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Review

# Use of Tandem Mass Spectrometry for Multianalyte Screening of Dried Blood Specimens from Newborns

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***Background:*** **Over the past decade laboratories that test for metabolic disorders have introduced tandem mass spectrometry (MS/MS), which is more sensitive, specific, reliable, and comprehensive than traditional assays, into their newborn-screening programs. MS/MS is rapidly replacing these one-analysis, one-metabolite, one-disease classic screening techniques with a one-analysis, many-metabolites, many-diseases approach that also facilitates the ability to add new disorders to existing newborn-screening panels.**

***Methods:*** **During the past few years experts have authored many valuable articles describing various approaches to newborn metabolic screening by MS/MS. We attempted to document key developments in the introduction and validation of MS/MS screening for metabolic disorders. Our approach used the perspective of the metabolite and which diseases may be present from its detection rather than a more traditional approach of describing a disease and noting which metabolites are increased when it is present.**

***Content:*** **This review cites important historical developments in the introduction and validation of MS/MS screening for metabolic disorders. It also offers a basic technical understanding of MS/MS as it is applied to multianalyte metabolic screening and explains why MS/MS is well suited for analysis of amino acids and acylcarnitines in dried filter-paper blood specimens. It also describes amino acids and acylcarnitines as they are detected and measured by MS/MS and their significance to the identification of specific amino acid, fatty acid, and organic acid disorders.**

***Conclusions:*** **Multianalyte technologies such as MS/MS are suitable for newborn screening and other mass screening programs because they improve the detection of many diseases in the current screening panel while enabling expansion to disorders that are now recognized as important and need to be identified in pediatric medicine.**



The recent and continuing impact of tandem mass spectrometry (MS/MS)[1] in newborn screening and other clinical chemistry applications is considerable. Unquestionably, the use of MS/MS to detect metabolic disorders in newborns is one of the most important advancements in neonatal screening since the introduction of a bacterial inhibition assay for phenylketonuria (PKU) by Dr. Robert Guthrie more than 40 years ago *(1, 2)*. Sweetman *(3, 4)*, Levy *(5)*, and Bennett and Rinaldo *(6)* have chronicled the technical developments of MS/MS newborn screening in *Clinical Chemistry* with their editorials. Advancements in and validation of this innovation are still occurring more than a dozen years after initial studies first suggested that MS/MS might be an effective tool in screening for multiple metabolic disorders in a single analysis. This ongoing quantitative validation and enhancement indicates that

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[1] Nonstandard abbreviations: MS/MS, tandem mass spectrometry; PKU, phenylketonuria; MCAD, medium-chain acyl-CoA dehydrogenase; QA, quality assurance; GC, gas chromatography; C2, acetylcarnitine; FC, free carnitine; FAB, fast atom bombardment; FIB, fast ion bombardment; Pre, precursor ion scans; BE, butyl ester; GA-I and -II, glutaric acidemia type I and II, respectively; C5DC, glutarylcarnitine; C8, octanoylcarnitine; NL, neutral loss; QC, quality control; SRM, selected-reaction monitoring; MSUD, maple syrup urine disease; TNT, transient neonatal tyrosinemia; CPT I and II, carnitine palmitoyltransferase I and II, respectively; C16, hexadecanoylcarnitine; C18, octadecanoylcarnitine; C4, butyrylcarnitine; SCAD, short-chain acyl-CoA dehydrogenase; C5, isovalerylcarnitine; MADD, multiple acyl-CoA dehydrogenase deficiency; C3, propionylcarnitine; MCHAD, medium-chain hydroxyacyl-CoA dehydrogenase; C10, decanoylcarnitine; C14, tetradecanoylcarnitine; C14:1, tetradecenoylcarnitine; MCT, medium-chain triglyceride; VLCAD, very-long-chain acyl-CoA dehydrogenase; C18:1, octadecenoylcarnitine; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; C16OH, hydroxyhexadecanoylcarnitine; TFP, trifunctional protein; C10OH, 3-hydroxydecanoylcarnitine; PA, propionic acidemia; MMA, methylmalonic acidemia; and C5OH, 3-hydroxyisovalerylcarnitine.

MS/MS is comprehensive, versatile, and effective when used for mass screening.

As a newborn-screening assay, MS/MS is not merely a method for detecting medium-chain acyl-CoA dehydrogenase (MCAD) deficiency *(7)*, nor is it simply an improved method for accurate neonatal detection of PKU with a false-positive rate 10-fold lower than the best method previously available *(8)*. MS/MS technology offers a new vision to newborn-screening programs that now have the ability to screen for 30 or more metabolic disorders in a single analysis from one small disk of dried blood. With more than 3 million infants screened worldwide and more than 500 confirmed disorders detected from this screening, MS/MS newborn screening is a demonstrated clinical screening technology *(9–17)*. Furthermore, MS/MS has ushered in the concept of multiple-metabolite analyses for the detection of numerous metabolic disorders in a single analytical run; this approach is being used with increasing frequency in new genomic applications and recent approaches to biochemical and newborn screening.

In this review, we provide an understanding of the current applications of MS/MS to metabolic disease detection together with references to key historical developments. A substantial portion of the review will be focused on individual analytes, namely amino acids and acylcarnitines, and how they are used in data interpretation and diagnosis. A brief discussion of the complexities of and novel approaches to quality assurance (QA) in newborn screening by MS/MS are included with a final section on the future of MS/MS in newborn screening that emphasizes the important role of its integration and support of other technologies and methods. Table 1 provides a list of common terms used in the application of MS/MS and suggested abbreviations that will be used throughout the text.

**Table 1. Suggested abbreviations for the application of MS/MS in newborn screening.**

| Category | Term | Abbreviation |
|---|---|---|
| Mass spectrometry | Tandem mass spectrometry | MS/MS |
| | Electrospray ionization | ESI |
| | Precursor ion scans | Pre |
| | Neutral loss scans | NL |
| | Product ion scans | Pro |
| | Selected-reaction monitoring | SRM |
| | Mass-to-charge ratio | *m/z* |
| Metabolites[a] | Phenylalanine | Phe |
| | Free carnitine | FC |
| | Octanoylcarnitine | C8 |
| | Glutarylcarnitine | C5DC |
| | 3-Hydroxyisovalerylcarnitine | C5OH |
| | Tetradecenoylcarntine | C14:1 |
| | 3-Hydroxyoctadecenoylcarnitine | C18:1OH |

[a] Examples of suggested formats for specific metabolites are included. Amino acids should use the three-letter convention already established. It should be assumed all acylcarnitines are of the L-isomeric form. Acylcarnitines are indicated with an uppercase C followed by the number of carbons comprising the fatty acyl group. Dicarboxylic acids are indicated with an uppercase DC, aliphatic hydroxy fatty acyl groups with an OH, and unsaturated species with a colon followed by the number of unsaturated sites.

## Historical Developments Leading to the Application of MS/MS to Newborn Screening

Before 1980, diagnosis of inborn errors of organic acid or fatty acid metabolism usually required gas chromatographic (GC) analysis of urine extracts with relatively nonspecific detectors. Identification of a metabolite in urine was based solely on its retention time. The addition of mass spectrometry (MS) to GC applications in the late 1970s dramatically improved the analysis of organic acids by providing mass spectral identification of each compound at a particular retention time. This combined technique, known generally as GC/MS, became a gold standard for identification of metabolic disorders from urine specimens *(18–20)*. The subject of numerous articles and reviews, this application remains the keystone for clinical diagnosis of organic acidemias *(21–24)*. Much of the current knowledge about organic acidemias and fatty acid oxidation defects is based on findings provided by GC/MS analysis of urine from affected individuals.

In the mid-1980s, recognition that patients with carnitine deficiency were frequently diagnosed with a metabolic disorder heightened suspicion of a possible connection between carnitine and disorders of fatty and organic acid metabolism *(25, 26)*. At that time, routine investigation of a possible metabolic disorder included measurement of plasma and urine carnitine concentrations. Methods used to measure carnitine, acetylcarnitine (C2), and total carnitine often involved enzymatic and radioenzymatic assays, whereas measurement of acylcarnitines usually involved HPLC *(27)*. Carnitine measurements generally included free, nonesterified, and total carnitine (free plus esterified carnitine). Quantification of esterified carnitine was imprecise, calculated by subtraction of the free carnitine (FC) concentration from total carnitine. Efforts to understand carnitine deficiencies, metabolic disorders, and Reye syndrome and their correlation with free, total, and esterified carnitine led to recognition that esterified carnitine may be diagnostic. Efforts were made to further investigate carnitine and acylcarnitine deficiencies to gain a better understanding of the role of carnitine and its fatty acid esters in metabolic diseases *(28–31)*. As a result, the establishment of new methods for carnitine and acylcarnitine measurement became important. Eventually, this search for new methods led to the development of MS/MS for newborn screening.

Roe and coworkers *(32–34)* recognized the function of carnitine and acylcarnitines and the therapeutic value of their measurement in the investigation of disorders of fatty acid oxidation. Before the availability of MS/MS, two approaches to the investigation of carnitine and acylcarnitines included either hydrolysis of fatty acyl residues from carnitine with subsequent analysis by GC/

MS, or extensive sample preparation for chemical modification of acylcarnitines to facilitate their analysis by GC/MS. Although both approaches presented sample preparation and time limitations, they nonetheless served to provide integral information about the role of acylcarnitines in metabolic diseases.

The limitations of previous methods led to the search for a simpler and more direct method for the measurement of acylcarnitines. As a result, a new form of MS for nonvolatile specimens, known as fast atom bombardment (FAB) MS, emerged *(35–38)*. With FAB MS, samples are ionized after bombardment by a stream of atoms. Unlike the electron impact ionization used in GC/MS, FAB MS produces little fragmentation during the ionization process. This feature is often referred to as "soft ionization". After ionization, the mass spectrometer detects and quantifies ions in a complex mixture. For the analysis of complex biological extracts, however, overlapping and suppressed masses lead to mass spectra that are not interpretable and thus uninformative. Consequently, further modifications led to the addition of liquid chromatography for separation of acylcarnitines from one another and from other compounds present in a sample *(37)*. Rapid advances in MS technology and the introduction of more affordable quadrupole MS/MS systems eventually produced instruments with enhanced selectivity and sensitivity that in turn eliminated the need for chromatographic separation. Furthermore, newer and simpler forms of ionization similar to FAB, such as fast ion bombardment (FIB), permitted more reliable and reproducible analyses. A concerted effort to develop a MS/MS newborn-screening assay using filter-paper blood spots began in 1990 *(39)*. The fundamental technologic developments in MS/MS application to metabolite analysis initiated in the early 1990s still exist today. More than a dozen years of improvements in ionization, automation, and data processing have enabled newborn metabolic disease screening for patient volumes ranging from a handful to more than a thousand per day in some laboratories.

In the early days of MS/MS applications to newborn screening, most public health laboratories dismissed MS/MS as a viable technique because of the high initial costs of the instrumentation (~$400 000.00). Curiously, the costs of reagents and peripheral equipment for MS/MS were extraordinarily inexpensive, thus enabling a high-volume screening laboratory to realize substantial savings over older classic technologies. As a result of further improvements in the reliability of the technology without increased costs per instrument in nearly 10 years, confirmation that this technology can detect many diseases in a single assay both reliably and accurately, increased competition from the private sector, and increased pressure by the parents of affected children, public health laboratories began to utilize the technology. However, it is clear that the cost of the instrumentation is not the only issue at hand. The expertise required to manage and interpret the results is scarce, takes substantial time to achieve without previous training, and is still subject to the whims of government politics and the economy.

## Essential MS/MS

Reviews are available that include descriptions of MS and MS/MS in basic understandable terms *(12, 40, 41)*. It is important to understand that a mass spectrometer is in essence a detector, albeit a powerful and versatile one. As a detector it identifies the mass (weight) of individual molecules and their fragments. Results of this detection are displayed in a graph known as a mass spectrum. In a mass spectrum, positions along the *x*-axis represent masses (*m/z*), whereas the height of a peak along the *y* axis represents the quantity of ions. A mass spectrometer when used without purification or chromatography is not entirely selective, and data may be uninterpretable because of compounds with similar integer masses produced by intact and fragmented ions. A tandem mass spectrometer, however, provides a solution to the issue of selectivity for many analyses without time-consuming chromatography.

A tandem mass spectrometer comprises five basic components: an ion source, a mass analyzer (MS1), a collision chamber (also known as a collision cell) where intact molecules encounter an inert gas and fragment, a second mass analyzer (MS2) that separates the ions and fragments produced in the collision chamber, and a detector. The use of computer algorithms allows for several types of MS/MS. As shown in Fig. 1, five different MS/MS scan functions can be achieved in a single analysis. For those compounds that share common fragment ions or neutral molecules, MS/MS is an efficient tool, hence the application of amino acid and acylcarnitine analysis in metabolic disease detection *(12, 42, 43)*. What is important to note is the ability of MS/MS to analyze different chemical families in a single analysis in a short period of time (~2 min). This provides for a broad spectrum analysis and high throughput, lending itself to a highly cost-effective instrument from the analytical perspective and cost benefits from the clinical perspective when it is used to screen for multiple disorders.

## MS/MS Analysis of Acylcarnitines and Amino Acids in a Blood Spot

An acylcarnitine is a fatty acyl ester of L-carnitine. The "family" of acylcarnitines analyzed by MS/MS include fatty acyl groups for saturated and unsaturated species ranging from the 2-carbon acetylcarnitine (C2) through the 20-carbon aliphatic groups *(27)*. In addition, hydroxy fatty acids and dicarboxylic acid acylcarnitines are detected *(43)*. Certain drug therapies and other compounds, including valproic acid, pivalic acid, and benzoic acid, also produce an acylcarnitine. All acylcarnitines are analyzed exclusively by use of precursor ion (Pre) scans *(44)*.

As described in numerous publications, the fragment

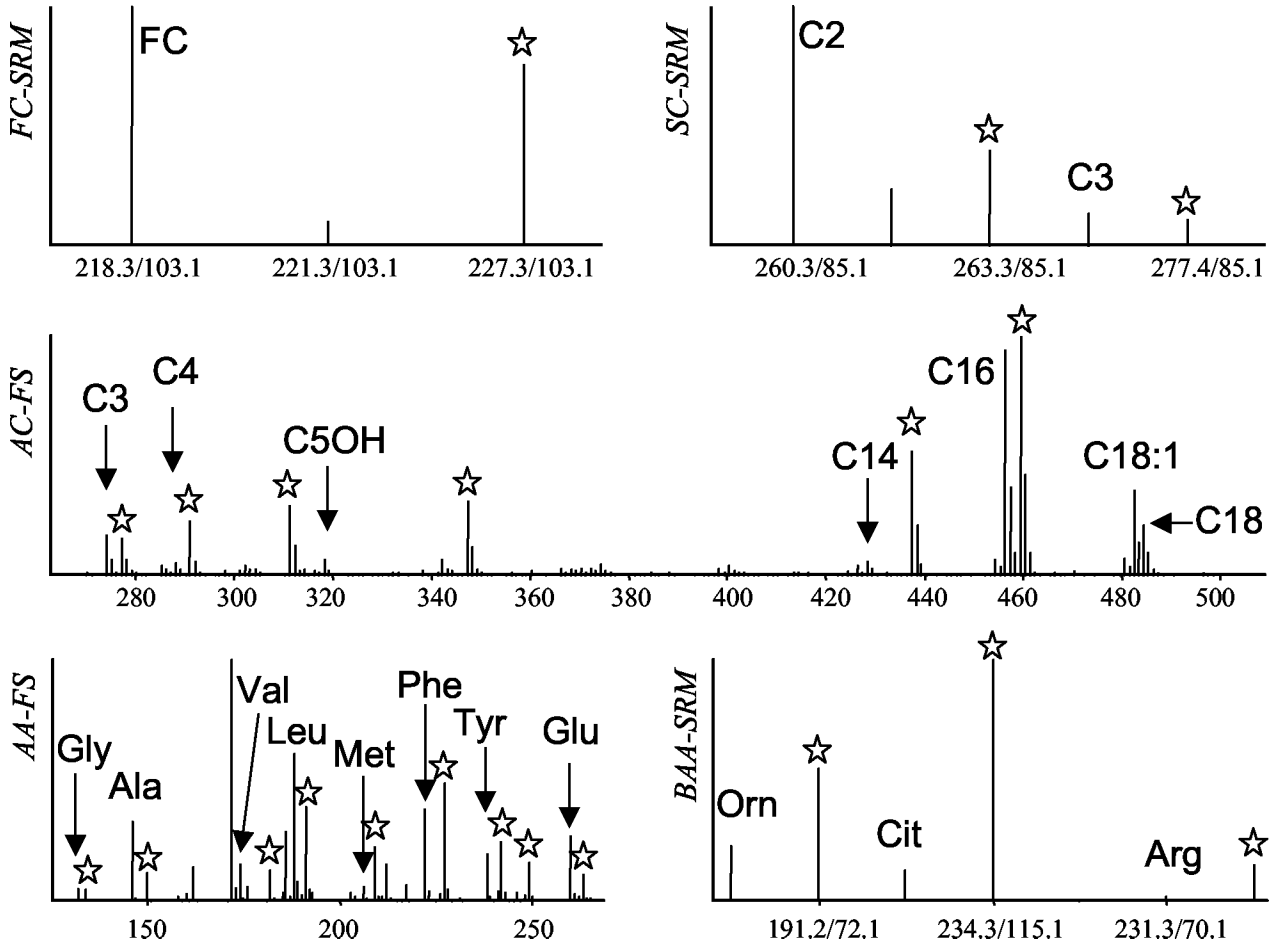


Fig. 1. Five MS/MS scan functions obtained in a 2-min analysis.

Internal standards are marked with a ☆. The *top left panel* is a Pre 103 analysis of FC and its internal standard in SRM mode. The *top right panel* is a Pre 85 analysis of C2 and C3 in SRM mode. The *middle panel* is an Pre 85 full-scan analysis of acylcarnitines. The *bottom left panel* is a NL 102 full scan of amino acids. The *bottom right panel* is a NL 119 scan of ornithine and citrulline as well as NL 161 or arginine in SRM mode.

common to all acylcarnitines is *m/z* 85 for both underivatized and butyl ester (BE) derivatives. The structure for the fragment ion has been illustrated previously *(7)*. This fragment ion is essentially the carnitine backbone produced by losses of BEs, quaternary ammonium, and the fatty acids. It is therefore easily observed why this fragment is common to both derivatized and underivatized fatty acylcarnitines of various chain lengths. Methyl ester derivatives produce a different, although similar, common fragment at *m/z* 99 *(45)*, primarily because the methyl ester group does not fragment readily. Although historically important in clinical screening methods, methyl ester derivatives are rarely used in newborn-screening applications. Some newborn-screening laboratories routinely use direct analysis of extracted acylcarnitine without derivatization. Validation articles are expected within the next year or two. Nevertheless, in our own investigation, butyl esterification was superior with regard to sensitivity and specificity. Its only limitation is the use of acidic reagents in the laboratory, which requires the use of a hood and appropriate safety measures. Furthermore, as noted in this review, nearly all published validation studies have used BE derivatives *(7, 46, 47)* with a few notable exceptions *(48)*. The top panel of Fig. 2 shows the acylcarnitine profile of a newborn with glutaric acidemia type I (GA-I), obtained using butyl esterification during sample preparation, whereas the bottom panel shows an acylcarnitine profile of the same newborn obtained without butyl esterification. Note that the profiles are quite similar in appearance but with different mass values. Note two important points: the added shift of the mass of glutarylcarnitine (C5DC), which is attributable to the fact that it is a dicarboxylic acid, and the lower sensitivity of detection relative to standards such as octanoylcarnitine (C8) produced by the increased likelihood of a net neutral or negative charge from underivatized carboxylic acid groups.

L-Carnitine (FC) produces fragments at both *m/z* 85 and 103 *(49)*. Generally, the fragment at *m/z* 103 includes the aliphatic hydroxyl group (−OH), which requires higher energy to fragment and produce the ion at *m/z* 85 and thus is generally present at higher abundance. For fatty acyl-

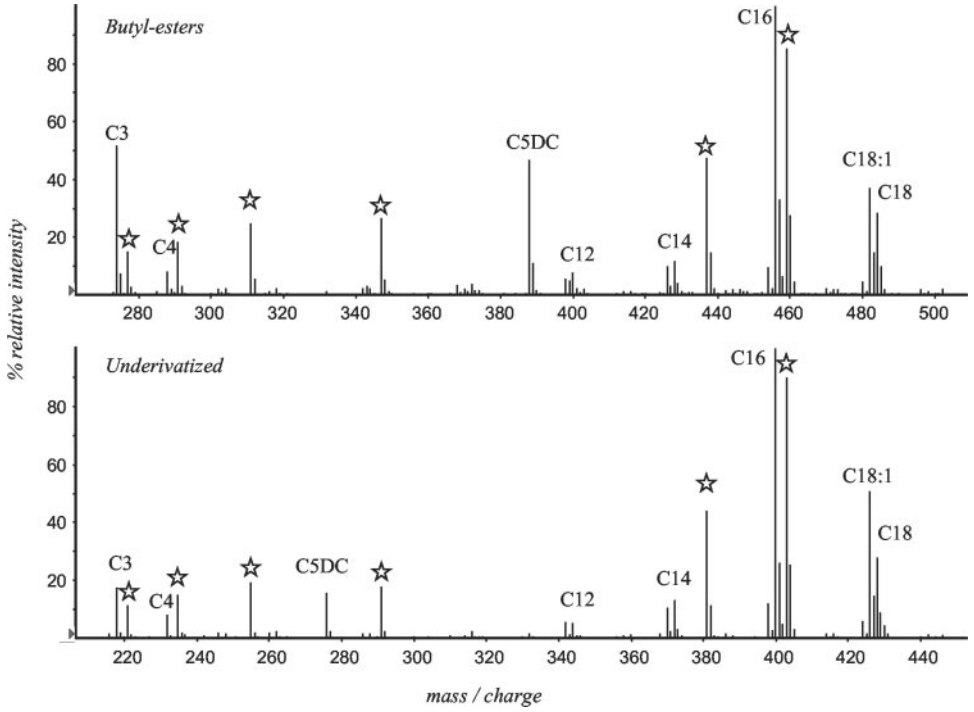


Fig. 2. Pre 85 MS/MS scans of acylcarnitines in a newborn confirmed to have GA-I.

(*Top*), blood specimen prepared by butyl esterification. C5DC is detected at *m/z* 388. (*Bottom*), blood specimen prepared without derivatization. C5DC is detected at *m/z* 276.

carnitines, this fragment is not produced because the aliphatic −OH group is part of the fatty acid lost. It is equally common to analyze FC using Pre 85- and Pre 103-Da MS/MS scan functions. For underivatized specimens, only *m/z* 85 can be used *(48)*. No studies to date have demonstrated advantages of either method other than higher sensitivity for precursor ions of 103 Da or reduce hydrolysis for methods that do not utilize derivatization. Methyl ester derivatives cannot be used for FC analysis.

Only α-amino acids fragment *(50)* to produce the neutral formate (56 Da) or butyl formate molecule (102 Da). This accounts for the observation that other amino acids, i.e., γ- or δ-amino acids and non-amino carboxylic acids (fatty acids) do not produce the common formic acid neutral loss (NL) in MS/MS. Furthermore, only selected amino acids can be measured in a quantitative and selective manner. It is important to emphasize "screening" vs quantification in MS/MS analysis of amino acids. Generally, quantitative analysis requires an amino acid analysis by HPLC. Nevertheless, identification of PKU by use of the phenylalanine/tyrosine (Phe/Tyr) ratio may be more important than a precise measurement of phenylalanine for the diagnosis and characterization of PKU *(8, 51, 52)*.

Two amino acid analyses that are not included in the routine NL 102 MS/MS panel in newborn screening because of the chemical characteristics of the compounds are cysteine and homocysteine. These compounds form disulfides easily, producing two sites of protonation and hence a doubly charged molecule. MS/MS analysis is complicated by the presence of doubly charged disulfide ions overlapping the mass values of singly charged thiols. Methods developed to analyze homocysteine use reducing agents such as dithiothreitol to ensure uniform compounds and sensitivity *(53, 54)*

Amino acids with a basic functional group are analyzed with different scan functions. These amino acids include arginine, ornithine, and citrulline. Because these amino acids have a basic functional group that fragments easily, the most common loss is a combination of butyl formate plus ammonia or basic group. Hence, for ornithine and citrulline, a NL of 119 Da scan is used. The 119-Da mass difference includes 102 from formate and 17 from ammonia. Therefore, a NL of 119 scan has been used to detect citrulline and ornithine. Arginine does not lose ammonia, but rather an arginino group. It is detected using a NL of 161 scan. Some of these molecules can also be analyzed in a NL 102 analysis because of fragmentation occurring in the electrospray ionization source. Some ions that fragment in the ion source produce stable molecules that do not contain a particular labile group, i.e., ammonia is lost from citrulline. Because the butyl formate remains, this new molecule will still produce a NL of 102 scan. Hence, for citrulline, a peak is detected at *m/z* 215 (232 − 17) in a NL scan. It is by this process that citrulline is also detected at a mass 17 less than its molecular mass. In summary, citrulline is detected at *m/z* 232 by a 119 Da scan function and a citrulline byproduct at *m/z* 215 by a NL 102 scan function *(40)*. Many other scan functions have been developed to detect amino acids with higher abundance and enhanced selectivity *(55, 56)*.

### High-Throughput Analyses

Equally important to rapid MS/MS analysis is simple, efficient, and automated sample preparation. In fact, most clinical chemists will point out that measurement of components in a specimen by any method (e.g., MS, HPLC, RIA, enzyme immunoassay, and ELISA) can only be as good as the quality and means of preparing that specimen. Well known to experts in newborn screening, but rarely encountered by clinical chemists, is the dried-blood specimen known as a filter-paper blood spot. The quantity of blood present in the paper varies by hematocrit, diameter of blood spot, degree of saturation, degree of hemolysis, and other minor factors related to blood collection in addition to the rigid controls of the cotton fiber paper and the environment that is present when applying blood (humidity and temperature). Because of these numerous factors, a dried blood spot is a highly imprecise specimen compared with liquids such as urine, blood, and plasma. It cannot be overemphasized that a highly accurate, selective, sensitive instrument such as a tandem mass spectrometer can only be as accurate as the volume and condition of the blood specimen itself. This is an important issue in quality assurance/quality control (QA/QC) aspects of MS/MS, but there are approaches and methods that reduce analytical inaccuracy while maintaining diagnostic accuracy *(57–61)*.

### Preparation for MS/MS Analysis

One or two disks of either 3.2 mm (1/8-inch) or 4.8 mm (3/16-inch) diameter are punched from the areas of dried blood on the filter paper into tubes or the wells of 96-well microtiter plates. Theoretically, single 3.2- or 4.8-mm disks of dried blood whose original hematocrit was 55% contain ~3.4 and 7.6 μL of blood, respectively *(57)*. Specimens are extracted in pure methanol containing known concentrations of stable isotopically enriched amino acids and acylcarnitines. These standards are commercially available individually or as a mixture for newborn-screening applications. Only methanol-extracted acylcarnitines and amino acids mix with the standards. Extraction efficiencies appear to be ~90%, with specific findings for certain analytes having been published *(7, 47, 50, 62, 63)*. It is noteworthy that this MS application of isotope-labeled internal standards is not as accurate as traditional isotope-dilution MS methods. Traditional isotope-dilution MS techniques require liquid specimens for quantification. Therefore, this application is denoted a pseudo-isotope-dilution MS procedure to account for the loss of accuracy and precision from losses during extraction and the uncertainty of the true volume of blood present in the excised disk *(40)*.

Most methods *(14)* require the blood disk to be extracted over a 30-min period with or without gentle shaking. Traditionally, these extracts have been transferred manually or by automated liquid handling devices to new tubes or microtiter plates so that the blood-containing disk is separated from methanol. The methanol extract is dried under a stream of nitrogen with gentle warming of the plate, or nitrogen, or both using driers specifically designed for this purpose. Some laboratories simply use hot air, although concerns remain over oxidation caused by the presence of oxygen and water vapor in air rather than pure dry nitrogen delivered from pressurized cylinders or nitrogen gas generators. Alternatively, some laboratories use centrifugation under reduced pressure *(9)*.

The most common method for extracted metabolite preparation requires esterification (most commonly BEs). Early publications using FAB or FIB ionization demonstrated that this process facilitated ionization and improved sensitivity *(45, 64)*. As ionization methods improved, e.g., through advances in electrospray ionization, underivatized samples could also be analyzed for many acylcarnitines and amino acids (see Figs. 1 and 2). The derivatization step requires heating of the dried analytes with dry, acidified (3N) butanol, which is commercially available, for 15 min at 65 °C. Variations of this generalized procedure have been published with slight changes to the incubation time and temperature. Longer, warmer derivatization conditions produce more extensive hydrolysis of acylcarnitines, whereas cooler, shorter conditions lead to incomplete derivatization. The reaction is stopped by removal of the derivatization reagent by use of dry nitrogen.

Derivatized specimens are reconstituted in a liquid matrix suitable for the type of MS/MS analysis. Historically, this matrix was a viscous glycerol–methanol mixture that contained a surfactant when FAB or FIB ionization was used. Current methods using electrospray ionization require a volatile partially organic-based solvent that may or may not be slightly acidified. The most common solvent is an equal mixture of acetonitrile and water. Some laboratories have used other organic/aqueous mixtures. Furthermore, formic acid may be added in small percentages to acidify solvents and enhance ionization.

### Ionization Methods

Many of the early reports in the literature described FAB or FIB ionization methods with manual analysis. These methods of ionization primarily used manual sample introduction, in which the extract was dissolved in an equal parts methanol–glycerol mixture with a surfactant. This viscous mixture was placed on a probe, inserted in the FAB/FIB ion source, degassed, and then ionized by striking a stainless steel target with the glycerol drop with a beam of atoms or, more commonly, cesium ions. The entire process from sample to sample took ~3–4 min. A few reports described the attempt to automate this process by use of a flowing system called continuous-flow FAB or FIB *(65, 66)*. In this system, a less viscous glycerol–methanol solution was infused and flowed to a stainless steel tip where there was a constant bombardment. However, problems with carryover, sample retention, and

fluidics limited the success of this approach to fewer than 100 samples in any sustained environment.

The true advancement in high-throughput sample analysis came with electrospray ionization, in which a continuous flowing system could be sustained and the sample was injected into this flowing system. Electrospray not only solved the carryover and sample retention problems but improved the ionization efficiency of many acylcarnitines and amino acids. Its predecessor, thermospray *(67)*, also showed the advantages to this type of approach but was much less robust than either FAB or electrospray. Today, electrospray ionization is the primary means for specimen ionization in MS/MS *(68–71)*. FAB/FIB ionization has all but been replaced. It is noteworthy that the 2002 Nobel Prize in Chemistry was awarded to two mass spectrometrists (J. Fenn and K. Tanaka) for their development of soft ionization techniques, which include electrospray ionization *(41)*.

With electrospray ionization, typically 10 μL of sample extract is injected into a flowing stream operating at 20–50 μL/min. Typical injection rates between samples are 2 min, giving a potential 400- to 600-sample capacity per instrument per day. Practically speaking, however, this volume is typically 200–400 specimens per instrument per day because maintenance issues, repeat specimen analysis, and 8-h vs 12-h work days are included. Solvent delivery is via HPLC or syringe pump with no chromatography, a method called flow injection analysis. Most problems encountered in this type of analysis are particle or air obstructions. Use of an inline filter prevents particle obstructions, and use of degassed solvents prevents air obstructions. Many laboratories, including ours, have used unique approaches to reduce down time associated with flow injection analyses.

### Modifications, Enhancements, and Variations

Many recent publications have described alterations to the essential MS/MS newborn-screening methodology published in the 1990s *(7, 45, 50, 62–64, 72, 73)*. Some of these are sufficiently important to describe here. The core method was described in the previous section and includes minor variations. Perhaps the most important change is the analysis of amino acids and acylcarnitines without derivatization. This procedure avoids the use of acidified butanol and the derivatization step, saving ~30 min in total preparation time. However, sample throughput remains largely unaffected because the time-limiting step for large numbers of samples is MS/MS analysis rather than sample preparation. We have compared both derivatized and underivatized acylcarnitines and include one of them here (Fig. 2). Because there is sparse literature to date, we are providing a few observations that are consistent with what is known in laboratories experienced in MS/MS. Overall, both methods are comparable but with a few precautions that can be important. In general, in an identical analysis, sensitivity is less for underivatized acylcarnitines than for BEs. Simply stated, the ionization efficiency of a positive ion is greatest when the number of potential negatively charged sites is reduced, hence, derivatization of carboxylic acids to their BE forms *(40)*. Because the vast majority of ions have a positive charge and only a small percentage of ions are required for detection, the analysis of underivatized specimens is largely not problematic. Dicarboxylic acids, on the other hand, have two potential sites of negative charge, and acylcarnitines can exist as neutral or a with net negative charge. This produces a greater loss of sensitivity for dicarboxylic acid, for which glutaric acid is an important member of this family. Comparative sensitivity (peak ratios of C5DC relative to $d_3$-C8) is ~33–66% depending on ionization conditions. Dicarboxylic acids, because of multiple derivatization sites, demonstrate mass shifts that can affect interpretation, especially in profiles where C5DC is not produced in particularly high abundance (<0.5 μmol/L). Fig. 2 includes a GA-I specimen whose C5DC was particularly abundant and would not be affected by a loss of sensitivity because of no derivatization. However, the mass shift and smaller signal intensity of C5DC are clearly noted. Of greater concern is amino acid analysis, in which dicarboxylic acids produce mass shifts that may be more problematic. Often a mixture of mono- and diderivatized species is observed for a compound such as glutamic acid. Early data regarding sensitivity and derivatization issues in the analysis of acylcarnitines have been reported recently regarding FC analysis in plasma *(74)*.

The other area of importance regarding flow injection analysis is source design and high-throughput analysis. In newborn screening, small fibers from the paper and other particles are often present in the extraction solvent. Methods that directly sample this solvent will be more prone to injector clogging than procedures that transfer solvent to another well, dry the solvent, and reconstitute in a clean solvent. Still better, methods that derivatize with an acid reagent may in fact dissolve some of the fibers. Some laboratories have used filters in the flow stream, but this generally causes an increased dead volume that must be compensated by higher flow rates (~50 μL/min). We have minimized dead volume by use of an inner fused-silica capillary that is directly connected to the injector and proceeds to the end of the electrospray ionization electrode. We have achieved low flow rates (18 μL/min) and have had no problems with fibers creating flow interruptions.

### Result Processing and Interpretation

A mass spectrometer provides two interrelated items of information: the number and mass values of ions striking its detector. The quantity of ions (ion count, ion intensity) for a particular mass (produced by a precursor, product, or NL) can be tabulated or graphically represented as a spectrum. Therefore, each mass selected in various scan functions has an associated ion count value, a quantity known generally as ion intensity. Results are tabulated in

a list or displayed in a bar chart known as a mass spectrum. A MS/MS analysis comprises numerous scans that may be cumulative or averaged over a specific period of time, generally 2 or 3 min in this application. An individual scan includes acquisition of one set of data from one or more acquisition functions such as selected-reaction monitoring (SRM) scanning, Pre scanning, product ion scanning, or NL scanning, for ~1–2 min. As the compounds pass into the mass spectrometer, the number of ion counts increases to a maximum value and then decreases. The total number of ions, regardless of mass values, is defined as the total ion count. Data are taken specifically from the top 50% of the elution profile of the total ion count to maximize sensitivity. Generally this represents approximately one-third of the total data acquisition period. Monitoring the peak shape of a total ion chromatogram is important because it provides information on the relative quality of the injection and the analysis and provides an index for sample carryover.

In the early 1990s, results were primarily interpreted by visual examination of the mass spectra, not unlike how organic acid interpretation is still carried out today. Although mass intensity lists could be derived, this was a tedious task and was used mainly for abnormal results or research. With improvements in software and interest by the major manufacturers of mass spectrometers, software was developed to assist in data processing and quantitative calculations. The earliest version was a simple macro-type program that automated many of the tasks performed. Rasheed and coworkers *(55, 73)* used a more advanced program in their laboratory, known as CAMPA. This was followed by further developments that essentially exported the ion intensity data into spreadsheet- or database-compatible formats. Many programs enabled calculation of ratios of the ion intensities of two masses, often the mass of a metabolite and its internal standard. Incorporation of mathematical functions enabled further calculations to produce numbers relating to concentration. In addition, the ability to set flags (high and low limits) facilitated more rapid and accurate interpretation. Furthermore, calculation of molar ratios (concentration ratios) and raw ion intensities have served to improve the sensitivity of diagnostic screening for metabolic diseases.

Interpretation is perhaps the most critical component of recognition of a disease. Software is a tool that can be used to facilitate this process, but it does not replace the experience needed to recognize complex profiles. Current approaches *(12, 75)* are described in recent publications and will be described in further detail in subsequent sections

## Quality Assurance

Many factors contribute to the success of a newborn metabolic screening program. In actuality, blood sample collection after birth and before hospital discharge marks the beginning of the newborn-screening process. Proper analyses rely heavily on blood-spot quality. Consequently, an improperly collected blood spot may disrupt the entire screening process for a newborn. A properly collected newborn blood specimen requires several elements, including suitable collection time, appropriate application of heel-stick blood to filter paper, sufficient drying before packaging, and timely shipment to the screening laboratory. A disturbance in any part of this sequence can lead to poor or insufficient sample analysis. Proper collection time avoids the possibility of dubious results affected by age. Careful spotting of the blood sample on filter paper avoids inaccurate analysis results caused by undersaturation, oversaturation, and abraded specimens. Ample drying of a sample and its prompt receipt by a laboratory can prevent sample degradation *(76)*.

Blood-spot materials have been developed by several organizations to be used in newborn-screening laboratories for QA/QC. A few key reports have been published describing the features of these materials as developed for MS/MS *(57–59, 77)*. One of the challenges of developing QA/QC materials for MS/MS arises from the complexity of multianalyte systems that measure not one, but many metabolites for which a positive screening result may not be based solely on increases in a single metabolite. The development and use of such materials present unique problems. For example, although preparation for MS/MS of materials specifically containing multiple metabolites makes for efficient use of QC specimens, these same QA/QC materials are not appropriate for other methods that have reduced selectivity (i.e., phenylalanine and tyrosine can be measured selectively by MS/MS, whereas phenylalanine and tyrosine interfere with each other in fluorometric assays). Another dilemma involves disorders for which multiple acylcarnitines indicate disease. For example, with MCAD deficiency, should all metabolites that are increased in this disease be included or only the major metabolite, C8? In proficiency testing, could a disease state be artificially created by simply adding concentrations of specific metabolites into a blood spot? How could a newborn blood specimen be mimicked using blood collected from adults? Recent research suggests that the complexities of developing a QA/QC program for use of blood with multianalyte technologies will be quite challenging. Issues related to QA themselves require a review and cannot be completely discussed here. As experience is gained in this area, reports will likely suggest novel QA/QC methods and approaches that will accommodate multianalyte screening using MS/MS.

## Disorders of Amino Acid, Fatty Acid, and Organic Acid Metabolism

A review of the literature reveals that many disorders of fatty acid oxidation, organic acid metabolism, and amino acid metabolism are detectable by use of MS/MS. Recently, however, there has been much confusion about

which disorders are detectable by MS/MS and how it should be used in their detection. The number of diseases detectable by MS/MS, as reported in the literature, generally numbers around 20, with ranges from 10 to >40 *(16, 17, 72, 78)*. Our discussion here concerns primarily newborns, although much of the literature describes children whose disorders were detected at an older age when they became unwell. In this review, an analytical, metabolite perspective will be used rather than a disease perspective, as has been used elsewhere *(12)*. Also note that for simplicity the discussion of each individual analyte occurs from the perspective of its BE derivative unless otherwise noted.

## AMINO ACIDS

*Leucine.* Newborn screening by MS/MS is not free of challenges. A relevant example is leucine analysis for maple syrup urine disease (MSUD). Routine newborn screening for MSUD began in 1964 with use of the Guthrie bacterial inhibition assay technology *(79)*. In MS/MS analysis for MSUD, the metabolite of primary interest, leucine, has a mass of 188 Da, which is the same mass as the BE of one of its isomers, isoleucine. The mass spectrometer cannot differentiate between these two compounds. Fortunately in MSUD, leucine is dramatically increased and there is also an increase in other branched chain amino acids, including isoleucine and valine. Thus, MSUD detection using MS/MS relies on either one or all of these increases. Because *m/z* 188 is both leucine and isoleucine and is generally extremely increased when MSUD is present, MS/MS newborn screening is likely to detect this disease even if it is nonspecific for leucine. It is important to note that this method cannot be used to accurately quantify leucine and isoleucine without chromatography. Furthermore, the presence of hydroxyproline and creatine will also contribute ions at *m/z* 188, making precise measurements by MS/MS difficult. Any laboratory hoping to use MS/MS for newborn screening should perform its own validation experiments to illustrate the high selectivity of these instruments. The success of MS/MS as a reliable newborn-screening tool for MSUD detection has been detailed *(62, 80)*.

*Phenylalanine.* PKU is a disorder of amino acid metabolism (frequency of ~1:12 000–1:15 000) resulting from a deficiency in the enzyme phenylalanine hydroxylase, which catalyzes the oxidation of phenylalanine to tyrosine. Phenylalanine is substantially increased in affected individuals, producing mental retardation when untreated. Most current methods are poorly selective, such as fluorometry, or poorly quantitative, such as the bacterial inhibition assay. MS/MS surpasses both methods by selectively detecting the BE of phenylalanine at *m/z* 222 as well as the BE of tyrosine at *m/z* 238 *(50)*. By measuring multiple amino acids, MS/MS permits a more accurate diagnosis of PKU based on the molar ratio calculation of phenylalanine to tyrosine, a demonstrated indicator of PKU and other hyperphenylalaninemias *(8)*. Furthermore, MS/MS can distinguish with a reasonable degree of certainty a true PKU vs a hyperphenylalaninemia attributable to circumstances surrounding infants in neonatal intensive care units that may be supplemented with amino acids. In some cases, a hyperphenylalanine may be tetrahydrobiopterin-dependent and thus distinguishable by second-tier assays from other cases of increased phenylalanine *(81)*. Several recent reports *(51, 52, 82, 83)* have confirmed many of the findings established in the 1993 and 1998 publications regarding MS/MS analysis for phenylalanine *(8, 50)*.

*Tyrosine.* As with phenylalanine, a NL 102 scan function is used in measuring tyrosine. Increased tyrosine may indicate several metabolic states, including three variations of tyrosinemia, types I, II, and III, and a transient form, transient neonatal tyrosinemia (TNT) *(84)*. Immature enzyme systems or vitamin C deficiencies are thought to lead to TNT. Deficiency of the enzyme fumarylacetoacetase leads to hepatorenal tyrosinemia (type I) *(85)*. Deficiency of another enzyme, tyrosine aminotransferase, causes oculocutaneous tyrosinemia (type II) *(85)*. Tyrosinemia type III arises when the enzyme 4-hydroxyphenylpyruvic acid oxidase is deficient *(85)*. Although the amino acid tyrosine, detected at *m/z* 238, may indicate a case of tyrosinemia when significantly increased, it is not possible to distinguish among the three primary types of tyrosinemia by MS/MS. In the experience of our laboratory, one case of the least common form of tyrosinemia, type III, has been detected and confirmed by other clinical methods. Unfortunately, not all cases of tyrosinemia will exhibit a significantly increased tyrosine concentration during the neonatal period. Several false-negative cases of tyrosinemia type I have been reported in laboratories relying on tyrosine analysis alone that collect samples on day 2 of life. Succinylacetone analysis may be the only reliable method for detection of type I *(86)*. In addition to potential false-negative rates, many positive results (often considered false-positive results) arise from the high incidence of TNT in the general newborn population *(87)*.

*Methionine.* Methionine is detected by a NL 102 scan function at *m/z* 206. When increased, methionine is the metabolite used in MS/MS analysis to identify cases of isolated hypermethioninemia or homocystinuria, which often result from deficiencies of methionine adenosyltransferase and cystathionine β-synthase, respectively *(63)*. Unfortunately, methionine concentrations tend to be very low during the newborn period even when a related disorder is present *(63)*. This phenomenon may be attributed to several factors, including early specimen collection and lower dietary protein intake. Notably, our laboratory detected an abnormal methionine concentration at <24 h of age in a patient diagnosed prenatally with homocystinuria *(12)*. Methionine measurement for homocystinuria detection is illustrative of the challenges

MS/MS screening presents because it may not always be significantly increased and thus readily detectable by this assay in the newborn period *(12)*. Clearly, in two cases of homocystinuria detected by our newborn-screening program, the concentrations of methionine were <20 mg/L, values below most traditional cutoff concentrations for homocystinuria detection with methionine quantification. Caution is advised, however, because the concentration of methionine may not be increased in all cases *(88)*.

*Citrulline.* Citrulline is yet another clinically relevant amino acid detectable by MS/MS *(89, 90)*. It is detected by use of a NL 119 scan at *m/z* 232 and may also be analyzed by use of a NL 102 scan at *m/z* 215, a mass produced by source-induced dissociation of ammonia, a feature common to basic amino acids. In acute neonatal citrullinemia, citrulline is substantially increased. In cases of argininosuccinic aciduria, citrulline may be mildly or moderately increased. New research suggests that low concentrations of citrulline are observed in infants affected with carbamoyl phosphate synthase deficiency and perhaps ornithine transcarbamylase deficiency. However, it is our experience that the potential false-positive and -negative rates for detecting carbamoyl phosphate synthase or ornithine transcarbamylase deficiencies are high with MS/MS and may limit its usefulness. The false-positive and -negative rates for acute neonatal citrullinemia and argininosuccinic aciduria appear to be low because we have diagnosed several cases in newborns.

*Other amino acids.* Other amino acids that can be measured routinely by MS/MS include glycine (*m/z* 132), alanine (*m/z* 150), serine (*m/z* 162), valine (*m/z* 174) *(62, 80)*, histidine (*m/z* 212), glutamic acid (*m/z* 260), glutamine (*m/z* 186) *(56)*, arginine (*m/z* 231) *(89, 90)*, and ornithine (*m/z* 189) *(89, 90)*. Many are detected by use of NL 102 or other scan functions, whereas others require specific scan settings. Few have been useful in identifying disorders in routine newborn screening.

### FATTY ACID ACYLCARNITINES

MS/MS is uniquely suited to measure acylcarnitines in blood. Several reports have described how these unique markers reflect the status of mitochondrial β-oxidation *(91–94)*. Fig. 3 illustrates the roles of carnitine, acylcarnitines, and fatty acids in fatty acid oxidation and elucidates biochemically the cellular location of defects in β-oxidation and the metabolites likely affected. A series of icons representing various structures are used rather than complex compound names to provide a visual understanding of the process of β-oxidation. For other reviews on the matter, please refer to a review by Rinaldo et al. *(94)* or appropriate chapters in *The Metabolic and Molecular Bases of Inherited Disease (84)*.

It is clear that no single metabolite will entirely provide the information needed to diagnose or screen for a metabolic defect. Nearly all metabolic disorders present a pattern of metabolites, and it is these patterns that makes a profile highly presumptive for a particular disorder. But more importantly, confirmatory tests using other methods are absolutely necessary to provide adequate information for a diagnosis. A further complicating factor for acylcarnitine analysis by MS/MS is age relative to disease state. It is known that the concentrations of FC and acylcarnitine decrease in the first week of life, making interpretation features distinctly different for older infants vs newborns. In addition, many metabolic defects may deplete carnitine dramatically, producing few diagnostic acylcarnitine metabolites *(95)*. The significance of a FC deficiency becomes greater in older infants; it therefore is critical to recognize this deficiency state. With all of the complex interactions, proficient and extensive knowledge of mitochondrial β-oxidation is therefore critical to understanding which acylcarnitines are produced in a particular defect and how the profiles suggest various disorders.

In the next section, we will use a approach to discuss MS/MS analysis and disease that will differ from those of other reviews. We will emphasize metabolites and which disease they may indicate, compared with the disease and which metabolite is produced. This approach emphasizes the analysis rather than diagnosis, which involves many other tests, confirmation, clinical history, and other factors. One final note is that most of the knowledge of acylcarnitine profiles and disease association was discovered in infants who were ill, as much of the literature reveals. Newborn-screening patterns were discovered to be clearly different in infants who were not well at the time of discovery and who were subject to stresses after birth. A pattern clearly emerged that distinguished newborn metabolic profiles from those of older infants (>7 days). With rare disorders, it has taken the cumulative experience of nearly 2 million infants to discern with a reasonable degree of scientific certainty abnormal vs normal patterns in newborns, infants, and children. Below is a discussion of what is known to date from the experience of our laboratory and the experiences of numerous other laboratories practiced in acylcarnitine analysis.

*FC.* FC is detected by use of a Pre 85 scan or Pre 103 scan at *m/z* 218 for BEs. Many laboratories acquire FC within the full scan of acylcarnitines (*m/z* 218–500), whereas others analyze FC as a separate analyte with its own optimized settings and scan function, such as SRM. Monitoring FC is important for several reasons in addition to disease detection *(49, 96)*. For example, increased FC in QC samples suggests hydrolysis of acylcarnitines during storage. FC is also produced during derivatization from hydrolysis of acylcarnitines. Instability of acylcarnitines and production of FC is a problem not just for MS/MS but for all FC analyses and is a topic in many publications describing analysis of FC *(96–98)*.

According to Wilcken et al. *(99)*, MS/MS profiles that show no increases in acylcarnitine but very low FC

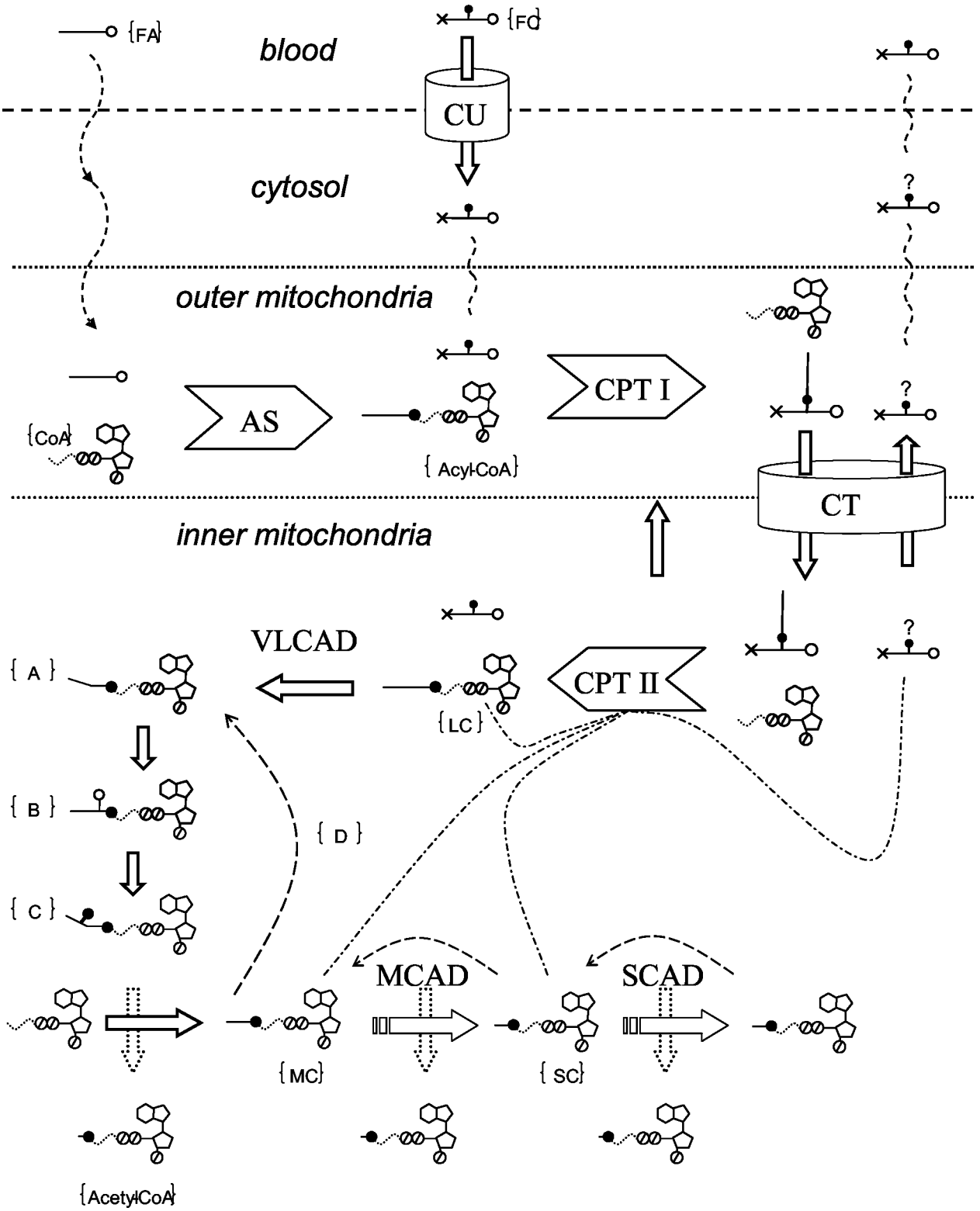


Fig. 3. Illustration of β-oxidation and the roles of carnitine and acylcarnitines in metabolism.

*FA*, fatty acids; *LC*, long-chain acyl-CoA; *MC*, medium-chain acyl-CoA; *SC*, short-chain acyl-CoA; *A*, product of acyl-CoA dehydrogenase; *B*, product of enoyl-CoA hydratase; *C*, product of 3-hydroxyacyl-CoA dehydrogenase; *D*, product of β-ketothiolase; *CU*, carnitine uptake; *AS*, acyl-CoA synthase; *CT*, carnitine translocase. The second round of β-oxidation at the appropriate chain lengths is represented by *dashed arcs*.

indicate a possible carnitine transporter defect. The authors discussed four known cases of carnitine transporter defect, confirmed by other methods such as fibroblast uptake studies; they believed that two of the cases had FC concentrations low enough to be detected by MS/MS newborn screening *(99)*. In our experience, MS/MS identification of a low FC concentration may not be entirely reliable for diagnosis of primary carnitine deficiencies in the newborn period. Measuring FC is nevertheless important in secondary carnitine deficiencies, e.g., carnitine palmitoyltransferase I (CPT I) deficiency *(46, 10)*. In CPT I deficiency, production of the long-chain acylcarnitines hexadecanoylcarnitine (C16) and octadecanoylcarnitine (C18) is inhibited because of a defect in the enzyme CPT I, which usually would transfer long-chain fatty acyl-CoAs to carnitine for acylcarnitine production *(46)* (see Figs. 1 and 3). Thus, C16 and C18 concentrations are often low, whereas the FC concentration is significantly increased in CPT I deficiency *(46, 100)*. However, decreased C16 and C18, along with increased FC, alone are not sufficient criteria for CPT I detection. Instead, the ratio of FC to the sum of C16 and C18 is believed to be sufficient for differentiation between patients with CPT I deficiency and those receiving supplementation or suffering from other conditions that may affect fatty acid metabolism *(46)*.

*C2.* No known disorders are recognized solely by an increase or decrease in C2, which is measured at *m/z* 260 by a Pre 85 scan. However, an increase in C2 may reflect ketosis or other conditions in which there is a generalized increase in short-chain acylcarnitines. A deficiency of C2 coupled with low FC increases the probability of a primary or secondary carnitine deficiency. Note that glutamate is also detected at *m/z* 260 (BE form), and therefore special calculations are required to accurately quantify C2 and differentiate Glu and C2.

*Butyrylcarnitine (C4).* C4 and an isomer, isobutyrlcarnitine, are detected at *m/z* 288 by a Pre 85 scan. An increase in only C4 can be produced by a deficiency of short-chain acyl-CoA dehydrogenase (SCAD), as well as isobutyryl-CoA dehydrogenase *(101)*. These two compounds, which are isomers, represent two different disorders, a problem encountered when using MS/MS for other metabolites, such as leucine and isoleucine, and for isovalerylcarnitine (C5). From a screening perspective, this is acceptable, but from a diagnostic perspective it is not. Clearly, repeat testing that remains positive will require confirmatory tests, such as GC/MS analyses for organic acids to elucidate which disorder produces the increased C4. These are not the only causes of an increased C4, however, especially when this increase is present with other increased acylcarnitines. The concentrations of other acylcarnitines may suggest which disease is likely. For example, an increase in C4 together with medium- and long-chain acylcarnitines may reflect multiple acyl-CoA dehydrogenase deficiency (MADD). An increase in C4 with primarily increases in C2 and propionylcarnitine (C3) may be produced by carnitine administration, which is now occurring routinely in some hospitals. C4 may also be produced with other short-chain acylcarnitines, reflecting a state of ketosis, or in some instances a sample collected from a child who recently died. In general, expressing the ratio of C4 to another short-chain acylcarnitine is helpful in differentiating a true SCAD or other metabolic disorder from healthy patients, reducing false-positive results.

*Octanoylcarnitine.* Octanoylcarnitine (C8) is an 8-carbon (mid-range of fatty acid chain lengths) fatty acylcarnitine derived from octanoic acid. It is detected at *m/z* 344 by use of a Pre 85 scan function. A defect in MCAD, the enzyme responsible for oxidizing octanoyl-CoA, will lead to its accumulation as well as the accumulation of hexanoyl-CoA, decanoyl-CoA, and decenoyl-CoA *(102)*. In MCAD deficiency, C8 and the aforementioned associated acylcarnitines are produced in higher concentrations (Fig. 4a), evidence of which can be detected in blood and other biological fluids, including plasma, serum, bile, and urine, and are increased compared with controls. Most MCAD-deficient newborns produce C8 at very high concentrations *(7)*, especially those who are homozygous for the common mutation 985A>G (Fig. 4a). Typically, homozygotes produce a C8 concentration in the range of 4–10 μmol/L, although some compound heterozygotes will also produce these concentrations. Generally, compound heterozygotes produce lower C8 concentrations, in the range 1–4 μmol/L in newborn specimens *(15)*. Although C8 is the metabolite of primary interest in detecting MCAD deficiency by MS/MS, it is important to note that severity of disease is not easily predicted based solely on C8 concentration *(95, 103)*.

A poorly defined group of infants will be detected with C8 concentrations ranging from 0.35 to 1 μmol/L. It is thought that many in this group have one copy of the most common mutation causing MCAD deficiency (Fig. 4b), A985G, and a "milder" mutation, such as T199C *(104)*. However, there are cases of newborns with <1 μmol/L of C8 that have become metabolically decompensated, making it somewhat difficult to precisely define a cutoff for MCAD deficiency.

The age of an infant affects the C8 concentration in blood because the concentrations of carnitine and acylcarnitines decrease rapidly after the first few days of life *(7)*. Typically the concentration by day 7 has decreased considerably in many infants. Therefore, separate criteria must be used to interpret data from infants more than 7 days of age. This general rule should be applied for all disorders of fatty acid or organic acid metabolism. Furthermore, the effects of total parenteral nutrition are unknown at this time and may account for lower concentrations of C8.

Not all increases in C8 result from MCAD deficiency. Other disorders of fatty acid oxidation, such as MADD

and the poorly understood medium-chain hydroxyacyl-CoA dehydrogenase (MCHAD) deficiency, may also produce an increase in medium-chain acylcarnitines. Generally, an increased C8 concentration is mild in these disorders compared with MCAD deficiency and is accompanied by other important metabolite markers. MADD, also referred to as glutaric acidemia type II (GA-II), is characterized by variable acylcarnitine profiles that in addition to increases in medium-chain acylcarnitines such as C8 (see Fig. 4c), may also display increases in various short- and long-chain acylcarnitines *(75)*. Other metabolites that are usually increased in the blood of newborn MADD patients include C5, decanoylcarnitine (C10), dodecanoylcarnitine, tetradecanoylcarnitine (C14), and tetradecenoylcarnitine (C14:1). MS/MS analysis of dried blood specimens from two siblings presumptive for MADD, one of whom did not survive past the second day of life, revealed abnormal acylcarnitine profiles indicative of MADD in the newborn specimens of each, as did a specimen taken during metabolic crisis at age 9 from the sibling who survived, but a specimen taken from the surviving sibling after treatment with carnitine did not *(105)*. Unfortunately, referring to MADD as GA-II can be misleading because glutaric acid, although sometimes increased in urine organic acid profiles of MADD patients, is often not increased in their blood acylcarnitine profiles. Although only a few cases of MADD have been identified prospectively by MS/MS newborn screening *(106, 107)*, it is likely an efficacious tool for both presymptomatic detection of MADD and postsymptomatic diagnosis as has been demonstrated *(105)*.

Certain drug treatments, such as valproic acid (Depacoat®) and medium-chain triglyceride (MCT) oil (Fig. 4d), also produce an 8-carbon acylcarnitine (Fig. 4) in blood, as well as C10 and hexanoylcarnitine (C6), or interfere with the metabolism of medium-chain fatty acids, producing iatrogenic MCAD. As reported by Van Hove et al. *(103)*, the distinguishing factor between an increase in C8 attributable to valproic acid therapy or MCT oil supplementation and one attributable to MCAD deficiency is the ratio of C8 to C10, which is significantly increased in MCAD-deficient patients but not in those receiving valproate or MCT oil. This ratio is also reliable in distinguishing MCAD deficiency from other metabolic conditions that produce an increase in C8, such as MADD *(75, 103)*. Fortunately, valproate is not commonly administered to newborns and therefore is not usually an issue in newborn-screening programs. Carnitine administration will increase short- and medium-chain acylcarnitines (Fig. 4e).

It is worth noting that occasionally interfering substances are present in blood at very high concentrations. If these compounds have a minor fragmentation pathway that produces a product ion at *m/z* 85, they will be detected in an acylcarnitine profile. Often these patterns are recognizable, such as with EDTA, where metabolites are produced at *m/z* 272, 349, 405, and 461. Occasionally, a compound is present that is 1 Da less than the metabolite of interest, and if sufficiently high in concentration, its natural isotope contribution (1 amu higher in mass ([M+1]) will cause interference. This is the case with a unknown substance routinely detected at *m/z* 343. Its

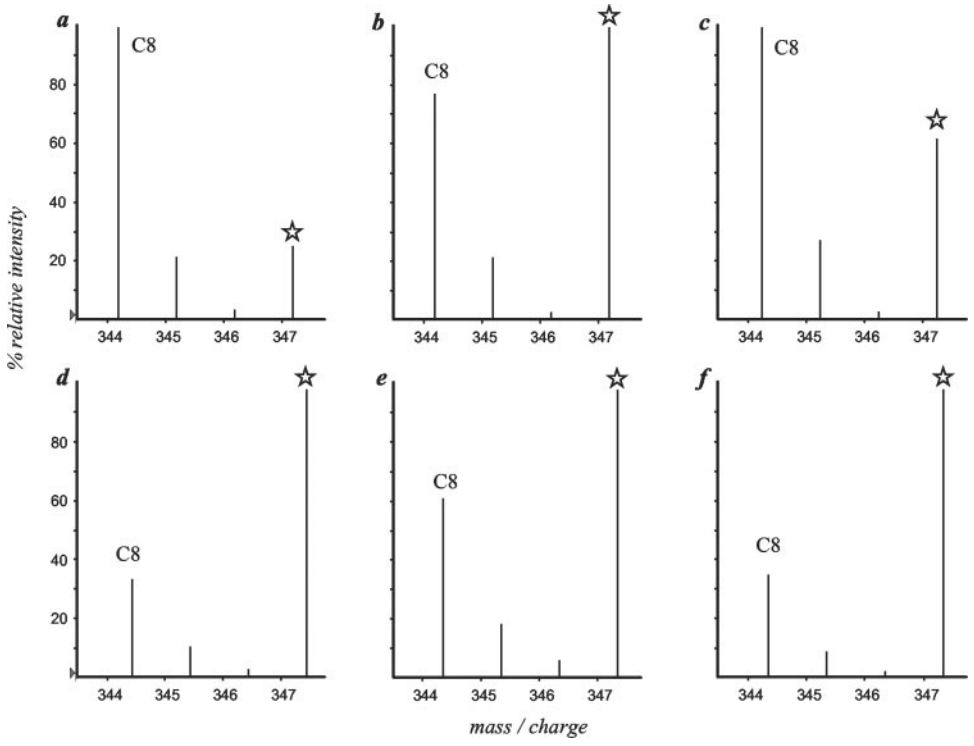


Fig. 4. Pre 85 MS/MS scans of C8 and its internal standard [$^2H_3$]-C8.

(*a*), MCAD deficiency, homozygous A985G; (*b*), MCAD deficiency, heterozygous A985G; (*c*), MADD; (*d*), treatment with MCT oil; (*e*), treatment with carnitine; (*f*), isotopic interference from a unknown substance detected at *m/z* 343. The internal standard is indicated with a ☆.

isotope is detected at *m/z* 344 and can lead to a false result if performing SRM vs full-scan analysis (Fig. 4f).

*Tetradecenoylcarnitine (C14:1).* C14:1, another long-chain acylcarnitine, represents the metabolite most reliably indicative of very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency *(75, 108)*. C14:1 is detected at *m/z* 426 by a Pre 85 scan analysis. In addition to C14:1, C14 is increased in VLCAD deficiency with potentially other acylcarnitines as well, including hexadecenoylcarnitine, C16, octadecenoylcarnitine (C18:1), and C18 *(75, 109)*. Detection of VLCAD deficiency is best in the newborn period because diagnostic acylcarnitines rapidly decrease in specimens collected after 7 days. Clearly, presymptomatic diagnosis of VLCAD deficiency by use of MS/MS dried-blood spot analysis is possible *(108)*, but caution is needed for older infants. It is important to note than increased C14 and C14:1 may be produced in other disorders as well, such as carnitine palmitoyltransferase II (CPT II), carnitine/acylcarnitine translocase (CACT), MADD, and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency. In addition, too much blood on a card because of poor specimen application may increase C14 along with other acylcarnitines, increasing the risk for a false-positive result.

*Hexadecanoylcarnitine (C16).* C16, also referred to as palmitoylcarnitine, is a long-chain acylcarnitine detected at *m/z* 456 by a Pre 85 scan. A significant increase is most commonly indicative of CPT II or carnitine/acylcarnitine translocase deficiency, but may also accompany other fatty acid disorders, including VLCAD deficiency and even MADD. In CPT II deficiency, C16 and other acylcarnitines, C18 and C18:1, are increased. Although C14 may be increased, the relative concentration of C14 to C16 is low compared with VLCAD, in which the ratio is reversed relatively speaking. In CPT I deficiency, C16, C18, and C18:1 are low relative to an increased FC. Although several cases of CPT II or translocase deficiency have been detected, relatively few cases of CPT I deficiency have been documented as being detected in newborn screening.

*Hydroxyhexadecanoylcarnitine (C16OH).* C16OH, detected at *m/z* 472, is one of several long-chain hydroxyacylcarnitines present in significantly increased concentrations in both trifunctional protein (TFP) and LCHAD deficiency. LCHAD and TFP deficiencies are indistinguishable by acylcarnitine profile alone *(110, 111)*, although molecular analysis is available for the most common LCHAD mutation. In addition to C16OH, hydroxyoctadecenoylcarnitine (*m/z* 498) and hydroxyoctadecanoylcarnitine (*m/z* 500) are usually also increased in LCHAD or TFP deficiency *(75)*. Occasionally, 3-hydroxytetradecanoylcarnitine is increased. These increases may be accompanied by milder increases in C14, C16, C18:1, or C18 *(75)*. In cases of treatment or milder presence of the long-chain hydroxy metabolites of interest, an increased ratio of C16OH to C16 is invaluable in ascertaining TFP or LCHAD deficiency. It is noteworthy that interference is possible by an unidentified compound that produces a two peaks at *m/z* 472 and 473.

*Other fatty acid oxidation metabolites.* 3-Hydroxybutyrylcarnitine (*m/z* 304 by a Pre 85 analysis) is increased in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency *(112)*. It is also increased in a typical postmortem specimen together with C4. In MCHAD deficiency, 3-hydroxydecanoylcarnitine (C10OH; *m/z* 388) is potentially increased. Increased C10OH was observed in a patient confirmed to have one copy of the common mutation for MCAD and one copy of a MCHAD mutation.

### ORGANIC ACID ACYLCARNITINES

*C3.* The short-chain acylcarnitine C3 is an extremely important diagnostic marker for certain organic acidemias. It is detected at *m/z* 274 by use of a Pre 85 scan function. An increase in C3 concentration may indicate the presence of either a propionic acidemia (PA), a methylmalonic acidemia (MMA), any of various cobalamin defects, or deficiency of vitamin $B_{12}$. Propionyl-CoA carboxylase is the primary enzyme deficient in cases of PA. For MMA, C3 will accumulate at lower concentrations than in PA as a result of either a defect in methylmalonyl-CoA mutase or adenosylcobalamin. Extremely high concentrations of C3 (>10 μmol/L) generally indicate acute PA. Moderate increases in C3 (>6–10 μmol/L) usually indicate a MMA or cobalamin defect. This rule, however, is an approximation, and organic acid analysis and other confirmatory tests are required to accurately diagnose any of these disorders *(47)*.

In addition to the C3 concentration, relative ratios of C3 to C2 or C3 to C16 are important to improving the diagnostic efficacy of the screening test as well as reducing the false-positive rate. Some MMA disorders may not produce significant concentrations of C3 and will not be detected. One case has been documented *(47)*. In some cobalamin defects it is also thought that both C3 and methionine will be increased. It is important to recognize that the potential for false positives in newborn screening for PA and MMA may be significant depending on the cutoff concentration of C3 and the relative ratios of C3 to C2 and C3 to C16. One important question that frequently arises is whether methylmalonylcarnitine is increased in this MMA. The answer to this question is no. In all confirmed disorders we have not observed an increase in methylmalonylcarnitine (*m/z* 274), but we frequently observe an increase in healthy newborns.

*C5/2-Methylbutyrylcarnitine.* C5, *m/z* 302 by a Pre 85 analysis, is indistinguishable from 2-methylbutyrylcarnitine by this method. An increased concentration is indicative of either isovaleric acidemia *(113)* or 2-methylbutyryl-CoA dehydrogenase deficiency *(114–117)*. Because these

compounds are isomers, neither of these disorders can be distinguished by MS/MS measurement of C5. Notably, C5 and 2-methylbutyrylcarnitine are not the only substances that can produce an increased signal at *m/z* 302. Pivalic acid, commonly present in many pharmaceutical preparations, also produces a signal at the C5 mass *(113)*. A moderate increase in C5 is also observed in newborns with MADD. A generalized increase in short-chain acylcarnitines produced by carnitine supplementation may also increase C5 above reference values. As described for C4 and other acylcarnitines, a repeat analysis will not elucidate which of the disorders is present with the exception of ruling out a false result attributable to supplementation or pivalic acid. Certainly long-term follow-up and confirmation require organic acid analysis by GC/MS to differentiate these diseases.

*3-Hydroxyisovalerylcarnitine (C5OH).* Increases in C5OH or 2-methyl-3-hydroxybutyrylcarnitine are detected at *m/z* 318 by a Pre 85 scan and may indicate one of four disorders. C5OH is increased in 3-methylcrotonyl-CoA carboxylase deficiency, 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, and 3-methylglutaconyl-CoA hydratase deficiency, whereas 2-methyl-3-hydroxybutyrylcarnitine is increased in β-ketothiolase deficiency and 2-methyl-3-hydroxybutyrl-CoA dehydrogenase deficiency *(118)*. β-Ketothiolase may also be characterized by an additional increase at mass 300 that represents tiglylcarnitine. Infants born to mothers affected with 3-methylcrotonyl-CoA carboxylase deficiency will exhibit a high concentration of C5OH by MS/MS but will have normal urinary organic acid profiles. With time, the concentration of C5OH decreases, but initial repeat analysis will still be abnormal for the disorders. Many mothers have been diagnosed with this disorder after screening of their infants. It is noteworthy that a small fraction of infants with biotinidase deficiency will have increased C5OH. However, screening for biotinidase deficiency by MS/MS is unreliable.

*C5DC.* C5DC is a dicarboxylic acylcarnitine detected as a BE at *m/z* 388 (*m/z* 276 without butyl esterification) by a Pre 85 scan (Fig. 2). Increased C5DC is indicative of GA-I (Fig. 2). Often in cases of GA-I, the molar ratio of C5DC to C16 is also increased, thus becoming another criterion for identifying presumptive cases. Relatively rare in the general population, GA-I occurs more frequently in populations that are more commonly consanguineous, such as the Amish of Lancaster County, PA *(119)*, and the Canadian aborigines of Manitoba/Northwestern Ontario *(120)*. Infants with MADD or GA-II may also have increased C5DC. Because the analysis of newborn specimens is more sensitive than those from older infants, GA-I may be missed entirely by MS/MS analysis if the age of the infant is more than 7 days. This has been documented in a study by Smith et al. *(121)*. The case described unfortunately is only partially correct. The value in the original newborn blood specimen was significantly increased, and an organic acid analysis was requested. Repeat analysis showed only an increased C5DC-to-C16 ratio, which is typical for newborns. However, because this analysis was part of a pilot study and the age of the infant at collection was not identified, the interpretation was typical. This case documented in the report, albeit inaccurately, points out the necessity to ordering follow-up analysis using other methodologies, such as an organic acid analysis, after the first analysis as well as correlation of a repeat specimen with the original analysis. More importantly, it points out the importance of age-related cutoff values. It is also important to note that *m/z* 388 is also the *m/z* value for C10OH. This metabolite is increased in MCHAD and can easily be confused with C5DC.

### OTHER METABOLITES

Malonic acidemias are characterized by an increase in malonylcarnitine, detected at *m/z* 360 *(122)*. Other metabolites of interest, although not reflective of disease, are benzoic acid, which forms benzoylcarnitine (*m/z* 322). Interestingly, EDTA, as described above, is also detectable at *m/z* 272, 349, 405, and 461. A compound sometimes found at *m/z* 424 is an unidentified substance that is often present at high concentrations in infants treated with certain food supplements. In fact, this compound, together with an abnormal amino acid profile with generalized increases in leucine, phenylalanine, methionine, and tyrosine, strongly suggests treatment with a specific nutritional supplement.

## Specificity and Sensitivity

It is important to understand the complexities of interpretation of multiple analytes because ultimately the goal is to assist in the early diagnosis of a metabolic disorder. We have included Table 2 to correlate diseases with the expected metabolites. This table also includes the estimated frequency of disorders based on results from >1.5 million analyses. Of the various factors that can affect interpretation, the most important is the method of sample preparation, i.e., derivatization scheme. Perhaps the second most important factor is age of the infant. It has been documented that the concentration of acylcarnitines *(75)* decreases rapidly after birth. In our laboratory, we use automated interpretation for infants <7 days of age and a different procedure that includes visual interpretation of all profiles for infants older than 7 days. Other factors affecting interpretation include the quality of the blood specimen, gestational age, drugs, vitamins, and nutritional supplements.

The number of analytes and diseases that MS/MS can be used to screen is vast. On average, the method is highly specific and sensitive, but there are notable differences of sensitivity and specificity for different analytes and the diseases they detect within the same method; this was described in part by Wilcken et al. *(123)*. MS/MS has a

**Table 2. Primary and secondary metabolites, related disorders, and frequencies.**

| Analyte | Secondary analytes | Disorder | Frequency[a] |
|---|---|---|---|
| Phenylalanine | Tyrosine | PKU[b] | +++++ |
| Leucine | Isoleucine, valine | MSUD | +, (+++)[c] |
| Methionine | | Homocystinuria | + |
| Tyrosine | | Tyrosinemia II[d] | + |
| Citrulline | | Citrullinemia | ++ |
| Citrulline | Ornithine | ASA[e] | + |
| C8 | C6, C10, C10:1 | MCAD deficiency | +++++ |
| C8 | C4, C5, C10, C12, C14, C16 | MADD (GA-II) | + |
| C14:1 | C14, C16 | VLCAD deficiency | + |
| C4 | | SCAD deficiency | + |
| C16OH | C18:1OH, C18OH, C16, C14, C14:1 | LCHAD deficiency | + |
| C16 | FC, C18, C18:1 | CPT II deficiency | + |
| C16 | FC, C18, C18:1 | CPT I deficiency | + |
| C3 | C2 | PA | ++ |
| C3 | C2 | MMA | +++ |
| C5DC | | GA-I | ++, (+++)[c] |
| C5 | | IVA | + |
| C5OH | | 3-MCC deficiency | ++++[f] |
| C5OH | | HMG deficiency | + |

[a] Approximate frequency of disease detection in the newborn period (<7 days) based on our experience with 1.5 million analyses, supporting publications, and frequency of detection in other laboratories. The frequency ranges are as follows: +++++, <1:25 000; ++++, 1:25 001–1:75 000; +++, 1:75 001–1:125 000; ++, 1:125 001–1:250 000; +, >1:250 001.

[b] Classic and nonclassic.

[c] Frequency in our laboratory because of specific population groups (Amish/Mennonites).

[d] Frequency of type I attributable to false negatives in most laboratories performing analysis.

[e] ASA, argininosuccinic aciduria; C12, dodecanoylcarnitine; C18:1OH, hydroxyoctadecenoylcarnitine; C18OH, hydroxyoctadecanoylcarnitine; IVA, isovaleric acidemia; 3-MCC, 3-methylcrotonyl-CoA carboxylase; HMG, 3-hydroxy-3-methylglutaryl-CoA lyase.

[f] Includes affected infants and carriers with affected mothers.

high specificity and sensitivity for detecting the diseases of highest frequency, notably MCAD deficiency and PKU. These assays have false-positive rates of <0.05% in our laboratory. However, other disorders, such as the tyrosinemias and certain organic acidemias such as MMA and cobalamin disorders, have a substantially higher false-positive rate (~0.2%). Nearly all increased tyrosine results that require reanalysis will be within reference values when a second specimen is received. This is because of the large number of infants with TNT, premature infants, and cases or infants with liver disease. For MMAs, increased concentrations of C3 are quite common, but a second specimen often reveals a concentration within reference values. Questions remain as to whether a $B_{12}$ deficiency could create transient abnormal results, as is the case with vitamin C and TNT. Perhaps the increasing use of DNA analysis as a second-tier assay for presumptive positive results will improve the sensitivity of the overall screening procedures.

It is increasingly apparent, however, that traditional methods of determining the sensitivity and specificity of screening methods provide confusing information when applied to multianalyte methods such as MS/MS. For example, in their recent abstract, Wilcken et al. *(123)* state that "more cases of inborn errors of metabolism are diagnosed by screening with MS/MS than are diagnosed clinically"; they further state that "it is not yet clear which patients with disorders diagnosed by such screening would have become symptomatic if screening had not been performed". Our experience supports the first finding as true. However, the second statement is of concern in that the answer may never be found. It is known for MCAD deficiency that in approximately one-third of affected individuals, the symptoms are not sufficiently serious to lead to a clinical diagnosis. Screening for MCAD deficiency will not reveal which cases would be serious and which would not, an issue that was debated even before the routine implementation of MS/MS. In fact, Wilcken et al. *(123)* in their discussion contradict their abstract by stating that "the diagnosis of cases that might never come to clinical attention should not be used as an argument against this expanded program of newborn screening". However, we do know that a significant number of undiagnosed individuals with fatty acid oxidation defects will die as result of not having been screened *(75)*. Because most newborn screening has traditionally resided in the bureaucracy of "public health", decisions are made by policy makers rather than healthcare providers. The newborn screen is also the first "snapshot" of the status of "body chemistry". This metabolism may be abnormal regardless of whether a metabolic disease is present, e.g., liver disease or tyrosinemia. Detection of a high tyrosine concentration is important to the healthcare provider but not to a state-mandated newborn-screening program that is interested solely in detecting an inherited metabolic disease. In this context, this result may be considered a false-positive result. The next decade will see this debate continue, especially as more diseases are added to screening panels.

## Multianalyte Concepts in Screening and Disease Confirmation

It is clear that a single metabolite such as C8 can be a marker for several disease states. Differential diagnosis is made by examination of other metabolic markers as well as confirmatory tests, such as GC/MS analysis of organic acids, enzyme studies, fibroblast studies, or molecular analyses. Many of these issues have been encountered in this application, and it is for this reason that MS/MS has emerged as a model for a multianalyte newborn-screening approach. However, MS/MS is no longer the only multianalyte assay being used in screening. Other assays are or soon will be available in areas of genomic and biochemical screening applications. One example of such a technology detects multiple analytes by a multiplexed fluorescent microsphere immunoassay that is both multi-

platform and multiply versatile, but potentially problematic because it relies on an immunoassay *(124–126)*. It is well known that small molecule analysis by immunoassay often lacks specificity. It is further believed that multiplexing an immunoassay requires caution because of vastly different substrate concentrations in the same mixture (poor dynamic range). Both problems are overcome by MS/MS, which can detect picomolar concentrations of acylcarnitines and millimolar concentrations of amino acids in the same analytical run with high specificity. Some also consider molecular microarray technology a feasible platform for newborn screening *(127–129)*. Although either of these methods may become efficacious in the future, neither of them has been demonstrated beneficial to newborn screening, as has MS/MS. It is noteworthy that MS/MS can also serve in a reverse role by confirming diseases found by other technologies, especially older, classic methods or methods with high false-positive rates.

## Related Applications

MS/MS has numerous other applications outside of newborn metabolic screening. In fact, its first use was for high-risk screening; it was later adapted to newborn screening *(32, 37, 39, 130)*. Furthermore, its use extends to diverse areas, including pharmaceutical discovery and environmental testing. Investigators have also recognized the potential value of MS/MS in other clinical applications *(40, 49)*. One of its unique clinical uses arose as a result of its use in newborn metabolic disease screening. Suspicion that inborn errors of metabolism may play a role in a significant number of unexplained premature infant and child deaths grew substantially during the last decade *(131–136)*. Some laboratories are now applying the methods used for newborn metabolic disease detection to investigate cases of sudden unexpected and unexplained death in infants and children *(75)*. Such testing plays a critical forensics role in that it sometimes explains the cause of death in a group of cases that may otherwise be left unsolved. Screening of newborns and children after the first week of life who are considered to be at high risk of developing a metabolic disease because of family history or who unexpectedly become ill is another area where MS/MS has been applied successfully. Most postmortem and high-risk specimens are collected as blood dried on filter paper, as is the case with newborn-screening specimens. MS/MS has the versatility, however, to analyze other biological fluids in postmortem and other samples, including bile, plasma, serum, cerebral spinal fluid, vitreous humor, urine, tissues *(49, 93, 137)*, and amniotic fluid (for prenatal diagnosis) *(138, 139)*. It is noteworthy that the use of frozen plasma in the inborn errors of metabolism laboratory is still preferred over the dried blood spot, although we believe that with time, the utility of the dried specimen may take a more prominent role.

A few words of caution are required when evaluating new MS/MS methods in newborn screening. New MS/MS methods are being published at an extraordinary rate *(140)*. Currently in the journal *Clinical Chemistry*, the authors of an average of two reports per issue indicate the use of MS/MS. Many of the methods demonstrate the ability of MS/MS to detect a particular analyte with a low false-positive rate. What they do not always directly state is whether they are appropriate for routine newborn screening. Being able to perform an metabolic analysis in a blood spot does not translate to high-throughput newborn screening. In other words, a method must be appropriate for newborn screening, where many hundreds of samples are analyzed per day in a cost-effective manner. Nevertheless, these methods are appropriate in the confirmation of a disease and often, with newer technologic advancements, are adapted as newborn-screening techniques. Certainly these methods are entirely valid and important in diagnostic laboratories.

## Future of MS in Newborn Screening

The future of MS/MS, or more broadly, MS, in newborn screening is expansive and comprehensive. What exactly does this mean? A mass spectrometer is a detector and quite versatile. It can be coupled to many types of chromatographic systems or, as is the case of MS/MS, perform a mass separation followed by a second mass separation. There are also other types of MS, such as time-of-flight and tandem time-of-flight MS, that are useful in analyzing large molecules such as proteins or polynucleotides. Given the scope of possible metabolites and varieties of methodologic approaches, it is virtually certain that MS will be used to analyze one or more of the compound classes that remain important in disease states that are treatable in infants. Many of the technologic advances will be in specimen preparation rather than in use of the mass spectrometer itself. After all, a mass spectrometer is just a detector, but a powerful one indeed.

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