. We have successfully used molecular analyses to screen for the common MCAD and LCHAD mutations for confirmation. In addition, we have obtained original newborn spots that were available in some cases or retrieved a different specimen, such as bile. For acylcarnitine analysis, the original newborn specimen is considered the ideal specimen for identification of several inborn errors of metabolism. If a molecular analysis by our laboratory was unavailable or the original newborn specimen or a bile specimen was unavailable, we relied on pertinent pathology, such as fatty liver, cardiomyopathy, or circumstances of death. This information was then provided to the Medical Examiner, who would then provide additional testing if necessary.

Clearly, limitations in our follow-up program must be taken into consideration. Confirmation of many disorders requires molecular analyses, enzyme assays, and cell culture studies that were not available in our laboratory. Furthermore, our program relies on the Medical Examiner to implement confirmatory testing not provided by our laboratory. The complexity of follow-up programs cannot be underestimated. With the data now available from this large-volume screening program, further emphasis and support in the postmortem follow-up of suspected metabolic diseases can be justified. Toward that end, we have made every effort to maximize disease recognition while maintaining a low false-positive rate. Recognition of an abnormal profile is the critical and clearly essential step that is required before follow-up and confirmatory testing can be implemented.

In conclusion, the importance of developing a routine postmortem metabolic screen in the investigation of premature or unexplained death in infants and children cannot be understated. Toxicology screens are obtained routinely from postmortem specimens to rule out accidental or deliberate poisonings or drug overdoses. With the ability of MS/MS-based methodology to provide a metabolic screening panel, routine screening of infant and child deaths should become a standard protocol for Medical Examiners. In utilizing such services, Medical

Examiners provide benefits to future siblings who might then have very early screening. In addition, parents may be provided genetic counseling and family planning. Postmortem metabolic screening also provides a public health service because the number of deaths attributable to inborn errors of metabolism may begin to be assessed. It is hoped that high-volume postmortem screening and the data presented here will begin to demonstrate the importance of even more extensive testing procedures to determine the cause of infant and child death. These data may be developed to find profiles that require additional sophisticated testing, such as enzyme analysis, metabolic studies of skin fibroblasts, and molecular analysis of known mutations. Blood or bile screening of postmortem samples is just the beginning of an exciting new area, and although it will clearly not detect metabolic diseases that do not demonstrate abnormal acylcarnitine or amino acid profiles, it is the beginning of a multianalyte procedure to search for the cause of death when no other information may be available.

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