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THE APPLICATION OF TANDEM MASS SPECTROMETRY TO NEONATAL SCREENING FOR INHERITED DISORDERS OF INTERMEDIARY METABOLISM

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■ **Abstract** This review is intended to serve as a practical guide for geneticists to current applications of tandem mass spectrometry to newborn screening. By making dried-blood spot analysis more sensitive, specific, reliable, and inclusive, tandem mass spectrometry has improved the newborn detection of inborn errors of metabolism. Its innate ability to detect and quantify multiple analytes from one prepared blood specimen in a single analysis permits broad recognition of amino acid, fatty acid, and organic acid disorders. An increasing number of newborn screening programs are either utilizing or conducting pilot studies with tandem mass spectrometry. It is therefore imperative that the genetics community be familiar with tandem mass spectrometric newborn screening.

INTRODUCTION

The number of inherited disorders of intermediary metabolism that can be identified through newborn screening (NBS) has increased more than fivefold in a single decade. The lion's share of this improved identification can be attributed to just one innovation, the use of tandem mass spectrometry (MS/MS) for amino acid (AA) and acylcarnitine (AC) analysis in blood obtained from newborns. In addition to expanding screening to include disorders of fatty acid oxidation (FAO) [e.g., medium chain acyl-CoA dehydrogenase (MCAD) deficiency] and disorders of organic acid metabolism [e.g., glutaric acidemia type I (GA-I)], MS/MS has also improved the sensitivity and specificity of classical NBS for AA disorders such as phenylketonuria (PKU). The use of MS/MS in the neonatal detection of metabolic diseases is revitalizing NBS, as well as presenting the medical and genetics communities with unique opportunities for early treatment. NBS using MS/MS may prove to be the archetype for new technologies that will expand and enhance future

biochemical genetics applications in an era when the impact of early and sensitive disease recognition will most likely have its greatest effect.

SCOPE OF REVIEW

The purpose of this review is to educate the genetics community about the applications of MS/MS to biochemical genetics and NBS. As the number of newborns who receive a metabolic screen using MS/MS-based systems multiplies, it is imperative that geneticists, pediatricians, metabolic specialists, family practitioners, and genetic counselors have a basic understanding of the technology including terminology, accuracy, and precision; the meaning and reliability of results; appropriate follow-up and confirmatory testing; and the experience of programs already utilizing MS/MS. Equipped with a thorough understanding of the application of MS/MS to metabolic disease testing, scientists and health care professionals alike will have better insight into developing new therapies, introducing new screening technologies, and providing better care to their patients.

ESSENTIALS OF MASS SPECTROMETRY

MS/MS is one type of analytical apparatus from a general class of instruments known as mass spectrometers (9). For many years, biochemical geneticists have analyzed urinary organic acids by capillary gas chromatography coupled with mass analysis using a single-sector quadrupole mass spectrometer (gas chromatography/mass spectrometry, GC/MS) (37, 61, 62). Essentially, a mass spectrometer is a "mass" detector and can be utilized in other analytical systems for the identification of products of other chromatographic separations such as high-pressure liquid chromatography (HPLC) (28, 44) and capillary electrophoresis (27). More recently, systems have been developed in which mass analysis alone (no chromatographic separation) is sufficient for the identification and quantitation of compounds (9, 13, 72). Illustrations of mass spectrometry without chromatography include the application of MS/MS to NBS, as described in this review, and other state-of-the-art systems that use matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) mass spectrometry to directly measure the mass of single nucleotide polymorphisms (35), proteins, glycoproteins, and other diagnostic markers (34, 39, 103).

All mass spectrometers share three essential processes: (*a*) ionization, (*b*) mass analysis, and (*c*) detection. However, only gas-phase ions can be analyzed by a mass spectrometer. Accordingly, the ion source of a mass spectrometer produces positive or negative charges on a molecule while also enabling the molecule to enter the gas phase. Ionization prior to mass spectrometric analysis can occur by two mechanisms (100). A molecule can be ionized either when a stream of high-energy electrons (emitted from an ion source) impacts a molecule or by acquisition or loss of a proton to or from a surrounding matrix. The former process, known as electron impact ionization, causes extensive fragmentation or breakdown of a molecule

into predictable and reproducible species, whereas protonation [soft ionization (36)] generally results in little or no fragmentation. After ions are formed, they are separated by mass and charge in the mass analyzer region of the mass spectrometer and subsequently counted by a detector. Analytical results are presented in a mass spectrum where the x-axis represents the mass to charge ratio (m/z) and the y-axis represents ion intensity, ion counts, or some other value pertaining to the amount of signal produced for a given mass.

BASIS OF ANALYSIS

In technical terms, an MS/MS system such as that used for NBS comprises two mass analyzers, of a particular type known as a quadrupole (four parallel rods), termed MS1 and MS2, separated by a collision chamber that induces molecules to fragment in a process known as collision-induced dissociation (CID). The collision chamber often comprises either a quadrupole or hexapole that, unlike MS1 and MS2, does not separate ions by mass. This chamber is used to fragment molecules that enter it from MS1 and enables these fragmented ions to enter MS2. Addition of a collision gas such as nitrogen (N_2) or argon will cause fragmentation of molecules that enter the chamber from MS1. The degree of fragmentation is controlled by the amount of gas in the chamber and the energy imparted to both the collision gas and the molecules entering the chamber. Fragments that are formed in this chamber enter MS2 where they are analyzed. The fragment ions are separated by mass and charge in the same manner as MS1. As a result, mass separations are detected and their signals quantified. Using computer algorithms, fragment ions measured following separation in MS2 can be correlated with their original intact molecular ion separated in MS1. In this manner, three types of MS/MS spectra can be acquired. These are product ion scans, precursor (Pre) ion scans, and neutral loss (NL) scans.

Several analogies to common situations may be used to describe the operation and purpose of MS/MS in analyzing complex mixtures quickly, accurately, and quantitatively. One useful analogy that helps to elucidate how MS/MS sorts molecules by taking advantage of their elemental composition and structural features is the formation of words themselves. Words and the letters that compose them are like molecules and elements. Letters are ordered in a specific manner to create meaningful words, just as atoms are arranged to create functional and stable molecules. Atoms in a molecule are arranged in recognizable groups that bestow certain characteristics upon a molecule. One example of this is an acidic molecule that is recognized as such by the mere presence of a COOH group. Words also share other common features, such as syllables. Molecules are broken into specific fragments in much the same way as words are broken into syllables. To illustrate this point, imagine trying to quickly ascertain how many words in this manuscript contain the suffix “ing.” Each word could be examined individually, or perhaps a computer could automatically search for all words containing this suffix and then display them. Subsequently, each word in this list could be further separated into

two fragments, the primary portion of the word and its "ing" suffix. This is not unlike the way in which a quadrupole MS/MS system operates. Whereas the first mass spectrometer separates and records each intact molecule ionized, the second mass spectrometer detects the fragments formed by each molecule. The computer controlling the mass spectrometry system, depending on the question (scan function) assigned, will thus display in a mass spectrum only those compounds that contain the fragment of interest.

MS/MS ANALYSIS OF AMINO ACIDS

The first use of MS/MS for NBS was to perform AA analyses, specifically for phenylalanine (Phe) (17, 48). Existing technology used by NBS laboratories before the introduction of MS/MS included bacterial inhibition assays (BIA), fluorometry, and HPLC (7a). This particular application of MS/MS enabled a critical comparison of established techniques with new technology. By the early 1990s, a movement was already underway to improve analytical methods for measuring Phe by non-BIA-based techniques because they traditionally returned high rates of false positive results. Therefore, during its development, the Phe assay by MS/MS was compared to both HPLC and fluorometric assays that were used routinely in many NBS programs (17, 19).

Physiological AAs (i.e., α-AAs) share a common structural feature, a formic acid chemical group, which enables their selective analysis by MS/MS. Hence, when subjected to CID, α-AAs produce a unique fragment ion, formic acid (42 Da). Early research showed that ionization of α-AAs was enhanced by esterification of free carboxylic acid groups. Also, studies have determined that butyl esters are ionized optimally. This reaction introduces a butyl ester at each carboxylic acid functional group in an α-AA. Upon exposure to CID, butylformate (102 Da) is lost. Because butylformate is a molecule (uncharged) rather than an ion, the product ion detected in MS2 is equal to the α-AA minus butylformate. MS/MS provides a specific scan mode, known as the NL scan function, to selectively detect this type of common loss (8, 9, 17). For α-AAs specifically, the MS/MS is set to look for product ions that differ from the precursor or molecular ions by 102 Da through the NL-102 scan function. Several noteworthy α-AAs, including Phe, tyrosine (Tyr), leucine (Leu), alanine (Ala), valine (Val), and methionine (Met), share this mode of fragmentation. The MS/MS spectrum for an NL-102 scan function of a control newborn specimen, as shown in Figure 1*a*, displays the protonated molecular ions of several α-AAs, including all those aforementioned. It is important to note that the mass of a protonated molecule is often denoted as $(M + H)^+$, where H (1 Da) represents the proton that generally associates with basic functional groups, usually those containing nitrogen, such as amines. Also note that the charge on an α-AA (z) is 1, hence the horizontal axis (m/z, mass/charge) of a mass spectrum generated by MS/MS essentially represents the mass (m). The vertical axis represents the number of ions detected either absolutely or as a percentage relative to the largest amount detected (relative intensity).

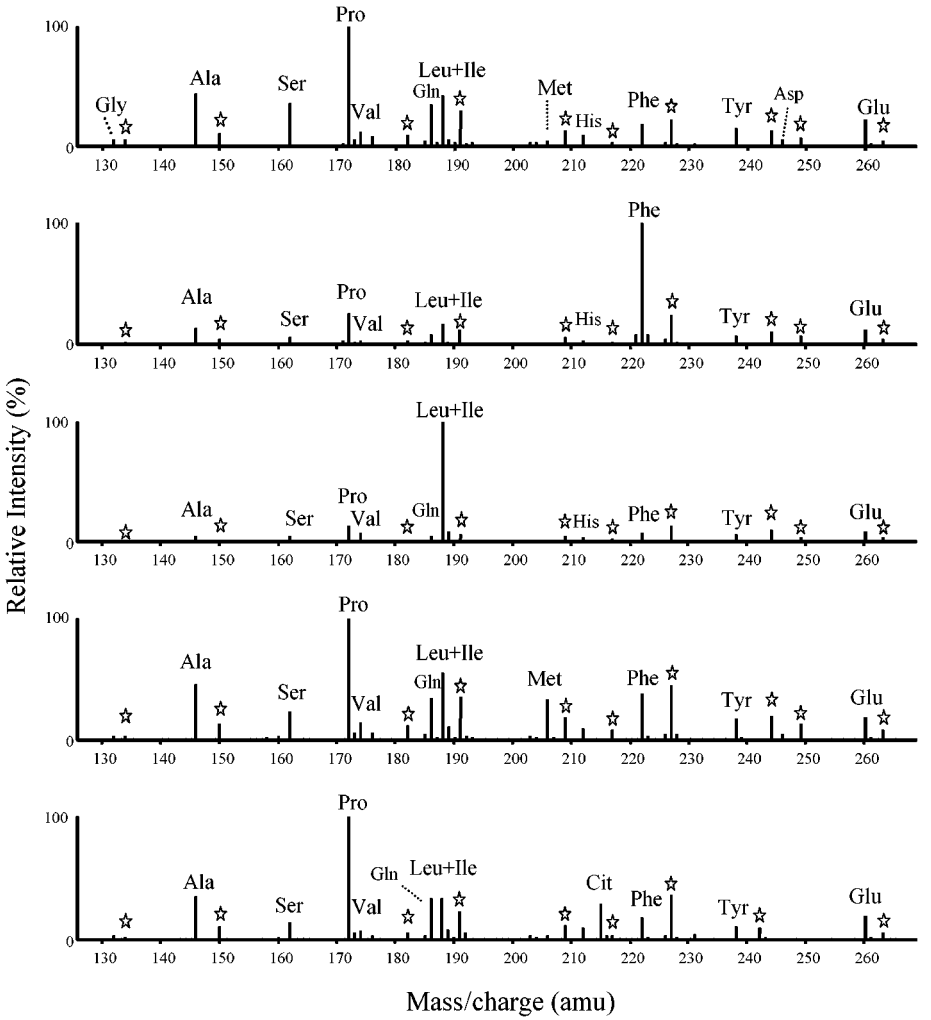

Figure 1 NL-102 MS/MS spectra of newborn specimens: (*a*) control, (*b*) PKU, (*c*) maple syrup urine disease (MSUD), (*d*) homocystinuria, and (*e*) acute neonatal citrullinemia. The stars represent stable isotope internal standards.

SPECIMEN PREPARATION

Sample preparation prior to AA MS/MS analysis requires extraction from dried-blood spots (DBSs). To begin, blood specimens are punched as single or multiple punches of 3/16- or 1/8-inch disks from blood dried on filter papers. These blood spot–containing filter paper cards are referred to as Guthrie cards (10, 18, 58). These disks are then placed into microtiter plates or tubes, where methanol

containing isotope-enriched internal standards is added. These standards are used to quantify Phe, Tyr, and the other α-AAs that are extracted into the methanol. It is important to note that quantification of the compounds in a DBS is different from clinical analyses that use liquid blood, plasma, or urine samples. Traditional isotope dilution techniques require addition of a stable isotope standard to a liquid blood sample, where it can uniformly mix with the unlabeled compounds in the original sample (13). Because the chemical properties of the labeled standards and the unlabeled compound are nearly identical, all extraction and derivatization procedures will be nearly the same for batch species. Because the blood spot is a dry sample, a liquid standard cannot be uniformly mixed with the original sample. As a result, adding the standard to the methanol extracts causes the loss of a certain degree of accuracy and precision because of variations in both blood volumes in the disk and extraction efficiency (2). The remainder of the sample preparation after extraction, however, is identical for both standard and metabolite. Therefore, we essentially use a partial isotope dilution technique, which we refer to as pseudoisotope dilution (9).

Following blood sample extraction, the solvent is evaporated under a gentle stream of N_2. For procedures that utilize derivatization to form butyl esters, acidified butanol (3N HCl in n-butanol) is added. After heating for 15 min, the excess derivatization agent is removed under a gentle stream of N_2. Just prior to analysis, the dried, derivatized, or underivatized specimen is reconstituted in a solvent generally containing approximately 50% organic solvent (acetonitrile) and 50% water. In some applications, a small quantity of a volatile low-molecular-weight organic acid (formic acid) is added to assist in protonation and ionization. For analysis, small aliquots of each reconstituted specimen are injected approximately every 2–3 min into a flowing stream of solvent. The stream terminates in a spray that is charged to over 5000 V. This is known as electrospray ionization (ESI). ESI is the most-recently developed and highly efficient form of ionization by MS/MS systems (100).

MS/MS, as currently employed in nearly all biochemical genetics and NBS laboratories for the measurement of small molecules, is configured with ESI and quadrupole mass analyzers (18, 58, 72, 83, 86, 90, 99, 106). Early MS/MS systems used fast-atom or fast-ion bombardment (FAB/FIB) ionization configurations (8, 48, 51, 52, 60, 89). These early modes of ionization required manual sample introduction, making analyses slow and labor intensive. Automated sample introduction had limited success until the advent of ESI. ESI has demonstrated both robustness and versatility, in that the potential number of new compounds that may be analyzed using the same instrument is considerable (68, 70, 72). Consequently, this method for mass analysis has remained virtually unchanged since its original application to NBS in the early 1990s (7a, 9).

AMINO ACID DISORDERS

By the early 1990s, most states in the United States as well as many other countries were performing NBS for PKU (81). Later, a few programs added one or two other disorders: either maple syrup urine disease (MSUD) (59), homocystinuria (14),

or tyrosinemia (40, 63). Because MS/MS can measure the α-AAs associated with PKU (Phe and Tyr) in the same analytical run as those associated with MSUD (Leu and Val) and homocystinuria (Met), it is possible to detect all four of these disorders using one method (14, 15, 17). Certainly, for those states mandating testing for all four disorders, a single MS/MS analysis presented some obvious advantages. Below is a discussion of individual disorders and the application and experience of MS/MS analysis to amino acidopathies.

Phenylketonuria

As described previously, MS/MS is capable of simultaneously identifying and quantifying several α-AAs. The primary metabolites of quantitative importance in PKU are Phe and Tyr. This defect is characterized by a deficiency of phenylalanine hydroxylase, the enzyme responsible for conversion of Phe to Tyr (7, 21, 82). A deficiency of or defect in this enzyme results in both the time-dependent accumulation of Phe and a possible decline in the level of Tyr. An MS/MS AA profile of a specimen from a newborn with PKU is shown in Figure 1*b*. A dramatic increase in Phe relative to its standard, other α-AAs, and the control specimen (Figure 1*a*) is noteworthy.

Detection of this disorder by NBS is historically based on the analysis of Phe from a sample collected after 48 h of age (19, 25, 87). Technology used to screen for PKU in the past was adapted to the concentration of Phe expected at this age. In the mid-1990s when there was a trend in early discharge of infants from hospitals at less than 24 h of age, the aforementioned time of Phe accumulation was significantly shortened to the point where the concentration of Phe was potentially not high enough to be detected by established cutoff concentration. Subsequently, cutoff concentrations were lowered to insure earlier detection, causing the number of false positive results to skyrocket.

For this problem, two challenges faced NBS laboratories: time of sampling and technology. Clearly, time was a factor, and so a standard for sampling was established at an Early Hospital Discharge conference held in Washington, D.C., in 1995 (87). The group decided that the 24-h blood sample should be standard and that any specimen collected prior to this point would not be considered a newborn screen. This standard posed a problem in obtaining any specimen in states where discharge after delivery frequently occurs before 24 h, as was the case in California (19).

With regard to the challenge in technology, instrumentation used in many NBS laboratories during this period was neither sufficiently precise nor necessarily accurate to differentiate abnormal PKU results from controls at the Phe concentration expected at 24 h, or less, of age. Some thought that perhaps the application of MS/MS would improve analysis for PKU by enabling more precise measurement of Phe coupled with simultaneous measurement of Tyr, thus also permitting the calculation of the ratio of Phe to Tyr (Phe/Tyr) (19, 80). The Phe/Tyr ratio would serve two purposes, the first being to allow for more precise cutoffs for PKU detection because this ratio would be expected to increase at a faster rate than Phe alone, and the second being to improve the overall coefficient of variation (CV) for measurement of Phe in a blood specimen. By using MS/MS, CV values that

would otherwise be high owing to variations in blood volume and various other factors are, in fact, reduced (2, 10, 17, 19). Moreover, the Phe/Tyr ratio normalizes variations in the quantification of Phe and Tyr arising from differences in blood volume in individual blood spots.

An important study (19) that compared both fluorometry and MS/MS analysis of early discharge specimens demonstrated that MS/MS could reduce the PKU false-positive rate by almost 100% as a result of a combination of both a more precise Phe measurement and the contribution of the Phe/Tyr ratio. It is surprising to note that more than 90% of the falsely elevated Phe measurements as determined by fluorometry were normal when measured using MS/MS. It is a well-known fact that immunologically based assays, including fluorometric techniques, have problems with selectivity and cross reactivity that are exacerbated for small molecules of similar structure, i.e., Phe and Tyr. Therefore, the inherent accuracy of Phe measurement using MS/MS alone, a characteristic that would be expected for other metabolites as well, represents the single greatest contribution to improvement of PKU screening. Measurement of the Phe/Tyr ratio further reduced the remaining false-positive results, namely, those cases arising from hyperalimentation, where α-AA values were artificially elevated by direct central venous α-AA administration. The ratio of Phe/Tyr is usually normal in these cases, except in instances where the administered α-AAs are low in Tyr (Tyr is more difficult to mix into pharmaceutical preparations as compared to other α-AAs). When the Phe/Tyr ratio is abnormal owing to hyperalimentation, however, both the calculation of other α-AA ratios such as Phe to Leu and the simple observation of multiple α-AA elevations suggest a treatment issue rather than a disease.

When fully implemented, these interpretation schemes will produce a false-positive rate for PKU of less than 0.01% (7a, 13, 18). Clearly, cost savings can be realized as a result of lower false-positive rates, particularly in states with large numbers of early-discharge infants. Some cost calculations may show that MS/MS applied to Phe analysis alone does not satisfy any cost-benefit criteria. Because there are dozens of other disorders that can be screened simultaneously, however, an argument based on PKU identification alone is not valid.

Maple Syrup Urine Disease

One of three other amino acidopathies historically screened by public health NBS programs is MSUD. The primary metabolite accumulating in this disorder, Leu, is often accompanied by increased concentrations of Val, isoleucine (Ile), and alloisoleucine (alloIle). Patients have a defect in branched-chain α-keto acid decarboxylase, resulting in increased concentration of keto acids and their corresponding α-AAs (59). For treatment to be most effective, this devastating disorder must be detected as early in the newborn period as possible. Because this disease can be fatal if treatment is not started early, a presumptive positive result requires that a child be referred to a tertiary care center for immediate clinical evaluation and resampling. Like PKU, the traditional technology in use by most NBS laboratories

for measuring Leu in the early 1990s was the BIA. Unfortunately, this method returned a high false-positive rate for MSUD, creating even more problems and expense for those programs screening for this disorder because a positive result requires immediate referral to a tertiary care center.

The first applications of MS/MS to Leu analysis by MS/MS were reported in the mid 1990s (15). An MS/MS AA profile of a specimen from a newborn with MSUD is shown in Figure 1*c*. A dramatic increase in Leu + Ile relative to their standards, other amino acids, and the control specimen (Figure 1*a*) is observed. Comparisons of MS/MS and the BIA showed a reduced false-positive rate for MSUD by MS/MS. There are some differences, however, in the quantitative measurement of Leu as compared to Phe. Although an NL-102 scan function is used to measure Leu and other branched-chain AAs such as Val, it is important to note that Leu measurement may not be entirely accurate because Leu, Ile, and alloIle share the same molecular weight. These metabolites are detected at m/z 188 and have similar fragmentation patterns. All three are simultaneously elevated in MSUD, and the signal intensity at m/z 188 represents the sum of their contributions. They therefore cannot be differentiated by MS/MS without a chromatographic method to separate these AAs.

Similarly, m/z 188 ions with an NL of 102 in control samples also represent the combined concentration of Leu + Ile. Other m/z 188 ions with an NL of 102 such as hydroxyproline and creatine do not contribute substantially to the total signal in control patients. Furthermore, in the case of a newborn diagnosed with MSUD, the ions at m/z 188 represent not only Leu and Ile, but norleucine and alloIle, which also become significant in these patients. Precise measurement of any of these species individually requires the addition of a chromatographic technique. Nevertheless, the ability of MS/MS to detect an elevation of Leu (or the sum of these accumulating branched-chain species) as compared to controls is excellent (18, 59). Yet, just as hyperphenylalaninemia may be confused with PKU, hyperleucinemia may also be mistaken for MSUD. To reduce this problem, metabolite ratios such as Leu to Phe and Leu to Ala have been implemented. In fact, the false-positive rate now is as low as that for PKU, at less than 0.01%, in part because the ratios of Leu to Phe and Leu to Ala have assisted in reducing the contribution of hyperalimentation cases to the false-positive rate. The importance of lowering the false-positive rate for MSUD should not be underestimated when considering the cost of a suspected patient receiving a tertiary care referral.

Homocystinuria

Another disorder traditionally screened by many NBS programs is homocystinuria. This disorder is characterized by a primary elevation of homocysteine with secondary elevations of Met (7, 21, 82). Because homocysteine is readily oxidized to homocystine, the metabolite primarily indicative in this disorder that is currently routinely tracked by MS/MS without special sample preparation methods is Met (14). Elevation of Met is also time dependent, as are elevations of Leu and Phe in MSUD and PKU, respectively. However, because it is a secondary metabolite, the

rate of rise in its concentration may be delayed relative to the primary metabolites. This phenomenon makes the diagnostic concentration for Met in homocystinuria more difficult to pinpoint, and it also underscores the importance of waiting until 24 h of age for newborn blood sampling. The net result of sampling before 24 h of age is either a higher false-positive rate or the potential for a false-negative result. Recently, however, our laboratory detected an abnormal concentration of Met at 18 h in a patient that was prenatally diagnosed with homocystinuria.

Concerns regarding the application of MS/MS to NBS for homocystinuria and the potential for a false-negative result have been discussed frequently among newborn screening professionals (57). Nonetheless, application of MS/MS homocystinuria screening has produced an improvement in testing in terms of both precision and accuracy, as demonstrated by the third in a series of validation articles (14). Met is detected in the NL-102 scan function, along with other α-AAs, as described above. An MS/MS AA profile of a specimen from a newborn with homocystinuria is shown in Figure 1*d*. A less dramatic increase in Met relative to its standard, other AAs, and the control specimen (Figure 1*a*) is noted.

In addition to Met, a ratio of Met to Phe is calculated to increase analytical precision for DBS analysis. The prenatally diagnosed infant with homocystinuria mentioned earlier was followed from birth through 4 days of life, with blood samples collected approximately every 6 to 12 h. In this case, using cutoff levels of 1.0 mg/d-liter, this infant would have been diagnosed at 24 h of age. In fact, based on the data, homocystinuria would have already been detected between 12 and 24 h of age. Nonetheless, there is still the concern that, in a blood specimen acquired at less than 24 h of age, the Met may not yet have reached abnormal levels in a newborn with homocystinuria who has milder mutations.

New assays for directly screening homocysteine in plasma using MS/MS have been developed (30, 43). These techniques offer the potential to be adapted to newborn DBSs. Because this assay requires the presence of a reducing agent to prevent the oxidation of homocysteine, and because of the unknown interferences this reagent might cause in the analysis of other α-AAs, it is more likely that a separate analysis would be required, leading to additional costs per test.

Tyrosinemia

A limited number of laboratories in the United States and Canada have historically screened for tyrosinemias. Tyrosinemias are categorized into three distinct disorders as well as a transient form. These disorders are namely Type I, II, or III (84), in addition to transient neonatal tyrosinemia (TNT) (38). Tyrosinemia type I, also known as hepatorenal tyrosinemia, is caused by a deficiency of fumarylacetoacetase. Because Tyr is positioned several steps before this enzyme abnormality, its elevation over time is thought to be slow in accumulation. Owing to its proximity to the metabolic block, an abnormal metabolite, succinylacetone, is also produced in what are thought to be higher concentrations than Tyr (79). Studies have shown that screening both Tyr and succinylacetone, rather than Tyr screening alone, is

more efficacious for detecting this disorder. In children who are affected, liver disease is quite severe.

Tyrosinemia type II is also known as oculocutaneous tyrosinemia and results from a deficiency of Tyr aminotransferase. Because this blockade is proximal to Tyr, it is expected that Tyr concentration should be readily detectable. Tyrosinemia type III is the rarest of these disorders and is associated with 4-hydroxyphenylpyruvic acid oxidase deficiency. Finally, TNT occurs presumably as a result of either immature enzyme systems or vitamin C deficiency.

As described previously in the PKU section, Tyr is measured using an NL-102 scan function (17). Its MS/MS analysis shares similar beneficial attributes as those had by other α-AAs. The concern, however, is not with accurate measurement of Tyr, but rather whether this metabolite alone will enable successful detection of all tyrosinemias, especially tyrosinemia type I. One of the most serious limitations is the extremely high rate of false-positive results due to TNT. Furthermore, certain tyrosinemia cases may not have a significant elevation of Tyr in the neonatal period and therefore present the potential for false-negative results. Several cases of TNT have been observed in our program, along with one confirmed case of tyrosinemia type III. In a small pilot screening program for newborns in North Carolina, one case of tyrosinemia type II was detected as well (17). Although no false-negative results have been reported, known positive cases of tyrosinemia types I and II have been observed. Our laboratory has recently lowered the diagnostic criteria for an elevated Tyr and added a succinylacetone confirmatory test to improve diagnostic efficacy for tyrosinemias in a screening program utilizing MS/MS.

Basic Amino Acids

In addition to those AA disorders for which screening has traditionally been available, application of MS/MS technology to NBS has enabled detection of other important AA disorders, including those resulting from inborn errors of urea cycle metabolism. These include primarily argininosuccinic aciduria, also termed argininosuccinic acid (ASA) lyase deficiency, and citrullinemia, known also as ASA synthetase deficiency. Other related urea cycle disorders include argininemia, also called arginase deficiency, carbamoylphosphate synthetase (CPS) deficiency, ornithine transcarbamylase (OTC) deficiency, and N-acetylglutamate synthetase deficiency. The metabolite of primary interest in screening for ASA and citrullinema is citrulline (Cit). Cit contains a labile amino group that fragments together with butyl formate. This results in a net neutral fragmentation of 102 (butyl formate) plus 17 (ammonia), for a combined loss of 119. Hence, an NL-119 scan function will selectively detect Cit at m/z 232. Additionally, a peak may be observed at m/z 215 (M-17) in the NL-102 scan function because some instruments produce fragmentation of the ammonia functional group in the ion source prior to MS/MS analysis (9). This ion is essentially an MS/MS NL-102 product of Cit that has lost an ammonia group. Thus, we see in citrullinemia an increase in the mass 215 with

NL-102 analysis and an increase at m/z 232 with NL-119 analysis. Although Cit is the primary MS/MS diagnostic marker for ASA lyase deficiency and citrullinemia, ornithine (Orn) and arginine (Arg) are also determined because they may be secondarily elevated in these disorders.

In citrullinemia, Cit is dramatically elevated. An MS/MS AA profile of a specimen from a newborn with acute neonatal citrullinemia is shown in Figure 1*e*. Cit is clearly elevated relative to its standard, other α-AAs, and the control specimen (Figure 1*a*). In the profile of a newborn with ASA lyase deficiency, this increase in Cit is much milder. Moreover, elevations in the urea cycle metabolites (Orn and Arg) have been noted in older infants with ASA lyase deficiency, but not in newborns. Therefore, both citrullinemia and ASA lyase deficiency rely on detection via an increased Cit concentration. ASA lyase deficiency can be confirmed by observing a specific fragment characteristic of argininosuccinic acid in a product ion spectrum (73). Because both ASA and the internal standard for hexadecanoylcarnitine (C16) share the same mass, it was previously thought that each assay would interfere with the other. This is not the case, however. In fact, the ratio of a product ion of ASA to the product ion of the internal standard for C16 is used to confirm ASA lyase deficiency after a Cit elevation is detected.

The potential for MS/MS detection of other disorders such as CPS and OTC deficiencies may be realized in the near future by quantifying low Cit levels. However, further research is necessary. It is noteworthy that citrullinemia was once considered an untreatable metabolic disorder. In fact, the wisdom of screening for this disorder in our own program was questioned. Previous clinical histories were based on detection of citrullinemia after metabolic decompensation. Therefore, it was not known whether early detection would prove advantageous to a citrullinemiac. Fortunately, screening for citrullinemia has unexpectedly proven beneficial for some infants. In fact, two infants with citrullinemia detected by our laboratory have survived and are doing well after having been treated. These findings underscore the importance of rapid turnaround in NBS. They also highlight the need to evaluate unwarranted conclusions that disorders are untreatable, especially when these conclusions are based solely on experience gained in treating infants in crisis.

Argininemia due to arginase deficiency is another disorder potentially detectable by MS/MS, at least in older patients. It has been debated as to whether MS/MS could be used to detect arginase deficiency via Arg measurement in the newborn period. To date, only one case of severe arginase deficiency presenting with elevated Arg in the newborn blood specimen has been found using MS/MS, supporting the notion that arginase deficiency may not be readily detected in the newborn period. However, an alternative method based on direct enzyme assay has yet to yield a positive result in 500,000 newborns screened. Further research is necessary to determine the ability of MS/MS to detect argininemia and other urea cycle disorders such as hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome, which is characterized by elevated Orn and homocitrulline, both metabolites measured using MS/MS.

MS/MS ANALYSIS OF ACYLCARNITINES

The clinical significance of carnitine (CN) was originally recognized in the 1980s (20, 23, 75, 77). Once investigators ascertained the roles of CN and ACs in mitochondria, they realized the value of their measurement in the investigation of FAO and organic acid disorders. CN and ACs contain a quaternary ammonium functional group, making them preformed positive ions (cation) that are polar and nonvolatile. Upon recognition of their biochemical significance, direct analysis of CN and ACs by conventional mass spectrometric techniques at the time, namely, GC/MS, was virtually impossible because of their nonvolatility. Analysis required either chemical modification by removal of the quaternary ammonium group or hydrolysis of the fatty acid followed by fatty acid analysis using GC/MS (26, 42). Both methods were impractical. Later, advances in liquid chromatography/mass spectrometry (LC/MS) enabled consideration of the direct analysis of ACs (53, 104). First, mass spectrometry with FAB ionization and LC was used (60). Once again, however, direct analysis was impractical because selectivity was poor, primarily as a result of the hundreds of compounds present in blood. Introduction of MS/MS led to the development of an assay that did not require chromatography but could separate on the basis of masses of the intact ACs and the masses of their fragment ions (48, 50–52). Today, the fundamental MS/MS techniques for analyzing ACs introduced in the early 1990s still survive (12, 69, 70, 72, 83).

Fortunately, sample preparation for blood spot AC analysis is identical to that for AA analysis (see Amino Acid Disorders above). In terms of MS/MS analysis, however, unlike α-AAs, the common AC fragment is an ion rather than a neutral fragment (47, 48, 50, 55). Both butylester derivatives and underivatized CN and ACs share a common product ion, which is singly charged with a mass of 85 Da. Selective analyses of only those molecules that produce an 85-Da product ion is accomplished using another special scan mode known as the Pre ion scan. A typical Pre ion scan for 85-Da products in a control infant is shown in Figure 2*a*. All ACs ranging from the 2-carbon acetylcarnitine (C2) through the 18-carbon fatty AC are detected in a full scan spectrum (9). Hydroxyacylcarnitines and dicarboxylic acid acylcarnitines of various chain lengths are also detected in this full scan spectrum. Thus with a comprehensive scan of ACs, several disorders of fatty acid and organic acid metabolism are detectable. Of these, one of the most frequently occurring is MCAD deficiency, a disorder of FAO.

FATTY ACID OXIDATION DISORDERS

Carnitine transports long-chain fatty acids across the inner mitochondrial membrane. On the outer mitochondrial membrane, acyl-CoAs are converted to ACs via the enzyme CN palmitoyl transferase (CPT)-I and then transported across the inner mitochondrial membrane by the CN AC transporter (82). Inside the inner membrane, CPT-II converts the ACs back to acyl-CoAs, after which β-oxidation

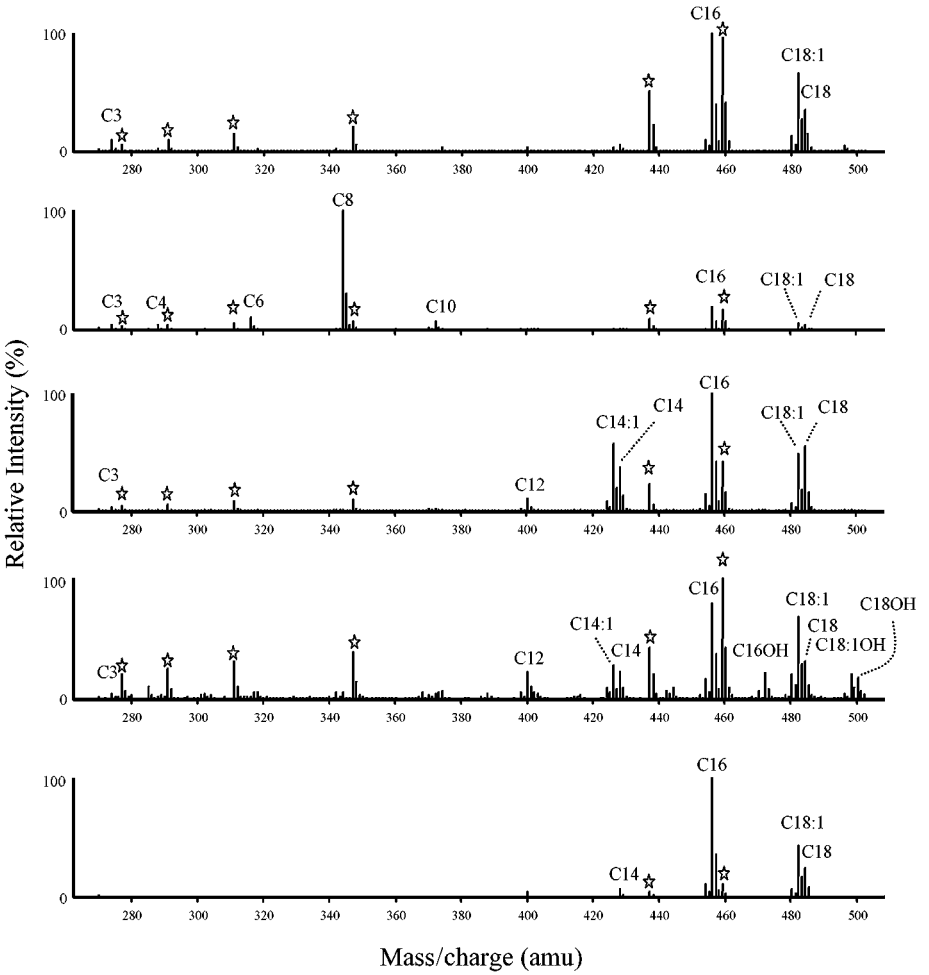

Figure 2 Pre 85 MS/MS spectra of newborn specimens: (*a*) control, (*b*) homozygous 985 A → G MCAD deficiency, (*c*) VLCAD deficiency, (*d*) LCHAD deficiency, and (*e*) CPT-II deficiency. The stars represent stable isotope internal standards.

occurs. Impairment of β-oxidation will result in an accumulation of fatty acyl-CoAs of various chain lengths inside the inner mitochondrial matrix. As a result of this accumulation, CN acyltransferases catalyze reformation of ACs, which are excreted across the mitochondrial membranes and into the extracellular matrix and blood. AC concentration in the blood reflects the concentration of ACs in mitochondria, hence representing an estimation of whether impairment of β-oxidation exists. The ACs found in abnormal concentrations in the blood will be directly dependent upon which enzymes are affected in a disorder of β-oxidation. MS/MS

can detect abnormal concentrations of numerous ACs. The pattern of elevated ACs can presumptively identify one or more disorders of FAO. It is important to note that each disorder requires confirmation by an independent method such as urinary organic analysis via GC/MS (31), molecular confirmation (3, 24, 105), enzyme studies, or skin fibroblast studies (78).

MCAD Deficiency

In MCAD deficiency, medium-chain-length fatty ACs accumulate. This accumulation results from a deficiency of the MCAD enzyme. The medium-chain fatty acids range in length from 6 to 10 carbons. The most prominent metabolite in MCAD deficiency is octanoylcarnitine (C8). Other metabolites presenting as abnormally elevated in MCAD deficiency include hexanoylcarnitine (C6), decanoylcarnitine (C10), and decenoylcarnitine (C10:1). A typical MS/MS spectrum of an MCAD-deficient newborn who is homozygous for the most-common mutation (985 A $\rightarrow$ G) is shown in Figure 2*b*. The ideal time for detection of this disorder is in the first few days of life when ACs are present in one's blood supply at their highest concentration. With the progression of time after birth, the concentration of ACs decreases significantly (9, 12, 46), making diagnosis of these disorders by AC analysis more problematic. This phenomenon is the opposite of that exhibited by AAs.

In MCAD deficiency, three or more metabolites are monitored, with the primary emphasis on C8 concentration. Generally, laboratories use a cutoff concentration of between 0.5 and 1.0 μ-mol/liter (16, 94). A typical MCAD-deficient patient has a C8 concentration greater than 2.5 μ-mol/liter. However, depending upon age and mutation, these C8 concentrations can be as low as 0.3 μ-mol/liter, a value that lies far below cutoffs used in several other laboratories. Therefore, effective detection of MCAD-deficient infants in NBS programs requires the calculation of additional identifiers including a complex series of medium-chain AC concentrations and a few calculated relative molar ratios. Furthermore, molecular analysis for the common mutations is helpful in confirming a diagnosis of MCAD deficiency (3, 24, 96). In screening approximately 1.1 million infants for inherited metabolic disorders, Neo Gen Screening has identified 65 cases of MCAD deficiency. Of these 65 cases of MCAD deficiency, 57 have been confirmed by molecular analysis. Their genotypes are listed in Table 1.

Although AC analysis for MCAD deficiency appears quite simple, an elevated C8 may also indicate other metabolic disorders or even administration of certain drug treatments. For example, in addition to MCAD deficiency, an elevated C8 may also be present either in patients receiving Valproate therapy or in cases of multiple acyl-CoA dehydrogenase deficiency (MADD) (16, 49, 94). Valproate therapy produces a C8 elevation when valproate metabolites compete with C6, C8, and C10 metabolites for the MCAD enzyme (49). Medium-chain triglyceride (MCT) oil supplementation may also elevate C8 because of excessive substrate loading. Examination of several pertinent ratios has indicated that the C8 to C10

TABLE 1 Genotypes of 57 MCAD-deficient newborns detected using MS/MS to screen more than 1.1 million newborns (Neo Gen Screening, Pittsburgh, PA, USA)

Mutation position and type	Number of patients identified
985 A → G/985 A → G	35
985 A → G/199 T → C (exon 3)	8
985 A → G/deletion 343–348	2
985 A → G/other[a]	5
985 A → G/unidentified	5
799 G → A/254 G → A	1
Unidentified/unidentified	1

[a]Other mutations:
244 insertion T (exon 4).
362 C → T (exon 5).
489 T → G (exon 7).
IVS 5 + 1 G → A.
IVS 8 + 6 G → T.

ratio is highly indicative of MCAD deficiency and is useful in distinguishing MCAD deficiency from other disorders or treatments.

VLCAD Deficiency

Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is characterized by an accumulation of long-chain-length fatty ACs (5, 74). Fatty ACs range from 12 to 16 carbons in length. These fatty ACs accumulate when the VLCAD enzyme is deficient. The most-prominent abnormally elevated metabolites indicative of VLCAD deficiency are tetradecanoylcarnitine (C14) and tetradecenoylcarntine (C14:1). Other metabolites significantly elevated are C16, octadecanoylcarnitine (C18), and octadecenoylcarnitine (C18:1) (101). A typical MS/MS spectrum of a VLCAD-deficient newborn is shown in Figure 2*c*. As is the case with MCAD deficiency, the ideal time to detect this disorder appears to be in the first few days of life when ACs are present in the blood at their highest concentration. With time, the concentration of long-chain ACs decreases significantly, making diagnosis of this disorder using an AC profile more difficult (9, 12). This AC decrease is even more dramatic with VLCAD deficiency than with MCAD deficiency. Most often, specimens collected after the seventh day of life show a dramatic decrease in the concentration of ACs; therefore, a VLCAD-deficient infant could easily be missed at this stage. Cutoff concentrations are based primarily on two metabolites, C14 and C14:1, with C14:1 characteristically exhibiting a greater elevation than C14. Cutoffs vary somewhat, with most laboratories using between 0.5 and 1.0 μ-mol/liter.

A typical VLCAD-deficient patient has shown C14:1 concentration greater than 2.0 μ-mol/liter. However, depending upon age and mutation, these concentrations can be found as low as 0.5 μ-mol/liter, which falls below cutoffs used in several laboratories. Therefore, as with MCAD deficiency, examination of a series of AC concentrations and relative molar ratios is necessary to establish a presumptive positive result. Furthermore, using molecular analysis for the common mutations is also helpful in confirming a diagnosis of VLCAD deficiency (92).

It is important to note that there is some confusion surrounding the naming of VLCAD deficiency. Historically, it was called LCAD deficiency, but with new evidence that there may be a different, more general LCAD enzyme, the disorders discussed here are now called VLCAD. No case of LCAD deficiency has been reported.

This MS/MS analysis of VLCAD deficiency appears to be quite simple on the surface. However, the combination of an elevated C14 with an elevated C14:1 may also indicate other metabolic disorders. For example, in addition to VLCAD deficiency, elevations of C14:1 and C14 may be produced by MADD. Also, CPT-II and translocase deficiencies may present with a mild elevation of C14. In both CPT-II and translocase deficiencies, however, the C16 elevation is generally more prominent (12).

SCAD Deficiency

Short-chain acyl-CoA dehydrogenase (SCAD) deficiency is characterized by an accumulation of short-chain fatty ACs, primarily 4-carbon butyrylcarnitines (C4) (95, 99). Their accumulation results from a deficiency of the SCAD enzyme. No other metabolites are significantly elevated in SCAD deficiency. As with those fatty acid disorders discussed previously, a C4 elevation does not always definitively and solely indicate SCAD deficiency (12). For instance, an elevation of C4 may also indicate the presence of MADD.

MADD (GA-II)

MADD is characterized by an accumulation of short-, medium-, and long-chain ACs as well as isovalerylcarnitine (C5) and often glutarylcarnitine (C5DC) (12, 68, 74, 82). The metabolite pattern is quite variable but generally always includes elevations of C8 and C8:1. With most diagnostic MADD profiles, ACs are mildly elevated as compared to disorders of other dehydrogenases such as MCAD, VLCAD, and SCAD deficiencies (67). This pattern of mild elevations makes the disorder one of the most difficult to detect using MS/MS. Certainly, in samples collected from infants more than 7 days of age, alterations in the AC profile are very subtle, and it may easily appear normal. Using special interpretation schemes like those used for the interpretation of postmortem metabolic profiles will help reduce false-negative and false-positive results. Urinary organic acid analysis and other confirmatory assays are required to definitively diagnose MADD.

LCHAD Deficiency

Long-chain hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency is characterized by an accumulation of long-chain hydoxy ACs (45, 74). These compounds include hydroxy-hexadecanoylcarnitine (C16OH), hydroxy-octadecenoylcarnitine (C18:1OH), and hydroxy-octadecanoylcarnitine (C18OH). In addition, elevations of C16, C14, C14:1, and hydroxy-tetradecanoylcarnitine (C14OH) may be present (93). A typical MS/MS spectrum of an LCHAD-deficient newborn is shown in Figure 2*d*. Unlike many of the other deficiencies described, the abnormal accumulations of metabolites in LCHAD deficiency may be very mild, making this disease another challenging disorder to detect using MS/MS (45). In particular, because the concentration of C16OH is near the limits of detection, the MS/MS system must be running optimally to detect this species. Further, as with MADD, the concentration of C16OH approaches control values as the infant ages, making confirmatory testing by MS/MS alone insufficient. At the very least, the ratio of C16OH to other ACs must be assessed. It is interesting to note the disorder may be difficult to distinguish from a mild VLCAD deficiency, especially if the sensitivity of C16OH is poor.

LCHAD is one of the three enzymes to compose mitochondrial trifunctional protein. Consequently, with secondary elevations of C16, C14, C14:1, and C14OH, LCHAD deficiency cannot be distinguished from complete trifunctional protein deficiency by MS/MS. Confirmatory molecular analysis and fibroblast studies are required to correctly differentiate between LCHAD and total trifunctional protein deficiency (45). Molecular analysis for the most common LCHAD-deficiency mutation, 1528 G → C, can be performed by polymerase chain reaction and allele-specific cleavage analysis to detect the mutant and wild-type forms of the gene.

CPT-I and -II Deficiencies

CPT-II deficiency is characterized by an accumulation of long-chain fatty acids and a relative deficiency of short- and medium-chain ACs (74). In this disorder, primarily C16, C18, and C18:1 are elevated (12). Although C14 may be elevated, relatively speaking, it is much lower in concentration than C16, owing to a blockade of conversion of long-chain fatty ACs back to acyl-CoA inside the inner mitochondrial membrane. Translocase deficiency produces an identical AC pattern. In translocase deficiency, the defect is in the transport of ACs across the inner mitochondrial membrane rather than in the reconversion of the long-chain ACs to acyl-CoAs (102). Whereas concentrations of C16 are often high in normal newborns, in CPT-II deficiency, the patient's level of C16 is extraordinarily elevated during the newborn period. Further, affected infants often have a deficiency of CN. Therefore, the ratio of long-chain ACs to free CN is diagnostic. A typical MS/MS spectrum of a CPT-II-deficient newborn is shown in Figure 2*e*.

With regard to CPT-I deficiency, the reverse metabolic pattern is observed (85). Because this enzyme is responsible for conversion of CN to ACs, in CPT-I deficiency, CN will accumulate and long-chain ACs will not be produced. In this

disorder, one would expect a decrease in C16, C18, and C18:1, and an increase in the concentration of free CN. Again, the ratio of free CN to long-chain ACs is important in establishing a diagnosis.

Other Fatty Acid Oxidation Disorders

Other extremely rare disorders of FAO include medium-chain hydroxyacyl-CoA dehydrogenase (MCHAD) deficiency and short-chain hydroxyacyl-CoA dehydrogenase (SCHAD) deficiency. MCHAD deficiency has yet to be detected in a newborn. However, studies have determined that it is characterized by an elevation of hydroxy-medium-chain ACs and medium-chain ACs. SCHAD deficiency is characterized by a significant elevation of 3-hydroxy-butyrylcarnitine (C4OH) and a milder elevation of C4 (6, 74). It is important to note that this pattern is also found in typical postmortem specimens that have no FAO defects (12).

ORGANIC ACID DISORDERS

Organic acidemias are metabolic disorders that encompass the metabolism of fatty acids, AAs, and carbohydrates (61, 62). Many of the intermediates in organic acid metabolism are acyl-CoA intermediates that undergo β-oxidation. As a result, in certain organic acid disorders, the concentrations of the acyl-CoAs will be substantially elevated, resulting in high concentrations of the associated ACs. For those organic acidemias that produce an AC, the ability of MS/MS to detect the disorder is satisfactory (71, 91, 95).

Propionic and Methylmalonic Acidemias

Both propionic acidemia (PA) and methylmalonic acidemia (MMA) are disorders that produce characteristic elevations of propionylcarnitine (C3) (11, 58, 68, 71). A typical MS/MS spectrum of an infant with PA is shown in Figure 3*a*. Because these 3-carbon fatty ACs are the primary ACs elevated in these disorders, their elevation cannot be used to differentiate PA from MMA. Nevertheless, PA cases do, on average, exhibit higher concentrations of C3 as compared to MMA cases. The decision criteria for suspecting either PA or MMA rely on both the concentration of C3 and the ratio of C3 to C2, denoted as C3/C2 ratio (11). Previously, a moderate number of falsely elevated C3 results were observed because of a generalized elevation of short-chain ACs, especially C2. With a sufficient number of known and false-positive cases, reevaluation of diagnostic criteria has demonstrated the value of using the C3/C2 ratio. This molar ratio reduces the number of false-positive results and may also reduce the possibility of a false-negative result, especially in infants with certain cobalamin (Cbl) defects and in infants greater than 7 days of age. Correct diagnosis of PA and MMA, and further reduction of false-positive results, clearly requires confirmation by urinary organic acid analysis. Although we have excellent sensitivity for most of these disorders, there is and will remain the possibility of obtaining a false-negative result among the Cbl deficiencies. In

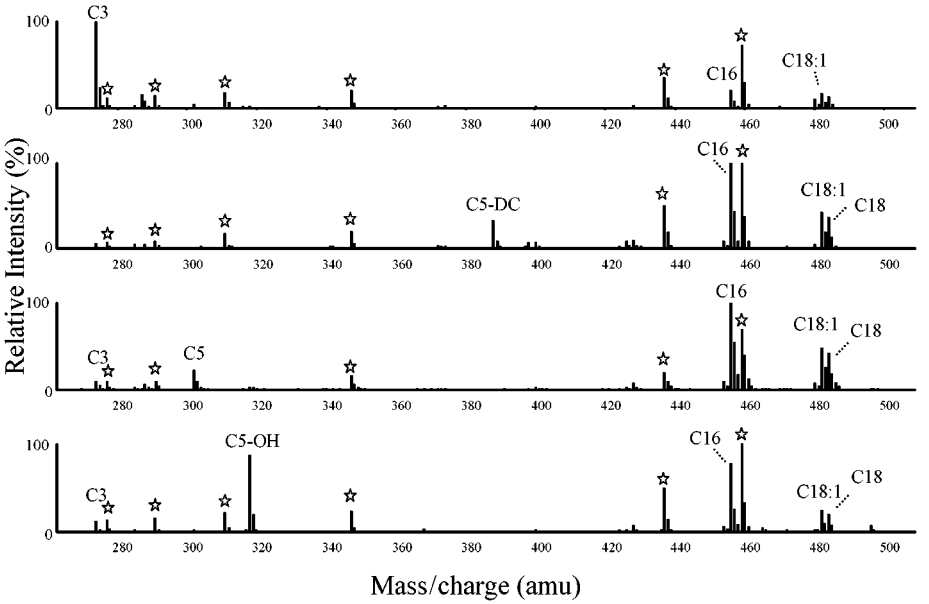

Figure 3 Pre 85 MS/MS spectra of newborn specimens: (*a*) PA, (*b*) GA-I, (*c*) IVA, and (*d*) 3-MCC deficiency. The stars represent stable isotope internal standards.

fact, we have documented one instance where an infant was diagnosed at almost 5 months of age with Cbl C deficiency. The original NBS result was within normal limits. This case documents the fact that in some metabolic diseases, especially milder variants, those abnormal concentrations may not be detectable in the early newborn period. Logic dictates that even if an NBS result is normal, a child may still be afflicted by a metabolic disorder. Therefore, vigilance dictates that if a metabolic disorder is suspected in a child owing to the presence of symptoms associated with a metabolic disorder, then another specimen should be analyzed using two methods.

Glutaric Acidemia Type I

Glutaric acidemia type I (GA-I) is a rare metabolic disorder that is expressed at high levels in a few notable population groups (4, 56). For instance, Pennsylvania has a frequency that is much higher than that exhibited in the general population, owing to its large Amish population, where GA-I is common (56). Overrepresentation of the Pennsylvania Amish community has enabled the development of screening for GA-I, even though glutarylcarnitine standards are unavailable at this time. GA-I is a very serious disorder that if undetected may result in permanent dystonia and dyskinesia (56, 88). However, NBS followed by early initiation of treatment prevents neurologic symptoms in more than 85% of affected children. Glutaric acid

accumulates in this disorder and is detected as a 5-carbon dicarboxylic AC termed glutarylcarnitine (C5DC). A typical MS/MS spectrum of an infant with GA-I is shown in Figure 3*b*. The concentration of C5DC is based solely on its relative ratio to the nearest internal standard, often that for C8. The amount of C5DC can vary considerably in the newborn period, from a peak that is just above the limits of detection to a peak of moderate intensity greater than the C8 internal standard. As with other ACs, the increasing age of an infant makes the metabolite more difficult to detect, thus making the ratios of C5DC to other ACs important. Experience has shown that initial positive results on known GA-I patients may be normal on repeat (12, 72, 88). An abnormal screen needs to be confirmed using urinary organic acid analysis. It is worth noting that, because C5DC is a dicarboxylic AC, it contains two negative charges. Esterification to butyl derivatives neutralizes these charges. However, because some laboratories do not derivatize their samples, their sensitivity for C5DC is disproportionately reduced, owing to the presence of this double-negative charge on a dicarboxylic AC. In these analyses, serious consideration of the method of derivatization is required if GA-I detection is to be part of the NBS panel using MS/MS.

Isovaleric Acidemia

Isovaleric acidemia (IVA) is a disorder of intermediate metabolism characterized by deficiency of mitochondrial isovaleryl-CoA dehydrogenase. In MS/MS analysis, an elevation of isovalerylcarnitine (C5) characterizes IVA. A typical MS/MS spectrum of an infant with IVA is shown in Figure 3*c*. The ratios of C5 to other ACs may be helpful. Clearly, an abnormal result must be confirmed by urinary organic acid analysis. It is interesting to note that pivalic acid metabolism also produces a characteristic C5 AC. Pivalic acid is a common counter ion in many drug preparations found outside the United States. An elevation of C5 may indicate drug therapy, especially in older children. It is, however, rare to see pivalic acid in newborn blood spots.

Disorders Characterized by an Elevation of 3-Hydroxy-Isovalerylcarnitine

There are three disorders of intermediary metabolism that are characterized by an elevation of 3-hydroxy-isovalerylcarnitine (C5OH). They include 3-methylcrotonyl-CoA carboxylase (3-MCC) deficiency (29, 32), 3-hydroxy-3-methylglutaryl-CoA (HMG) lyase deficiency (74, 76), and 3-methylglutaconyl-CoA hydratase deficiency. One other disorder, β-ketothiolase deficiency, is characterized in part by an elevation at the mass of C5OH, but tiglylcarnitine (C5:1) is also elevated (m/z 300) (54, 99). With β-ketothiolase deficiency, the elevated metabolite at m/z 318 is actually methylbutyryl hydroxyacylcarnitine, which shares the same mass as C5OH. Some of these disorders are accompanied by only mild symptoms, whereas others are more severe. A typical MS/MS spectrum of an infant with 3-MCC deficiency is shown in Figure 3*d*.

FREQUENCY OF AMINO ACID, FATTY ACID, AND ORGANIC ACID DISORDERS DETECTABLE BY MS/MS

The frequency of all disorders detected using MS/MS for more than 1.1 million infants screened at Neo Gen Screening is provided in Table 2. At least 75% of the screening population consists of infants born in Pennsylvania. The second-largest cohort includes infants from North Carolina who were screened as part of a 200,000-newborn prospective pilot screening program. Other areas and states with significant numbers of infants screened by our laboratory include the District of Columbia, Ohio, New Jersey, Mississippi, Louisiana, and Illinois. Our laboratory has used MS/MS to screen a greater number of newborns for amino acidopathies than for fatty acid or organic acid disorders. This trend can likely be observed in other NBS programs as well because state and regional NBS programs more commonly mandate screening for AA disorders. National (25, 106) and international (22, 33, 97) experience in the detection of amino acidopathies can produce similar findings.

SUMMARY

Since its initial application to NBS in the early 1990s, MS/MS has revolutionized the detection of inborn errors of metabolism. The inherent ability of MS/MS to detect and quantify multiple analytes from just one prepared blood specimen has

TABLE 2 Frequency of amino acid, fatty acid oxidation and organic acid disorders detected using MS/MS to screen more than 1.1 million newborns (Neo Gen Screening, Pittsburgh, PA, USA)

Disorder type and number identified (No.)					
Amino acid (N = 1,161,456)	**No.**	**Fatty acid (N = 1,019,602)**	**No.**	**Organic acid (N = 1,019,602)**	**No.**
PKU	68	MCAD	65	PA	6
HyperPhe	65	VLCAD	3	MMA	9
MSUD	12	SCAD	3	GA-I	13
HyperMet	4	MADD (GA-II)	1	IVA	4
Homocystinuria	1	LCHAD/TFP	2	3-MCC	15[b]
Citrullinemia	5[a]	CPT-II/translocase	2	HMG	1
Tyrosinemia III	1				
ASA	2				

[a]4 acute neonatal, 1 mild.

[b]11 maternal, 4 isolated.

enhanced early identification of inherited metabolic disorders by making DBS analysis more comprehensive, accurate, sensitive, and specific (25, 95). By virtue of its simultaneous detection of numerous AAs and ACs, MS/MS permits recognition of AA, fatty acid, and organic acid disorders in one brief analysis. In a growing number of NBS programs in the United States and around the world, MS/MS applications are being compared to, and in some cases taking the place of, classic BIA and fluorometric assays (7a, 41, 64–66, 96, 97, 99, 106). MS/MS presents unique opportunities because it is readily adaptable to screening for newfound disorders and conditions. It is important to note, however, that MS/MS alone is not a cure-all for any NBS program. Many factors contribute to the successful application of MS/MS to an NBS program, including sample preparation methods, proper instrument configuration and maintenance, profile interpretation, quality assurance (98), and overall experience. Clearly, however, its versatility will lend to the longevity of MS/MS in the field of NBS.

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