

## E.1 Introduction

Tandem mass spectrometry (MS/MS) is an analytical method that uses two mass analyzers to perform the separation and analysis of mixture components after ionizing them, usually by means of a “soft” ionization technique such as electrospray or fast ion bombardment. The method differs from the more familiar gas chromatography/mass spectrometry (GC/MS) method, used primarily for analysis of organic acids in urine, in several ways. MS/MS is applicable to polar compounds that are not readily amenable to GC/MS. By limiting or altogether avoiding the chromatography step, the analytical process is much faster and therefore capable of high specimen throughput. MS/MS is well suited to the quantitative analysis of specific metabolites or to groups of metabolites having similar chemical structure. It is thus a useful adjunct to GC/MS and other methods currently used in clinical diagnostic laboratories. Clinical applications of MS/MS are almost exclusively performed on a “triple” quadrupole mass spectrometer [1], equipped with electrospray ionization [2] and an automated sample introduction system. The central quadrupole in this type of instrument is actually a device that induces fragmentation and not a mass analyzer.

The first and most widely used MS/MS test developed for clinical diagnosis is the acylcarnitine profile [3]. This chapter will therefore focus primarily on this test and its diagnostic implications. Other valuable tests have recently been developed or are in the process of development by MS/MS, some of which are also discussed in this section. These include assays for free and total carnitine [17], selected amino acids including phenylalanine and tyrosine [4], methionine [5], homocysteine [6] and sulfocysteine, and for selected pyrimidines [7] and acylglycines [8]. Methods for bile acids [9, 10], steroids, plasmalogens [11], sphingolipids and phospholipids have also recently been reported. Although alternative methods are available for most of these tests, none can match the quantitative precision and rapid turn-around time of MS/MS.

One of the most encouraging and significant recent developments is the application of MS/MS in newborn screening for inborn errors of metabolism, first suggested over 10 years ago [20]. The development of microplate

batch analysis systems [14] rendered MS/MS practicable for analysis of the large numbers of samples (typically >600 per day) encountered in newborn screening laboratories. MS/MS has been in routine use for newborn screening in a few laboratories for the past several years. Preliminary reports [21, 22] are highly encouraging, and are prompting screening laboratories worldwide to adopt MS/MS. Many have recently adopted the new technology or are in the process of conducting pilot studies with a view to adoption.

Because of its versatility, enabling several important clinical diagnostic tests to be performed with a single apparatus, tandem mass spectrometry is one of the fastest growing analytical sciences, and further useful clinical diagnostic tests based on this technology are certain to appear in the literature in the near future. One of the most attractive features of this method is its cost-effectiveness, which has been commented upon in earlier reports [6, 17]. The cost-effectiveness of MS/MS is based partly on the reduction of consumable items such as kits and reagents, partly on the replacement of older technology, partly on the increased sample throughput facilitated by automated sample preparation and injection devices and partly on the ability to perform multiple tests simultaneously. In newborn screening, for example, over 20 inherited metabolic disorders can be screened in more than 500 samples per day using a single tandem mass spectrometer [14].

## E.2 Pre-analytical Conditions

### ■ Specimens

#### ● *Carnitine and Acylcarnitines*

Carnitine and acylcarnitine analyses are generally performed on random plasma or serum samples. The type of anti-coagulant used is unimportant. A whole blood specimen collected on cotton fiber filter paper, in the manner prescribed for collection of newborn's blood for the Guthrie test and other neonatal screening tests, is also applicable for acylcarnitine analysis. It is generally agreed that the preferred specimen for acylcarnitine analysis is one collected after at least a 6-h fast, since metabolites characteristic of the defects of fatty acid oxidation, for which the test is most often ordered (Table E.1), are likely to accumulate during the fasting state. Prolonged fasting is not recommended due to increased risk of metabolic decompensation. Diagnosis, however, is possible in the majority of cases without prolonged fasting. It is not necessary to "load" a patient with carnitine before the acylcarnitine test, unless the patient is markedly carnitine deficient.

Urine is of limited value and is not recommended for analysis of acylcarnitines [3]. Free and total carnitine determinations in urine are also generally of little diagnostic value.

**Table E.1.** Disorders associated with abnormal carnitine/acylcarnitine levels

| Acylcarnitine                        | Source | Change | Disorder to be considered<br>(in Chap. 14 except where<br>otherwise indicated)   |
|--------------------------------------|--------|--------|--|
| Carnitine (total)                    | P, B   | ↓↓     | Carnitine transporter deficiency<br>GA-I (12), MCAD, VLCAD, LCHAD  |
| Acetyl (C2)                          | P, B   | ↓      | Carnitine deficiency or insufficiency  |
| Propionyl (C3)                       | P, B   | ↑↑     | Propionic acidemia;<br>Methylmalonic acidemia  |
|                                      |        | ↑      | Holocarboxylase deficiency;<br>Biotinidase deficiency [7]  |
| Butyryl/Isobutyryl (C4)              | P, B   | ↑↑     | SCAD deficiency  |
|                                      |        | ↑      | SCAD deficiency (“mild” variant);<br>Isobutyryl-CoA dehydrogenase defi-<br>ciency [7]; multiple acyl-CoA dehy-<br>drogenase (MAD) deficiency |
| Tiglyl/3-Methylcrotonyl<br>(C5:1)    | P, B   | ↑      | 3-Oxothiolase deficiency; 3-MCC<br>deficiency [7]  |
| Isovaleryl/2-Methyl-<br>butyryl (C5) | P, B   | ↑↑     | Isovaleric acidemia [6]  |
|                                      |        | ↑      | 2-Methylbutyryl-CoA dehydrogenase<br>deficiency [7]; MAD deficiency  |
| 3-Hydroxyisovaleryl<br>(C5-OH)       | P, B   | ↑↑     | 3-Methylcrotonyl-CoA carboxylase<br>(3-MCC) deficiency [7]   |
|                                      |        | ↑      | 3-OH-3-methylglutaryl-CoA (HMG-<br>CoA) lyase deficiency [6]; Holocar-<br>boxylase deficiency;<br>Biotinidase deficiency [7]                 |
| 3-Hydroxy-2-Me-<br>butyryl (C5-OH)   | P, B   | ↑      | 3-Oxothiolase deficiency [7]   |
| Methylmalonyl (C4-DC)                | P, B   | ↑      | MMA [7]  |
| Glutaryl (C5-DC)                     | P, B   | ↑      | Glutaric acidemia, type I (GA-I) [12]  |
| Hexanoyl (C6)                        | P, B   | ↑      | MCAD deficiency; MAD deficiency  |
| Octanoyl (C8)                        | P, B   | ↑↑     | MCAD deficiency  |
|                                      |        | ↑      | MCAD deficiency; MAD deficiency  |
| Decenoyl (C10:1)                     | P, B   | ↑      | MCAD deficiency  |
| Methylglutaryl (C6-DC)               | P, B   | ↑      | HMG-CoA lyase deficiency [6]   |
| Decanoyl (C10)                       | P, B   | ↑      | MAD deficiency   |
| Dodecanoyl (C12)                     | P, B   | ↑      | MAD deficiency   |
| Tetradodecenoyl<br>(C14:2)           | P, B   | ↑      | VLCAD deficiency   |
| Tetradecenoyl (C14:1)                | P, B   | ↑      | VLCAD deficiency   |
| Tetradecanoyl (C14)                  | P, B   | ↑      | VLCAD deficiency; MAD deficiency   |
| Palmitoyl (C16)                      | P, B   | ↑      | VLCAD deficiency; CPT-II deficien-<br>cy; CAT deficiency; LCHAD defi-<br>ciency; MAD deficiency  |
|                                      | B      | ↓      | CPT-I deficiency   |

Table E.1 (continued)

| Acylcarnitine                 | Source | Change | Disorder to be considered<br>(in Chap. 14 except where<br>otherwise indicated) |
|-------------------------------|--------|--------|--|
| Linoleoyl (C18:1)             | P, B   | ↑      | VLCAD deficiency; CPT-II deficiency; CAT deficiency; LCHAD deficiency          |
| 3-Hydroxypalmitoyl (C16-OH)   | B      | ↓      | CPT-I deficiency   |
| 3-Hydroxypalmitoyl (C16-OH)   | P, B   | ↑      | LCHAD deficiency; TFP deficiency   |
| 3-Hydroxylinoleoyl (C18:1-OH) | P, B   | ↑      | LCHAD deficiency; TFP deficiency   |

In vitro testing using cultured skin fibroblasts with stable isotope-labeled substrates can be helpful in elucidating and confirming some defects of fatty acid [12] and branched-chain amino acid [13] catabolism.

Post-mortem specimens for acylcarnitine analysis, in descending order of usefulness for diagnosis of fatty acid oxidation disorders, are bile, blood and liver tissue.

For prenatal diagnosis, amniotic fluid can be used to identify acylcarnitines characteristic of certain branched-chain amino acid disorders. Cultured amniocytes or chorionic villous cells can be used with in-vitro substrate loading for diagnosis of fatty acid and branched-chain amino acid disorders as described earlier for fibroblasts [13].

### ● Amino Acids

Plasma, whole blood spotted onto filter paper, including Guthrie (newborn screening) cards, and urine samples are suitable specimen types, depending on the amino acid(s) of interest. Although LC/MS/MS methods are under development, the application of direct MS/MS for amino acid analysis is presently limited to a few components for which assays have been appropriately validated. At present, therefore, MS/MS is not a substitute for standard amino acid analysis using ion exchange or reverse-phase HPLC methods. The method is used primarily in newborn screening, as a means of identifying PKU and a few other amino acid disorders (Table E.2). It can also be used effectively in the follow-up of such patients, where a single amino acid level is often all that is needed. Limitations include the partial hydrolysis of glutamine and asparagine and the inability to distinguish between isomers, such as leucine, isoleucine and allo-isoleucine. Assays for specific amino acids include total plasma homocysteine, requiring special sample preparation [6], and urine sulfocysteine, for which dried urine on cotton fiber filter paper is acceptable. In both cases, a reversed-phase LC column is used on-line to the MS/MS.

**Table E.2.** Disorders associated with abnormal amino acid levels

| Compound              | Source  | Change | Disorder to be considered                                   |
|-----------------------|---------|--------|---|
| Glycine               | P, B    | ↑      | Non-ketotic hyperglycinemia                                 |
| Valine                | P, B    | ↑      | MSUD  |
| Leucine/Isoleucine    | P, B    | ↑      | MSUD  |
| Phenylalanine         | P, B    | ↑      | PKU, other hyperphenylalaninemias                           |
| Tyrosine              | P, B    | ↑      | Tyrosinemia   |
| Methionine            | P, B    | ↑      | Homocysteinemia and other hypermethioninemias               |
| Arginine              | P, B    | ↑      | Argininemia   |
| Citrulline            | P,      | ↑      | Citrullinemia, Argininosuccinyl-CoA-lyase (ASAL) deficiency |
| Argininosuccinic acid | P, B, U | ↑      | ASAL deficiency   |
| Sulfocysteine         | U       | ↑      | Sulfite oxidase deficiency, molybdenum cofactor deficiency  |

Note: only disorders for which validation studies for the MS/MS method have been carried out are included in this list.

### ● *Acylglycines, Purines and Pyrimidines*

Urine is preferred for these tests. The small volumes required make it practicable to spot the urine onto cotton fiber filter paper and allow it to dry out, for ease of shipment by mail. The limited experience in applying MS/MS for these analytes suggests that the levels of pathognomonic metabolites should significantly exceed control values for purines and pyrimidines regardless of the patient's clinical state (Table E.4). This is true also of acylglycines for certain disorders such as propionic acidemia, isovaleric acidemia and MCAD deficiency (Table E.3), although it appears that most other

**Table E.3.** Disorders of fatty acid and amino acid metabolism associated with abnormal urinary acylglycine levels (from [8])

| Compound                | Source | Change | Disorder to be considered (in Chap. 14 except where otherwise indicated) |
|-------------------------|--------|--------|--|
| Propionyl               | U      | ↑      | Propionic acidemia (PA), MMA, holocarboxylase synthetase def. (MCD) [7]  |
| Butyryl/Isobutyryl      | U      | ↑      | SCAD, MAD  |
| Isovaleryl              | U      | ↑      | Isovaleric acidemia [6]  |
| Tiglyl/3-Methylcrotonyl | U      | ↑      | PA, 3-MCC, HMG, MCD {6, 7}   |
| Hexanoyl                | U      | ↑      | MCAD, MAD  |
| Octanoyl                | U      | ↑      | MCAD, MAD  |
| Glutaryl                | U      | ↑      | GA-I [12]  |
| Hydroxyoctanoyl         | U      | ↑      | MCAD   |
| Phenylpropionyl         | U      | ↑      | MCAD   |
| Suberyl                 | U      | ↑      | MCAD, MAD  |

**Table E.4.** Purine/pyrimidine disorders associated with abnormal metabolite levels (from [7])

| Compound          | Source | Change | Disorder to be considered (in Chap. 23 except where otherwise indicated) |
|-------------------|--------|--------|--|
| Inosine           | U      | ↑      | Purine nucleoside phosphorylase deficiency (PNPD)                        |
| D-Inosine         | U      | ↑      | PNPD   |
| Guanosine         | U      | ↑      | PNPD   |
| D-Guanosine       | U      | ↑      | PNPD   |
| Orotic acid       | U      | ↑      | OTC deficiency   |
| Uracil            | U      | ↑      | OTC deficiency   |
| Xanthine          | U      | ↑      | Molybdenum cofactor deficiency   |
| Succinyladenosine | U      | ↑      | Adenylosuccinase deficiency (ADS)  |
| Thymine           | U      | ↑      | Dihydropyrimidine dehydrogenase deficiency (DPDH)                        |
| 5OH-Methyl uracil | U      | ↑      | DPDH   |

disorders of fatty acid oxidation are either not detectable at all or are not detected in all patient samples [8].

## ■ Patient Status/Patient Information

### ● *Carnitine and Acylcarnitines*

As is the case with all biochemical tests for inherited metabolic disorders, clinical information is an integral part of the test interpretation. Increasingly, special testing is referred by large contract laboratories that routinely do not provide this information. In this author's experience, more than 85% of test requests are now unaccompanied by any clinical information whatsoever. Therefore, it is incumbent on the referring physician to understand that the test results must be interpreted in the context of clinical status, and that direct communication with a qualified professional in the testing laboratory is often necessary, especially when the findings appear either ambiguous or unexpected.

The common clinical findings that would prompt a request for the MS/MS tests for acylcarnitines are listed in Table E.5. Note that there is considerable overlap of symptoms with those of organic and amino acidurias, and an acylcarnitine profile by MS/MS is arguably justifiable to include as part of the general metabolic screen for this type of disorder. Diagnosis of fatty acid oxidation disorders, especially long-chain defects, is greatly facilitated by the analysis of acylcarnitines. For this type of disorder, analysis of urinary organic acids by GC/MS is often unrevealing. A family history of unexplained infant death or near-death episodes is relevant. So is a history of pregnancy complications, especially acute fatty liver of pregnancy and

**Table E.5.** Acylcarnitine analysis

| Indications   |
|---|
| Clinical signs and symptoms                               |
| Respiratory distress                                      |
| Lethargy  |
| Coma  |
| Recurrent vomiting  |
| Failure to thrive   |
| Feeding difficulty  |
| Apnea   |
| Hypotonia   |
| Bradycardia   |
| Ventricular arrhythmias                                   |
| Cardiomyopathy  |
| Hepatomegaly  |
| Encephalopathy  |
| Seizures  |
| Dystonia  |
| Myopathy  |
| Rhabdomyolysis  |
| Renal tubular acidosis                                    |
| Polycystic kidneys  |
| Reye or Reye-like syndrome                                |
| “Near-miss” SIDS  |
| Presymptomatic indications                                |
| History of affected sibling(s)                            |
| History of sudden unexplained death or SIDS in sibling(s) |
| History of maternal pregnancy complications (AFLP, HELLP) |
| Routine clinical chemical indices                         |
| Acidosis  |
| Ketosis   |
| Hypoglycemia  |
| Hyperammonemia  |
| Elevated liver enzymes                                    |
| Elevated CK   |
| Other abnormal laboratory results                         |
| Dicarboxylic aciduria (excluding dietary MCT)             |
| Hydroxydicarboxylic aciduria                              |
| Abnormal newborn screen for acylcarnitines                |

“HELLP” syndrome, which have been linked to a fetus affected by more than one of these defects. Episodic symptoms and findings include lethargy, coma, seizure, respiratory distress, vomiting, hypoglycemia, hyperammonemia, cardiomyopathy, hepatomegaly, rhabdomyolysis, cardiac arrhythmias (especially in a neonate) and liver dysfunction.

There is a broad spectrum of clinical severity in patients affected by FAO disorders. Asymptomatic and chronically affected or deceased patients can occur within the same family. Therefore, when a diagnosis is made in a

family, it is prudent to promptly test any siblings. For these reasons in part, newborn screening tests for these disorders have recently been introduced in several countries [21, 22]. Experience has so far indicated a greater prevalence of this type of metabolic disease than would be predicted on the basis of previously diagnosed cases, suggesting that, as a group, these disorders have been significantly under-diagnosed.

#### ● *Amino Acids*

Because of the rather specialized applications of amino acid testing using MS/MS, general guidelines are not applicable. The individual chapters referring to each amino acid disorder are the best source of information. A new MS/MS test for sulfocysteine is now available, and should be included in the differential for intractable seizures in the neonate.

#### ● *Acylglycines*

Clinical presentation and specimen collection guidelines are analogous to those for urine organic acids analysis (Chap. C) and to disorders of fatty acids beta-oxidation (Chap. 14). It is reported that acylglycine excretion is less affected by the clinical status of the patient than organic acid excretion [8].

#### ● *Purines and Pyrimidines*

Inherited disorders of purine and pyrimidine metabolism exhibit a wide variety of clinical symptoms, including anemia, immunodeficiency, kidney stones, seizures, mental retardation, autism and growth retardation. Refer to Chap. 23 and ref. 7 for further details.

### ■ Specimen Collection

#### ● *Carnitine and Acylcarnitines*

As stated previously, the time of specimen collection has a bearing on the test results. A specimen collected when the patient is acutely ill or at least in the fasting state is more likely to be revealing. On the other hand, acute symptoms, especially those affecting liver function, and certain medications and dietary supplements may produce abnormal metabolites that can be misleading and confusing. Such clinical information should be provided to the reference laboratory whenever possible. Acylcarnitines are generally stable at room temperature for at least 24 h. Eventually, they degrade hydrolytically to free carnitine and the corresponding fatty acid. Stability is lower at pH values below and (especially) above 7.0. Short-chain species,



particularly acetylcarnitine, are the least stable. In order to obtain accurate values for free and total carnitine, plasma or serum should be separated, frozen and shipped on dry ice within a few hours of collection. Plasma should be stored at  $-20^{\circ}\text{C}$  prior to shipment. Under these conditions, acetyl and propionylcarnitine degrade to the extent of about 10 percent per year. For acylcarnitine analysis, plasma or serum should be frozen soon after separation and shipped on dry ice. Alternatively, especially where distance from the testing laboratory is an issue, whole blood or plasma can be spotted onto cotton fiber filter paper, dried in air at room temperature for 4 h and placed into a paper envelope for shipping, preferably overnight. Note that the reference laboratory will probably have determined control values based on a particular type of paper and that use of a different brand or type of paper will reduce the accuracy of the results. If in doubt, ask the reference laboratory for advice or ship frozen plasma.

Newborn's blood spots, collected on "Guthrie cards", are also useful for analysis of acylcarnitines and specific amino acids by MS/MS. In a situation where a child dies unexpectedly and there is no suitable post-mortem material available for biochemical testing, it is often possible to make a retrospective diagnosis from a single blood spot on the original Guthrie card. Newborn screening laboratories in several countries have recently expanded their service by the addition of MS/MS. Each laboratory sets its own normal ranges and makes its own decisions on the specific diseases to be screened for. Since there is currently no consensus on the protocols for setting control ranges or reporting abnormal values, normal and pathological ranges for neonates are excluded from this Chapter. The suggested method for establishing control values and the values published by Rashed et al. are a useful guide [14]. It is most important to understand that a child presenting with symptoms of a metabolic disorder that has reportedly had a normal newborn screen by MS/MS does NOT imply that that child cannot have any of the diseases that were screened for. As with any biochemical test performed on newborns, MS/MS is a screening test and can miss a diagnosis if a pathognomonic metabolite level does not exceed the control range.

The analysis of acylcarnitines by MS/MS is essentially molecularly specific, although it must be understood that isomeric species, such as the C5 species isovaleryl and 2-methylbutyryl carnitine, are not distinguishable (Table E.6). There are very few known direct interferences from drugs. It is known that antibiotics containing the pivaloyl group such as pivoxilsulbactam, used in some countries to treat urinary tract infections, can be passed to an infant in utero or by means of mother's milk and result in a falsely elevated signal for C5 acylcarnitine [15]. Numerous drugs, including anti-seizure medications such as valproate and various analgesics, can interfere with mitochondrial enzyme systems, including the fatty acid beta-oxidation pathway, and produce abnormally elevated levels of intermediates. Medium-

**Table E.6.** Reference values of acylcarnitines in plasma and whole blood

| Abbreviation | Acylcarnitine species    | Plasma | Whole blood |
|--------------|--------------------------|--------|-------------|
| C0 (free)    | Carnitine (free)         | 38±22  |             |
| C0 (total)   | Carnitine (total)        | 47±22  |             |
| C2           | Acetyl-                  | 2–16   | 2.5–23      |
| C3           | Propionyl-               | 0.75   | 1.93        |
| C4           | Butyryl/Isobutyryl-      | 0.43   | 0.44        |
| C4-OH        | 3-OH-butyryl-            | 0.21   | 0.25        |
| C5:1         | Tiglyl/3-Me-crotonyl-    | 0.03   | 0.03        |
| C5           | Isovaleryl/2-Me-butyryl- | 0.37   | 0.32        |
| C6           | Hexanoyl-                | 0.25   | 0.26        |
| C5-OH        | 3-OH-isovaleryl-         | 0.08   | 0.51        |
| C4-DC        | Succinyl/Methylmalonyl-  | 0.04   | 0.50        |
| C8           | Octanoyl-                | 0.22   | 0.15        |
| C5-DC        | Glutaryl-                | 0.03   | 0.03        |
| C6-DC        | Adipoyl/Methylglutaryl-  | 0.08   | 0.04        |
| C10:1        | Decenoyl-                | 0.30   | 0.16        |
| C10          | Decanoyl-                | 0.34   | 0.23        |
| C8-DC        | Suberyl-                 | 0.08   | 0.04        |
| C12:1        | Dodecenoyl-              | 0.24   | 0.14        |
| C12          | Dodecanoyl-              | 0.17   | 0.23        |
| C14:2        | Tetradecadienoyl-        | 0.15   | 0.11        |
| C14:1        | Tetradecenoyl-           | 0.26   | 0.22        |
| C14          | Tetradecanoyl-           | 0.10   | 0.30        |
| C14-OH       | 3-OH-tetradecanoyl-      | 0.03   | 0.03        |
| C16          | Palmitoyl-               | 0.27   | 0.24–2.63   |
| C16-OH       | 3-OH-palmitoyl-          | 0.03   | 0.03        |
| C18:2        | Linoleoyl-               | 0.27   | 1.02        |
| C18:1        | Oleoyl-                  | 0.42   | 0.31–2.78   |
| C18:1-OH     | 3-OH-oleoyl              | 0.03   | 0.03        |

Reference values for the acylcarnitines are derived from >500 patients, mostly pediatric (0.2–16 yrs), evaluated for metabolic disorders in the author's laboratory but with no manifest biochemical evidence of disease. Individuals with any markedly abnormal values were discounted. The analytical method used was tandem mass spectrometry with electrospray ionization. Internal standards used were stable isotope-labeled analogs of acetyl, propionyl, butyryl, octanoyl and palmitoyl carnitine. The values for straight-chain C2, C3, C4, C5, C6, C8, C10, C14, C16 and C18:1 species are in  $\mu\text{mol/l}$  and are derived from calibration curves using analytical standards; all other values are ratios of the signal for the compound to an appropriate internal standard. All values are mean + 2 std. dev. except where a range is given

chain triglycerides, employed as a supplement in various infant formulae, can elevate the levels of medium-chain acylcarnitines, especially C8 and C10, and dicarboxylic species (C6DC and C8DC). Patients receiving carnitine supplement have elevated C2, often with C3 and other species in a nonspecific pattern. Note that patients receiving high dose carnitine, especially by intravenous infusion, are likely to exhibit such grossly elevated

**Table E.7.** Artefacts and nonspecific abnormalities in plasma and blood acylcarnitine profiles

| Condition                     | Compound(s) involved           | Change |
|-------------------------------|--------------------------------|--------|
| MCT supplement                | C8, C10, (C6-DC, C8-DC)        | ↑      |
| Ketogenic diet                | C2, C4-OH                      | ↑↑     |
|                               | C12, C14:1                     | ↑      |
| Sunflower/olive oil challenge | C14:2                          | ↑      |
| Fasting ketosis               | C2, C4-OH, C12:1, C14:1        | ↑      |
| Lactic acidosis               | C2                             | ↑      |
| Valproic acid                 | C8, C10                        | ↑      |
|                               | C0 (total)                     | ↓      |
| Carnitine supplement          | C0, C2, C3, (+ others)         | ↑      |
| Benzoate supplement           | C0 (total)                     | ↓      |
|                               | Benzoylcarnitine               | ↑      |
| Other drugs (various)         | C8, C10, (C6DC, C8DC)          | ↑      |
|                               | C10:1, C12:1, C14:1 (+ others) | ↑      |
| Liver dysfunction             | C16DC, C18:1DC (+ others)      | ↑      |
| Dialysis (for renal failure)  | C0 (total)                     | ↓      |
| Short gut syndrome            | C0 (total)                     | ↓      |

carnitine and acylcarnitine levels that the result is uninterpretable. Prolonged fasting increases C2, OHC4, C12:1 and C14:1 [16]. Lactic acidosis also elevates C3 levels. Long-chain fat loading (sunflower oil or olive oil) causes elevations of C14:2 [16]. Patients receiving a ketogenic diet have markedly elevated levels of C2 and OH-C4 carnitine. Patients with urea cycle defects are often supplemented with benzoate and/or phenylacetate that produce signals corresponding to benzoylcarnitine and phenacetylglutamate, respectively. The masses of these species do not interfere with those of diagnostically important metabolites. It is pertinent to realize that patients who are severely ill can exhibit various nonspecific abnormalities in acylcarnitine patterns. However, none of the aforementioned interferences, summarized in Table E.7, should affect the diagnostic interpretation if performed by an experienced, qualified individual.

Urine samples for carnitine and acylcarnitine analysis should be frozen and shipped on dry ice (note however that urine is not recommended as a specimen for diagnosis, especially for defects of fatty acid oxidation). For convenience where distance from the testing laboratory is an issue, urine for these tests can also be spotted onto cotton fiber filter paper, allowed to dry and mailed in an envelope. Amniotic fluid should be frozen and shipped on dry ice.

Patient cells should be presented to the testing facility in flasks with appropriate medium (T-25 s or T-75 s) at or near confluency.

### ● *Amino Acids, Purines, Pyrimidines and Acylglycines*

Specimen collection for amino acids is comprehensively covered in Chap. B. Filter paper blood spots (PKU cards) are increasingly used for blood collection. Urine specimens for the testing of purines and pyrimidines, and for acylglycines are typically frozen and shipped on dry ice. They can also be applied to filter paper strips. For further details, refer to Chap. 21 and refs. 7 and 8.

## E.3 Analysis

### ■ Carnitine and Acylcarnitines

Analysis of carnitine and acylcarnitines by MS/MS is now considered routine in those laboratories that possess the appropriate technology. The ionization techniques commonly used with MS/MS are fast atom (or fast ion) bombardment and electrospray. Both are sufficiently sensitive to detect abnormally elevated concentrations of specific metabolites in all types of specimen, although the latter is much more widespread and is the more sensitive of the two, especially for long-chain acylcarnitines. The method is quantitative or at least semi-quantitative for most analytes, and uses stable isotope-labeled forms of the analytes as internal standards. The acylcarnitines are analyzed simultaneously in the positive ion mode as their methyl or butyl esters using a precursor ion scan function [14, 23] that detects the parent (molecular) ions. Free carnitine and total carnitine are determined by assaying the same specimen before and after alkaline hydrolysis, without derivatization, using  $^2\text{H}_3$ -carnitine as internal standard [17]. The value for “acylcarnitine” is determined by difference. This value includes the contribution of short, medium and long-chain acylcarnitines. Analysis time for each method is approximately 2 min.

Individual acylcarnitines are generally quantified using a mixture of isotope-labeled standards. In the author's laboratory, with the exception of acetyl carnitine, the normal value for each acylcarnitine is quoted as an upper limit, corresponding to the 99.5th percentile of the values from a large cohort (>500) of apparently unaffected patients. Neither analytical standards nor matching internal standards are available for several acylcarnitines of biological importance, including glutaryl, 3-hydroxyisovaleryl and 3-hydroxypalmitoyl carnitine. In these cases, the upper limit of the normal range is established from the ratio of the signal from the analyte to that of the internal standard nearest in molecular weight. Note that the control ranges for acylcarnitines in whole blood and in plasma or serum are different (Table E.6). Control values between different laboratories using the same methodology may vary somewhat according to the source of standards, internal standards and various other factors. The analysis report

should consist of a table of results from the patient specimen, with control values for comparison, and an interpretation from a qualified professional.

#### ■ Amino Acids

Most amino acids are analyzed directly in positive ion mode by MS/MS as their butyl esters, using a neutral loss scan function [14, 23]. Stable isotope labeled analogs are employed as internal standards. Fast ion bombardment and electrospray ionization are both applicable, although the latter is preferred. Analysis time is about 2 min. Sulfocysteine is analyzed by ESI-MS/MS without forming a derivative in the negative ion mode, using a stable isotope-labeled internal standard (Millington et al., unpublished). A short LC column is used on-line to separate the analyte from salts that suppress the MS/MS signal. Analysis time is about 6 min. Affected patients have much greater values than do normal or unaffected subjects.

#### ■ Acylglycines

Acylglycines are analyzed directly in positive ion mode as their methyl esters using a precursor ion scan function [8, 23]. Electrospray ionization is preferred. Analysis is about 2 min. Stable isotope-labeled internal standards are used for quantification.

#### ■ Purines and Pyrimidines

The target compounds are analyzed by HPLC-MS/MS, using electrospray ionization in the negative ion mode. The compounds are detected by multiple reaction monitoring, using stable isotope-labeled analogs as internal standards when available. The HPLC separation is required to distinguish between isomers, such as uridine and pseudouridine, or adenosine and deoxyguanosine, and to reduce the interference of salts. Analysis time is about 15 min.

### E.4 Interpretation and Normal Variation

#### ■ Carnitine and Acylcarnitines

Interpretation of carnitine values and other single analyte measurements by MS/MS is generally quite straightforward, since values are given alongside a normal control range. In plasma, the total carnitine values average about 47  $\mu\text{mol/l}$  (range: 25–70). With the exception of CPT-I deficiency and some instances of severe cardiomyopathy, there is little clinical significance to higher values; they reflect increased dietary intake or exogenous supple-

mentation. The same general comments apply to free carnitine values. It has been reported that plasma carnitine levels in neonates are initially elevated, then tend to fall by up to half the normal adult value and normalize after a few months [18]. Normally, the free carnitine value is at least 75% of the total, but elevated acyl/free carnitine ratios in plasma occur quite frequently. The usual reason is a temporary increase in acetylcarnitine due to increased formation of acetyl-CoA. This can occur after relatively short fasting periods, during ketosis and/or lactic acidosis.

In metabolic diseases, disease-specific acylcarnitines can accumulate and elevate the acyl/free carnitine ratio. However, a normal ratio is often seen in affected patients when under good metabolic control. Therefore, it is important to understand that neither the absolute concentrations nor the ratio of free and total carnitine values are *predictive* of metabolic disease. Therefore, if metabolic disease is suspected, it is recommended that the acylcarnitine profile test be ordered as well as the free and total carnitine assay. The acylcarnitine profile is a separate test, designed to recognize defects of intermediary metabolism in which abnormal acyl-CoA metabolites accumulate in mitochondria and are exported to the plasma as acylcarnitines.

The variation of acylcarnitine levels with age has not been systematically studied. It is known however from newborn screening studies that in neonates the levels of acetyl, propionyl and long-chain acylcarnitines are somewhat higher than in older children.

It should be noted that the control ranges for acylcarnitines in plasma and whole blood (Table E.6) are different. The main differences between whole blood and plasma acylcarnitine profiles are that concentrations of long-chain acylcarnitines are significantly higher in whole blood because of their association with erythrocyte membranes (Table E.6). Other species that are in significantly higher concentration in whole blood than in plasma include acetyl, propionyl, OH-C5 and C4-dicarboxylic species (C4-DC), the latter being a mixture of succinyl (mostly) and methylmalonyl carnitine. This difference becomes significant in the diagnosis of long-chain fatty acid disorders (except CPT-I) and of 3-MCC deficiency. Plasma is then preferred to whole blood because of the increased sensitivity to changes in disease-specific metabolite concentrations.

The normal acylcarnitine pattern in plasma consists of mostly acetylcarnitine (C2), with increasingly lower amounts of C3 (propionyl), C4 (a mixture of butyryl and isobutyryl), C5 (a mixture of isovaleryl and 2-methylbutyryl), OH-C4 (mostly hydroxybutyryl), OH-C5 (3-hydroxyisovaleryl) and typically minor amounts of others, up to C16 and C18:1. The concentration of acetylcarnitine in both plasma and whole blood is variable. During fasting, increased intra-mitochondrial production of acetyl-CoA and 3-hydroxybutyrate elevates the C2 and OH-C4 signals, sometimes markedly. Other conditions that can produce spurious changes in metabolite levels are discussed in E.2 and summarized in Table E.7

### ■ Amino Acids

In newborn screening laboratories, where the MS/MS method is typically used to screen for selected amino acid disorders, the control ranges are determined by each laboratory using essentially the same guidelines [14]. When applied to specimens from older patients, the normal ranges provided in Chap. B would be appropriate. Elevated levels are often seen in patients receiving TPN, but this is a generalized pattern easily recognized from the profile. For sulfocysteine, normal controls have  $<30$  nmol/mg creatinine (data from the author's laboratory). No significant chemical interferences have been reported.

### ■ Acylglycines

The major acylglycine in normal controls is acetyl, with minor amounts of C4, C5 and C6 [8]. Although this method has not been investigated as thoroughly as that of plasma acylcarnitines to establish normal variation, indications are that it is not significantly more prone to interference from drugs and dietary components [8].

### ■ Purines and Pyrimidines

There is limited experience with the application of MS/MS to this group of analytes, and control data using this method is lacking [7]. Indications are that affected patients will be readily distinguished from normal controls, and that the risk of chemical interference is low compared with other methods.

## E.5 Pathological Values: Differential Diagnosis

The association of abnormal metabolite levels and possible metabolic diseases are summarized in Table E.1. The degree of elevation in disease-specific metabolites is variable, and depends on several factors. With few exceptions (below), minor elevations (i.e.  $<1.5\times$  upper normal limit) of a single metabolite are not diagnostic.

Very low plasma total carnitine levels (i.e.  $<15$   $\mu\text{M}$ ) with a normal acylcarnitine pattern could signal any of a number of acquired deficiencies (diet or drug related) or a deficiency of the plasma membrane transporter. Patients with certain metabolic disorders, especially GA-I and fatty acid oxidation disorders such as MCAD and VLCAD, who have never received carnitine supplement can become markedly carnitine deficient, and their acylcarnitine profiles may be interpreted as normal if pathognomonic metabolite levels do not exceed the normal cut-off. Carnitine deficiency or in-

sufficiency should be suspected when the acetylcarnitine signal is abnormally low. It is prudent to repeat the analysis of acylcarnitines after carnitine supplementation in such patients with presumptive or manifestly very low carnitine levels.

Note that MS/MS is unable to distinguish between isomeric acylcarnitines. Therefore, elevations of C4 can be either from accumulation of butyryl or isobutyryl carnitine, C5 can be either isovaleryl or 2-methylbutyryl and so on. Some individual metabolites are characteristic of more than one disease. Propionylcarnitine is markedly elevated in both propionic and methylmalonic acidemia. 3-Hydroxyisovalerylcarnitine (OH-C5) is associated with both 3-MCC deficiency and HMG-CoA-lyase deficiency. Minor elevations in either or both of these metabolites are also consistent with holocarboxylase deficiency or with deficiency of the cofactor biotin (or biotinidase). The differential diagnosis of each of these conditions is generally made from a careful analysis of urinary organic acids, performed by capillary column GC/MS in a reputable facility. This is especially important in the follow-up of abnormal newborn screening acylcarnitine results.

Elevations of single metabolite levels are generally characteristic of disorders of amino acid catabolism. In propionic and isovaleric acidemia, the levels of pathognomonic metabolites are typically >5 times the upper limit of normal. Particular attention should be paid to glutarylcarnitine (C5DC). Even a minor elevation is likely to be significant, and should prompt immediate follow-up urine organic acids to check for metabolites of GA-I. Also, isolated C3, C4, C5 and OH-C5 elevations from 1.5 to 2 times the normal upper limit should prompt follow-up to include urine organic acids and a repeat acylcarnitine analysis.

Defects of fatty acid catabolism, with the exception of SCAD deficiency, generally have elevation of more than one characteristic metabolite. MCAD deficiency is characterized by accumulation of C6, C8 (mainly) and C10:1 species. LCAD and VLCAD are characterized by accumulation of C14:1, C14:2 and (usually) C16 and C18:1 species. LCHADD and TFP deficiencies are characterized by the accumulation of OH-C16, OH-C18:1 and usually at least one of the other long-chain species C14:1, C16 and C18:1. The CPT-II and CAT (carnitine/acylcarnitine translocase) deficiencies are characterized by marked elevation of both C16 and C18:1, but not C14:1. Multiple acyl-CoA deficiency (MAD) has several different etiologies, including electron transferring protein (ETF) deficiency, ETF-dehydrogenase deficiency and riboflavin deficiency. Disease patterns vary considerably. In severe forms of the disorder, a generalized marked elevation of multiple intermediates is observed. CPT-I should be suspected when both C16 and C18:1 are very low in whole blood, especially if free carnitine is normal or elevated.

Ratios of metabolite levels are a useful aid to diagnosis. In MCAD deficiency, for example, the ratio of C8 to C10 acylcarnitines is greater than 5:1 [19]. This should always be taken into consideration when evaluating pro-



files with elevated medium-chain species. In patients with VLCAD deficiency, the ratio of C14:1 to C12:1 is markedly elevated. These observations facilitate the differentiation of disease states from nonspecific abnormalities discussed in E.2 and summarized in Table E.7. Although acetylcarnitine is often reduced in patients with FAO defects, ratios of signals of specific acylcarnitines to that of acetylcarnitine are not generally reliable as an interpretative aid.

In vitro testing is helpful in making definitive or differential diagnoses when the results of acylcarnitine analysis are ambiguous. Such tests can include specific enzyme analysis, flux studies and metabolic substrates used in association with acylcarnitine analysis. These tests can also generally be applied to prenatal specimens, and are of course accessible to laboratories that are equipped with tandem mass spectrometers.

#### ■ Amino Acids

Current applications of MS/MS for amino acids are predominantly for screening purposes, especially in newborns, and follow-up confirmatory tests are necessary. Transient tyrosinemia, for example, is quite common in the newborn, and follow-up is perhaps advisable only if clinically indicated, or if tyrosine levels remain elevated 2–3 weeks after birth. In most cases, the amino acid elevations are marked ( $>5\times$  normal mean) and follow-up tests are ordered promptly, otherwise a repeat screen is ordered. Follow-up testing is not necessary if the second screen is normal. Sulfocysteine levels in affected patients are more than an order of magnitude higher than in normal controls. A summary of the amino acids for which validation of the MS/MS method has been carried out, and the disorders associated with their elevation, is provided in Table E.2.

#### ■ Acylglycines

As stated earlier, experience with the use of MS/MS for acylglycine analysis is limited, but should be expected to parallel that of previously published GC/MS methods. In many disorders, such as MCAD and MAD deficiencies, there is more than one pathognomonic metabolite. A list of metabolites and their associated disorders is provided in Table E.3. It should be noted that the method does not recognize patients with milder variants of SCAD and MAD deficiencies, and cannot diagnose long-chain fatty acid oxidation defects [8].

## ■ Purines and Pyrimidines

In the limited experience so far using MS/MS, patients with the known disorders listed in Table E.7 are readily distinguished from controls by the marked elevation of associated metabolites.

There is every reason to believe that this method will become a very useful tool to screen for such disorders.

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