TECHNICAL STANDARDS FOR CLINICAL GENETICS LABORATORIES (2021 Revision)

(For a General Overview of these Technical Standards, including Purpose and Disclaimer, see **Section A**)

F: CLINICAL BIOCHEMICAL GENETICS

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Note: This document contains hyperlinks between the table of content items on the first page and each section. It also contains hyperlinks to the original articles published in *Genetics in Medicine*, which are also included in appendices 1 to 3.

F1 Introduction

F1.1 Biochemical genetics testing broadly comprises the analysis of metabolites and/or proteins from physiological samples for the purposes of detecting inborn errors of metabolism (IEM). The IEM include primary enzymopathies as well as disorders of transport, processing, cofactor synthesis or utilization, and organelle biogenesis and/or function. More specifically, a biochemical genetics laboratory is concerned with the evaluation and/or diagnosis of patients with IEM and their families. Services to be offered typically include screening, diagnostic testing, monitoring of treatment, carrier testing and post-mortem screening.

F1.2 In most cases, the analytical methods used by biochemical genetics laboratories are similar to those of standard clinical chemistry laboratories. Accordingly, procedures for test validation, quality control, quality assurance, and monitoring of safety and equipment performance are generally the same in both settings. Importantly, the biochemical genetics laboratory differs from the clinical chemistry laboratory in the extent of interpretation required in order to provide a valid and meaningful result. Interpretation of biochemical genetics tests should be provided by an American Board of Medical Genetics and Genomics (ABMGG)-certified laboratory director, ideally taking into consideration the clinical history, results of other tests, and other relevant parameters.

F2 Personnel

See **Section B** for details of policies and requirements for director, supervisor, and staff members of clinical genetics laboratories.

F3 Facilities

See **Section C1** for guidelines pertaining to maintenance of facilities and equipment.

F4 Specimens

See **Section C2** for general guidelines for specimen collection and handling. For selected categories of testing, detailed specimen collection and handling instructions are provided in a series of published technical standards, see below (**Appendices 1 to 3**) or on the ACMG web site (**disease-specific technical standards or special topics**).

F4.1 Whenever possible, specimens for biochemical genetic testing should be accompanied by a reason for referral and information on the clinical, medication and nutritional status of the patient, so that results can be most meaningfully interpreted. The time relative to initiation of treatment should be noted when appropriate.

F5 Records

See **Section C5**.

F6 Quality Control/Assurance/Improvement

See **Section C6**.

F7 General Considerations

F7.1 Methods used in biochemical genetics laboratories cover a broad range of techniques and procedures including high-pressure liquid chromatography (HPLC), liquid chromatography-

tandem mass spectrometry (LC-MS/MS), gas chromatography/mass spectrometry (GC/MS), thin-layer chromatography, electrophoresis, as well as enzyme and analyte-specific assays that utilize immunologic, spectrophotometric, fluorometric and radiochemical techniques. In many cases, more than one analytical platform or testing method may be valid for the analysis of a given analyte or enzyme. The procedure selected for use in a given laboratory may depend on a number of factors including testing volume, budgetary constraints and pre-existing equipment, but in all cases, the procedure employed should be consistent with current laboratory practice.

F7.2 Turnaround times should be established and monitored for each test performed by the laboratory. Laboratory procedures utilized in clinical practice must be capable of providing rapid results when appropriate, particularly when the patient is acutely ill. If a rapid result cannot be produced in such circumstances, or if the expected turnaround time cannot be met in a situation that may negatively impact patient care, the referring physician or facility must be notified to permit consideration of, and plans for, alternative testing.

F7.3 Quantitative tests must be fully validated for assay performance, including accuracy, precision and analytical measurement range. Other important considerations include appropriate use of calibration material and internal standards. For both quantitative and qualitative tests, selection and validation of appropriate quality control material (positive and negative controls), and participation in proficiency testing schemes are also required. Validation methods and results, as well as thresholds for assay, quality control and proficiency testing performance, and corrective actions if thresholds are exceeded, must be documented by the laboratory. All

procedures for test validation and performance monitoring must be in accordance with the regulations set forth by CLIA as well as individual states. See also **Section C10**.

F7.4 The laboratory should provide reference ranges for each quantitative test, either established locally or adopted from reputable sources with adequate documentation of method equivalence. Test results should be evaluated according to patient age, and reference ranges should be established for discrete age ranges whenever age-related differences exist. Whenever possible, affected and, where appropriate, carrier ranges should also be determined. All ranges should be reviewed annually and updated as necessary. Documentation of all reference ranges and their corresponding validation data should be available in the laboratory for external review.

F7.5 Amino acid, urine organic acid, and acylcarnitine analyses are the most commonly ordered biochemical genetics tests (Appendixes 1 to 3). Biochemical genetics laboratories offer numerous additional analyte or enzymatic tests, often focused on a particular subset of metabolic diseases such as those affecting creatine metabolism, lysosomal storage, or peroxisome biogenesis and function. See the Medical Genetics Practice Resources on the ACMG web site for relevant technical standards.

F8 Quantitative Amino Acid Analysis (*Updated December 2018*)

Amino acid abnormalities are observed in a broad spectrum of inherited metabolic diseases, such as disorders of amino acid metabolism and transport, organic acidemias, and ureagenesis defects. Comprehensive analysis of physiologic amino acids in blood, urine, and cerebrospinal fluid is typically performed in the following clinical settings: evaluation of symptomatic patients in

whom a diagnosis is not known; evaluation of previously diagnosed patients to monitor treatment efficacy; evaluation of asymptomatic or presymptomatic (*at-risk*) relatives of known patients; follow-up testing for an abnormal newborn screen; and assessment of dietary protein adequacy or renal function in general patient populations. Currently, the most common analytical method to quantify amino acids is based on ion exchange chromatography using post-column derivatization with ninhydrin and spectrophotometric detection. Newer methodologies are based on liquid chromatographic separation with detection by mass spectrometry or spectrophotometry. Amino acid analysis by nonseparation methods, such as the flow injection–tandem mass spectrometric (MS/MS) method used for newborn screening, is considered inadequate for the diagnosis of *at-risk* patients. See **Genet Med 2018; 20(12):1499-1507** OR **Appendix 1**.

F9 Quantitative and Qualitative Organic Acid Analysis (*Updated March 2018*)

Organic acid analysis detects accumulation of organic acids in urine and other body fluids and is a crucial first-tier laboratory test for a broad spectrum of inborn errors of metabolism. It is also frequently ordered as follow-up for a positive newborn screen result, as recommended by American College of Medical Genetics and Genomics newborn screening ACTion sheets and algorithms. The typical assay is performed by gas chromatography–mass spectrometry. See **Genet Med 2018; 20(7):683-91** OR **Appendix 2**.

F10 Quantitative Acylcarnitine Analysis (*Updated February 2021*)

Acylcarnitine analysis is a useful test for identifying patients with inborn errors of mitochondrial fatty acid β-oxidation and certain organic acidemias. Plasma is routinely used in the diagnostic workup of symptomatic patients. Urine analysis of targeted acylcarnitine species may be helpful

in the diagnosis of glutaric acidemia type I and other disorders in which polar acylcarnitine species accumulate. For newborn screening applications, dried blood spot acylcarnitine analysis can be performed as a multiplex assay with other analytes, including amino acids, succinylacetone, guanidinoacetate, creatine, and lysophosphatidylcholines. Tandem mass spectrometric methodology, established more than 30 years ago, remains a valid approach for acylcarnitine analysis. The method involves flow-injection analysis of esterified or underivatized acylcarnitines species and detection using a precursor-ion scan. Alternative methods utilize liquid chromatographic separation of isomeric and isobaric species and/or detection by selected reaction monitoring. See **Genet Med 2021; 23(2):249-58** OR **Appendix 3**.

Appendix 1: Laboratory analysis of amino acids, 2018 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

ACMG TECHNICAL STANDARD | Genetics inMedicine

Laboratory analysis of amino acids, 2018 revision: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

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on behalf of the ACMG Laboratory Quality Assurance Committee

Disclaimer This laboratory standard is designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to this standard is voluntary and does not necessarily assure a successful medical outcome. This standard should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her own professional judgment to the specific circumstances presented by the patient or specimen.

Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with this standard. They also are advised to take notice of the date any particular standard was adopted, and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Amino acid abnormalities are observed in a broad spectrum of inherited metabolic diseases, such as disorders of amino acid metabolism and transport, organic acidemias, and ureagenesis defects. Comprehensive analysis of physiologic amino acids in blood, urine, and cerebrospinal fluid is typically performed in the following clinical settings: evaluation of symptomatic patients in whom a diagnosis is not known; evaluation of previously diagnosed patients to monitor treatment efficacy; evaluation of asymptomatic or presymptomatic (at-risk) relatives of known patients; follow-up testing for an abnormal newborn screen; and assessment of dietary protein adequacy or renal function in general patient populations. Currently, the most common analytical method to quantify amino acids is based on ion exchange chromatography using post-column derivatization with ninhydrin and spectrophotometric detection. Newer methodologies are based on liquid chromatographic separation with detection by mass spectrometry or spectrophotometry. Amino acid analysis by nonseparation methods, such as the flow injection–tandem mass spectrometric (MS/MS) method used for newborn screening, is considered inadequate for the diagnosis of at-risk patients. The purpose of this document is to provide a technical standard for amino acid analysis as applied to the diagnosis and management of inborn errors of metabolism.

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BACKGROUND

Amino acids and proteins

Amino acids serve as protein building blocks, metabolic intermediates, and substrates for energy production. By definition, amino acids contain an amino group and a carboxyl group, and often contain another functional group (e.g., sulfhydryl, hydroxyl, or secondary amino- or carboxyl-group). Proteins consist of 20 different amino acids, half of which are synthesized endogenously (nonessential), while the remaining amino acids are obtained from dietary sources (essential). For almost a century, the detection of amino acids depended on ninhydrin, a chemical that reacts specifically with primary and secondary amines to produce a purple color that can be measured spectrophotometrically. The development of the amino acid analyzer (based on ion exchange chromatographic separation of amino acids coupled with post-column ninhydrin derivatization) in the 1950s was an important advance that started large-scale investigations into

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inborn errors of metabolism. While only 20 amino acids are found in proteins, more than 100 amino acids occur in body fluids[1] and at least 76 of these are of biological interest.[2] Amino acids are essential for humans, and daily urinary losses are very small due to renal tubular reabsorption. Defects in amino acid metabolism and transport cause inborn errors of metabolism[3] and generalized aminoaciduria can be seen in patients with impaired kidney function. In addition, several amino acids act as neurotransmitters, and abnormal levels are significant for certain neurometabolic disorders. Because the normal concentrations of most amino acids in cerebrospinal fluid are quite low, analytical methods must be sensitive and accurate. As amino acid disorders include both catabolic and anabolic pathways, analysis should be capable of detecting pathophysiological elevations and reductions of amino acid levels.[2,4]

General description of amino acidemias and acidurias

Inherited defects of amino acid catabolism, biosynthesis, or transport have been known for many years, and new defects continue to be identified.[5–9] Cystinuria (MIM 220100) and alkaptonuria (MIM 203500) were among the first inherited metabolic disorders described by Archibald Garrod.[10] Phenylketonuria (PKU) (MIM 261600) (ref. [11]), the inherited amino acid disorder with the highest impact on public health, was first described in 1934 (ref. [12]) and prompted the initiation of routine newborn screening in the early 1960s (ref. [13]).

Amino acid disorders are clinically and biochemically heterogeneous. They are characterized biochemically by the accumulation or dissipation of pathological amounts of normal metabolites, or metabolites that are not present under normal physiological conditions, but are produced from alternative pathways in response to loss of a specific gene product's function (typically an enzyme or transporter protein). Primary disorders of amino acid metabolism include defects in the catabolic pathways of aromatic amino acids (e.g., phenylketonuria and tyrosinemia), sulfur-containing amino acids (classic homocystinuria, MIM 236200), branched-chain amino acids (maple syrup urine disease, MIM 248600), urea cycle intermediates (including ornithine transcarbamylase deficiency, MIM 311250; classic citrullinemia, MIM 215700; and argininosuccinic aciduria, MIM 207900), and several others. The broad category of amino acid disorders also includes transport defects such as cystinuria (MIM 220100), lysinuric protein intolerance (LPI, MIM 22700), citrin deficiency (MIM 605814 and 603471), and Hartnup disorder (MIM 234500) (ref. [3]). Several other amino acid disorders are characterized by abnormally low amino acid concentrations caused by the inability to synthesize a nonessential amino acid. Examples include asparagine synthetase deficiency (MIM 615574) (ref. [14]) and glutamine synthetase deficiency (MIM 610015) (ref. [15]), as well as remethylation[16] and serine deficiency disorders.[17] Recently, a defect in branched-chain ketoacid dehydrogenase kinase[18] has been reported that is characterized by increased catabolism leading to abnormally low concentrations of branched-chain amino acids.

Clinical findings associated with amino acid disorders are diverse but are often disease-specific. Signs and symptoms include overwhelming illness resulting from systemic biochemical derangements such as hyperammonemia, hypoglycemia, metabolic acidosis, or respiratory alkalosis. Several amino acid disorders also cause neurologic abnormalities including seizures, hypotonia, lethargy, coma, developmental delay, unexplained intellectual disability, failure to thrive, or autistic behaviors. Depending on the particular disorder, patients may present from birth to adulthood with one or more organ system affected.

It is important to take into consideration that certain amino acid conditions (e.g., aminoacylase 1 deficiency [MIM 609924] and aromatic L-amino acid decarboxylase [AADC] deficiency [MIM 608643]) may not be amenable to detection by amino acid analysis due to the nature of the intermediates produced. In the case of aminoacylase 1 deficiency, abnormal urinary excretion of N-acetylated L-amino acids can be detected by organic acid analysis using gas chromatography–mass spectrometry. Therefore, a comprehensive metabolic workup ideally should include at a minimum urine organic acid and plasma acylcarnitine analysis, in addition to amino acid analysis.

Secondary amino acid abnormalities

Amino acid abnormalities are not only associated with inborn errors of metabolism but are also sensitive markers of an individual's nutritional state and can indicate dysfunction of various organs including liver, kidney, and muscle. For example, liver is the primary organ for the metabolism of methionine and tyrosine, leading to their elevations in metabolic and nonmetabolic causes of hepatic dysfunction. Because acquired changes in amino acid concentrations may be subtle during the malfunctioning of a particular organ, amino acid analysis should have high accuracy and reproducibility to enable interpretation of these changes.[2]

Incidence

The combined incidence for all amino acidopathies is more frequent than 1 in 10,000 (ref. [9]). This estimate does not include other inborn errors of metabolism (e.g., organic acidemias or congenital lactic acidemias) that may need amino acid analysis for comprehensive diagnosis and treatment monitoring. The incidence of PKU in Caucasian populations is between 1 in 10,000 and 1 in 15,000 live births.[19] The incidence of urea cycle disorders is estimated to be at least 1:35,000 births,[20] with ornithine transcarbamylase deficiency being the most common urea cycle defect.[21]

Mode of inheritance

The majority of disorders of amino acid metabolism or transport are inherited as autosomal recessive traits. Exceptions include disorders such as X-linked ornithine transcarbamylase deficiency (MIM 311250), methionine adenosyltransferase

deficiency (MIM 250850, an autosomal dominant form of hypermethioninemia caused by heterozygosity for the p.R264H variant in the *MAT1A* gene),[22] and hawkinsinuria (MIM 140350, an autosomal dominant disorder of tyrosine metabolism caused by deficiency of 4-hydroxyphenylpyruvic acid dioxygenase).[23]

METHODS

The laboratory technical standard was informed by a review of the literature, including any current guidelines, and expert opinion. Resources consulted included PubMed (search terms: inborn errors of metabolism, amino acidopathies, amino acid analysis methods, ion exchange chromatography [& amino acid], ultra-performance liquid chromatography [& amino acid], tandem mass spectrometry [& amino acid & liquid OR gas chromatography]), the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories, Clinical and Laboratory Standards Institute (CLSI) guidelines, CLIA regulations, and the Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report (MMWR) on Good Laboratory Practices for Biochemical Genetics Testing and Newborn Screening for Inherited Metabolic Disorders. When the literature provided conflicting evidence about a topic or when there was insufficient evidence, the authors used expert opinion to inform the recommendations. Expert opinion included the coauthors of the document, members of the ACMG Laboratory Quality Assurance Committee, as well as the experts consulted outside of the Committee but acknowledged in this document. Any conflicts of interest for workgroup members or consultants are listed. A draft was delivered to the ACMG Board of Directors for review and member comment. The draft document was posted on the ACMG website and an email link was sent inviting ACMG members to provide comment. All comments were assessed by the authors. When appropriate, additional evidence was included to address member comments and the draft was amended. Both member comments and author responses were reviewed by a representative of the ACMG Laboratory Quality Assurance Committee and by the ACMG Board of Directors. The final document was approved by the ACMG Board of Directors. This updated standard replaces the previous version in Section F: Clinical Biochemical Genetics, American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories (2008 Edition, Revised 02/2007), section F7.5 (ref. [24]).

PREANALYTICAL REQUIREMENTS

Specimen requirements

Plasma is the preferred specimen for the evaluation of most primary disorders of amino acid metabolism. Lithium, or sodium, heparin is the preferred anticoagulant. Ethylenediaminetetraacetate (EDTA) plasma yields similar results to heparinized plasma with minor differences that are typically not diagnostically significant.[25–27] Yet, it should be noted that EDTA can react with ninhydrin and produce a ninhydrin positive contaminant that may lead to interference.[27] Sodium citrate–treated blood yields lower values for most amino acids. Laboratories should have established procedures for procuring specimens using their preferred anticoagulant. Fasting plasma samples are recommended to avoid erroneous interpretation of several disorders, particularly those involving amino acid synthesis that are characterized by low concentrations of certain amino acids.[2] Serum should not be used because clotting times may lead to analyte-specific artifacts.[27] Testing can also be performed on amino acids eluted from dried-blood spots (DBS).

Urine is the appropriate specimen for the identification of compounds that are efficiently cleared by the kidney (e.g., argininosuccinic acid [ASA]) or specific disorders of renal amino acid transport (e.g., renal Fanconi syndrome or cystinuria). However, urine is generally less reliable for first-tier investigations of primary amino acid disorders due to effective tubular reabsorption of a majority of amino acids, and susceptibility to interference from medications. Urine amino acid analyses may often be ordered erroneously in place of, or unnecessarily in addition to, plasma amino acid testing; therefore clinical indications should be preemptively reviewed (if available). When urine is analyzed, it should be collected without preservatives or fecal contamination.

The analysis of cerebrospinal fluid (CSF) is useful for diagnosing several biochemical genetic disorders, including asparagine and glutamine synthetase deficiencies, serine deficiency disorders, and most notably, glycine encephalopathy (nonketotic hyperglycinemia [NKH; MIM 605899]). In NKH, CSF should be collected and analyzed along with a simultaneously collected plasma sample, so that the CSF:plasma glycine ratio can be calculated.[28–30] It is noteworthy that CSF should be collected into preservative-free collection tubes; however, fluoride oxalate and lithium heparin tubes also suffice. CSF should be stored frozen if not immediately analyzed. Specimens contaminated with blood should be interpreted with caution as most amino acids are present in blood at much higher concentrations than in CSF. In such cases, a repeat CSF specimen may be advised.

Sample handling, shipping, and storage

Plasma should be centrifuged immediately to reduce the influence of other blood constituents on the free amino acid concentrations. Glutamate, aspartate, and taurine have high intracellular levels and increase by hemolysis. Plasma should be kept at –20 °C (for long-term storage, –70 °C) until analysis to slow the decomposition of glutamine.[2] Serum, if used, should be centrifuged after the standard time allowed for clotting (usually 30 minutes). However, the added time for clotting can lead to artifacts from the deamination of arginine to ornithine by red blood cell arginase, and by the release of oligopeptides. Postprandial samples should be avoided and information about current medications (e.g., antibiotics or

antiseizure medications) or dietary status may be informative. Following centrifugation, samples should be kept frozen (–20 °C; for long-term storage, –70 °C) until the time of analysis. CSF and urine specimens should be frozen as soon as possible after collection and stored frozen prior to analysis. Samples sent to the laboratory from an outside referral source should be shipped on dry ice via an overnight courier.

Finally, it is important to note that amino acids with terminal sulfhydryl groups (e.g., cysteine and homocysteine) readily form disulfide bonds leading to their association with plasma proteins,[31,32] particularly albumin.[33] Therefore, the accurate quantification of these amino acids necessitates separate techniques utilizing strong reducing agents.[34,35] This is particularly true for plasma homocysteine, an important biomarker for disorders of the methionine cycle, folate and vitamin B_{12} metabolism, and a risk factor for cardiovascular disease.

Preanalytical variables

Sample collection and handling are discussed in the previous section. Interference from coeluting, ninhydrin-positive compounds occur with certain antibiotics (e.g., ampicillin), contrast dyes, and other medications. Amino acid concentrations can also be influenced by anticonvulsants (e.g., hyperglycinemia with valproate use), nutritional status (e.g., intravenous [IV] nutrition or starvation), clinical status (e.g., fever, infections, and liver or kidney dysfunction), treatment of acute lymphoblastic leukemia (ALL) using L-asparaginase,[36,37] as well as bacterial contamination. These factors may affect results and should be taken into account when interpreting results.

Clinical indications for testing

Clinical presentations of different disorders of amino acid metabolism are variable and often nonspecific. Onset of symptoms may occur in the neonatal period or as late as adulthood. Amino acid analysis should be considered in a wide variety of clinical situations, such as:

1. Lethargy, coma, seizures, or vomiting in a neonate
2. Hyperammonemia
3. Failure to thrive
4. Electrolyte abnormalities, including metabolic acidosis or respiratory alkalosis
5. Lactic acidemia
6. Unexplained developmental delay or intellectual disability
7. Abnormal newborn screening results
8. A previously diagnosed sibling
9. Clinical presentation suggestive of a specific amino acid disorder
10. Monitoring treatment efficacy (e.g., dietary) of known patients

Amino acid analysis can also be used for dietary monitoring (including assessment of dietary protein adequacy or renal function) of metabolic patients on protein restriction and/or special formulas, irrespective of pathway affected, or in the general patient population not affected with an inherited metabolic disorder. Indeed, specific amino acid analytes can be used as biomarkers to assess the risk of developing more common medical conditions, including diabetes[38] and cardiovascular disease,[39] although these applications are outside the scope of these Standards.

The laboratory should be aware of the clinical indication(s) for testing, including the need for immediate analysis in a critically ill patient, so that testing can be triaged, results interpreted appropriately, and additional testing recommended as needed. Depending on the clinical situation, amino acid analysis is often performed together with urine organic acids, plasma carnitine (free and total), and plasma acylcarnitine profile analysis as part of a comprehensive metabolic evaluation. Ideally, these tests should all be performed by the same laboratory, and results integrated into a complete interpretation.

METHOD VALIDATION

Calibration and quantitation

The quantitation of amino acid concentrations should be performed in relation to known reference or calibration standards. Amino acid calibration mixtures are available from several commercial sources. Some manufacturers have omitted certain relatively unstable amino acids (e.g., asparagine) from these mixtures; in these cases, freshly prepared solutions of the missing compounds can be added to the commercial standards to form a complete set. Performance of calibration material should be verified either with weighed standards, or with amino acid calibrators obtained from an independent source (e.g., National Institute of Standards and Technology [NIST]). Instrument calibration should be verified, and/or the instrument should be recalibrated, at regular intervals as established by the laboratory per manufacturer's recommendations, and as required by CLIA '88. This should also occur with the introduction of new columns, reagent lots, or following service to the instrument or its components.

Quantitation using ion exchange chromatography (IEC) or ultraperformance or high-performance liquid chromatography (U/HPLC) coupled with spectrophotometric[40] or fluorometric[41] detection should be performed using at least one internal standard (IS). The selected standard should elute at a unique position in the chromatogram and not interfere with the analysis of other nearby compounds. Quantitative results should be calculated using an IS method that adjusts for the amount of IS in the patient sample relative to that in the calibration mixture.[25] For methods that employ mass spectrometric detection, stable isotope analogs for each amino acid should be used as ISs when available. The lack of ISs for specific analytes may reduce the performance of stable isotope dilution, mass-spectrometric methods for those analytes.[42] For the majority of amino acids with clinical significance, stable isotope standards are commercially available. The laboratory should establish protocols to determine and periodically verify their method's linear range, analytical

measurement range, and lower limit of detection (see CLSI document C24-A3: Clinical and Laboratory Standards Institute. Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions; Approved Guideline—Third Edition). Procedures should be in place for analytical values that fall outside of an assay's performance limits.

Reference ranges

Laboratory-specific reference ranges should be determined for each sample type (plasma, DBS, urine, and CSF), using guidelines as defined by a laboratory's policy and procedures for test validation (e.g., CLSI document EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition). It is important to note that amino acid levels obtained from different sample types (e.g., plasma versus DBS) are not comparable and should not be used interchangeably for monitoring purposes (e.g., phenylalanine). Anonymized samples from a general patient population are acceptable for generating a laboratory-specific reference range when patients with an identified diagnosis are excluded. These ranges should be categorized by age and sex when appropriate and verified on a regular interval as required by CLIA '88. When literature-based ranges are used, they should be verified using laboratory-specific methods. Because amino acid levels vary significantly with age, reference intervals should be age-specific.

Testing personnel

Laboratory personnel performing quantitative amino acid analysis should be documented to have received appropriate training and demonstrated competency in the performance of the test. In addition, laboratory personnel should satisfy CLIA '88 requirements for high-complexity testing and have, at a minimum, an associate degree in a laboratory science or medical laboratory technology from an accredited institution. More comprehensive requirements may apply to laboratories in some US states (see General Policies B: Personnel Policies of the ACMG Standards and Guidelines for Clinical Genetics Laboratories, 2008 Edition, Revised 02/2007 (https://www.acmg.net/acmg/Publications/Standards___Guidelines/Personnel_Policies.aspx).

ANALYSIS OF AMINO ACIDS

Analytical methods

Overview of methods

To analyze free amino acids in physiological specimens, deproteinization of the sample is necessary. For comprehensive, quantitative amino acid analysis, this is commonly achieved by protein precipitation using sulfosalicylic acid (SSA) or trichloroacetic acid (TCA), followed by centrifugation, and/or filtration. Supernatants or filtrates are diluted with buffer of appropriate pH prior to analysis. For limited amino acid analysis such as for newborn screening by flow injection tandem mass spectrometry, extraction of amino acids from DBS is achieved using acetonitrile, methanol, or an admixture of these solvents. Sample preparation should include the addition of at least one IS to control for run-to-run variation in sample extraction and analysis. For urine, the creatinine concentration is measured and useful for adjusting the sample volume prior to analysis. Postanalysis, amino acid concentrations are also often normalized to creatinine molality (e.g., mmol/mol creatinine). It is important to note that the Jaffé method, in which creatinine reacts with alkaline picrate to form a complex absorbing at 480–520 nm, is still commonly used to determine creatinine, despite well-recognized interference by bilirubin, protein, ketones, ketoacids, fatty acids, and other compounds.[43] HPLC[44] and particularly liquid chromatography–tandem mass spectrometry (LC-MS/MS)[45] methods for measuring creatinine offer significant improvements in sensitivity and specificity over the Jaffé method. Urine amino acid levels can also be reported from a 24-hour specimen collection (e.g., μmol/24 hours).

Analysis of the full physiologic amino acid profile can be achieved using several methods.[46] IEC with post-column derivatization using ninhydrin and spectrophotometric detection has been the most widely performed method for several decades and is still a commonly used clinical laboratory method. Alternative methods have been developed that take advantage of the selectivity of mass spectrometry and the reduced analytical time of ultraperformance liquid chromatography. When coupled with techniques such as ultraperformance liquid chromatography or gas chromatography, higher throughput and enhanced performance of amino acid analysis has been reported.[40,47–50] Such methods also require less sample volume when compared with IEC. However, it is recognized that mass spectrometric methods with additional complexities present relatively more challenges to laboratories that do not have the necessary expertise. Furthermore, each methodology may present a different source of variance (e.g., recovery of amino acids may be affected by the solvent used to precipitate protein in plasma samples). Thus, it is the laboratories' responsibility to determine the possible pitfalls of their specific test and to validate their methodology to address possible challenges and limitations.

A number of different instrument configurations are commercially available for amino acid analysis; these instruments have published methodologies that should be validated in the individual laboratory. Also see General Policies C7: Levels of Development of a Diagnostic Test, and C8: Test Validation of the ACMG Standards and Guidelines for Clinical Genetics Laboratories, 2008 Edition, revised 02/2007 (https://www.acmg.net/acmg/Publications/Standards___Guidelines/General_Policies.aspx).

Ion exchange chromatography (IEC)

IEC is the most commonly used method of amino acid analysis and recent method updates have improved observed matrix effects.[51] IEC requires derivatization of amino acids for detection. This can be accomplished prior to separation of amino acids (pre-column derivatization) using o-phthalaldehyde (OPA) or phenylisothiocyanate (PITC), or

following separation (post-column derivatization) using ninhydrin.

Post-column ninhydrin derivatization has been the preferred method for many years by a majority of laboratories, although methods based on newer technologies are now gaining in popularity. In this method, compounds are simultaneously detected at wavelengths of 570 nm and 440 nm using a dual-wavelength spectrophotometer. Laboratories may report values from the 570 nm channel only, from the sum of the two channels, or from a combination of both (e.g., hydroxyproline and proline from the 440 nm channel, and the remainder of the amino acids from the 570 nm channel). Identification of amino acids following chromatographic separation is based on retention time. Most systems are capable of resolving and quantifying roughly 40 amino acid peaks in a typical patient sample (the exact number varies as some systems do not resolve all amino acids from neighboring peaks). Laboratories, however, may elect to report a smaller subset of clinically relevant compounds.

Each chromatogram should be visually inspected for run performance, as well as for atypical peaks that may not be included in peak identification tables, including Δ-1-pyrroline-5-carboxylate, homocitrulline, ASA, and alloisoleucine (especially when the ratio of branched-chain amino acids is disturbed). For ninhydrin-based systems with spectrophotometric detection, an identification based on retention time comparison can be supported by standard spiking and by calculating the ratio of response at 570 nm to that at 440 nm. The 570/440 ratio can be established for all standard compounds, and compared with patient values to confirm peaks where coeluting interfering substances are suspected. In determining these ratios, peak baselines should be carefully examined such that potential artifacts from baseline fluctuations are eliminated.[52] Alternatively, where coelution is known to occur for amino acids that are diagnostically significant (e.g., homocitrulline and methionine on certain IEC systems), reinjection of the sample and analysis using an alternative gradient method may be useful to verify identity. Additionally, ASA identification can be confirmed by heating the sample at 90 °C and observing the change of retention time with anhydrous ASA formation. Quantitation should be based on the recovery of the IS in each specimen compared with the recovery of the IS in the calibration mixture.

Ultra Performance Liquid Chromatography (UPLC)

UPLC is increasingly being utilized due to its rapid separation of amino acids in approximately 35 minutes compared with 2 hours for a typical IEC analysis. One example of an UPLC application for plasma, serum, CSF, and urine amino acids analysis is a method that utilizes pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. This reagent rapidly reacts with both primary and secondary amines to produce stable chromophores that can be detected by ultraviolet spectrophotometry at 260 nm. Derivatized samples have a stable baseline, superb peak separation, and excellent peak symmetry for all amino acids including those that elute at the end of analysis. Using norvaline as an IS, correlation of this assay with IEC was satisfactory with adequate detection of most significant amino acids.[40] The superior resolution of UPLC compared with IEC confers better sensitivity for this method. Currently, the only known negative characteristic of this technique is the inability to separate ASA from ethanolamine with published methods. Laboratories using UPLC should consider validating a secondary analysis for specifically measuring ASA to confirm a suspected elevation. Noteworthy is the observation that alloisoleucine is separated from other branched-chain amino acids, negating the need for a special analysis to confirm the potential of maple syrup urine disease.

Tandem mass spectrometry methods

Within the last 15 years, methods utilizing liquid chromatography–electrospray–tandem mass spectrometry (LC-ESI-MS/MS) have been developed for amino acid analysis in plasma, urine, and CSF and applied to clinical practice.[47,49,50,53–57] LC-ESI-MS/MS methods offer the advantages of increased specificity and throughput as compared with traditional IEC-UV/VIS methods.[48] The main challenges for developing a robust LC-ESI-MS/MS amino acid test include the necessity to control for ion suppression or enhancement of the numerous analytes included in the analysis, and the separation of isobaric amino acid species. Additionally, these instruments usually require more frequent calibration than IEC-UV/VIS[57] and data review and processing may be cumbersome.[56]

LC-ESI-MS/MS methods have been published for the analysis of underivatized or derivatized amino acids. These methods used UPLC or HPLC with one or more column chemistries, stable isotope dilution or isobaric tagging, and detection by selected reaction monitoring (SRM).[46–50,54–56] Sensitivities as low as 1–2 μM and linearity of up to 1000–2000 μM have been reported.[47,49,50,55–57] Unlike IEC, LC-ESI-MS/MS methods are subject to matrix effects, and thus require the use of stable isotope, internal standards (IS) to compensate for variable recoveries and ionization efficiencies.[42,58] A stable isotope IS for each analyte is preferred; however, depending on the method, this may not be feasible. The lack of an IS may affect accuracy and precision for a specific analyte and is an important consideration for each laboratory. Published methods that employ stable isotope dilution often utilize commercially available [$^2H_2$] to [$^2H_8$] labeled amino acids for IS.[47,49,54] It is generally accepted that IS with a considerable degree of deuterium label may not reliably reduce all matrix effects in every sample due to the slight alterations in physicochemical properties, leading to small shifts in retention time and differences in ionization.[58,59] For several published LC-ESI-MS/MS methods, stable isotope–labeled analogs were not utilized for every amino acid measured, necessitating the application of surrogate IS for a subset of analytes. For these reasons, while LC-ESI- MS/MS methods have compared well with established amino acid analytical methods, there are limitations with the measurement of some target analytes.

The sample preparation procedure for underivatized, LC-MS/MS methods[47,49,50,57,60] is similar to IEC post-column derivatization methods in most cases: samples are combined with ISs and plasma proteins are precipitated using sulfosalicylic acid or organic solvent. Separation of underivatized amino acids has been achieved using reverse-phase chromatography with ion pairing reagents,[47,49,50,60] and with hydrophilic interaction chromatography using an amide UPLC column[57] for the analysis of 26 to 52 amino acids with 18- to 30-minute run times. As noted above, full quantification using stable isotope dilution was performed for only a subset of clinically significant amino acids, with reduced performance of several analytes without a corresponding IS. Other challenges with these underivatized methods included inferior performance of highly polar amino acids that are poorly retained on a reverse-phase column,[49] the requirement for extensive reconditioning of ion-pairing reverse-phase systems,[47] and the lack of separation of D-alloisoleucine from isoleucine with methods utilizing hydrophilic chromatography.[57]

A published LC-ESI-MS/MS method that utilizes amine-reactive isobaric tagging reagents (iTRAQ®; later aTRAQ™ Reagent 121) is an alternative approach to using stable isotope IS.[55,56] These reagents were initially used for multiplexed quantification of peptides and have been applied to measuring physiological amino acid concentrations.[53] These methods are based on the differential derivatization of IS with an isotopically unlabeled reagent (2,5-dioxopyrrolidin-1-yl-2(4-methylpiperazin-1-yl)acetate) and the derivatization of sample with $^{13}C_6$, $^{15}N_2$- stable isotope labeled reagent to generate isobaric tags. The differentially labeled sample and IS have identical chromatographic retention, and can be separated by MS/MS by the generation of product ions with an m/z difference of 8 amu. Pre-derivatized standards are combined with the sample after derivatization and serve to control for differences in injection volume and ionization efficiency. Two nonphysiologic amino acids are added to the sample before derivatization for monitoring sample extraction and derivatization efficiency. Analyte quantification can be obtained by comparison with IS as provided in specified amounts in each commercial kit. Alternatively, quantification using calibrators extracted with each run, and normalized using the ISs, improves accuracy by reducing variability due to different IS lots or tagging efficacy.[55,56] Chromatography is performed using a C18 (5 µm, 4.6 mm × 150 mm) column with a total analysis time of 18 minutes[55,56] with efficient separation of isobaric amino acids with the exception of alloisoleucine and isoleucine; for the accurate quantification of these branched-chain amino acids, it is necessary to modify the method to include the detection of the underivatized alloisoleucine and isoleucine, and the use of an underivatized stable isotope–labeled IS.[46,55,56] Thus, an aliquot is removed from the sample prior to the labeling (derivatization) reaction, mixed with IS, and recombined with the derivatized extract prior to injection on the column. An advantage of isobaric tagging compared with isotope dilution is the availability of a commercial kit that contains IS for each of the 42 analytes.[55,56]

The possible pitfalls of a specific LC-ESI-MS/MS method of choice should be determined by the laboratory and the assay should undergo a thorough and systematic validation before use in a clinical laboratory.

Quality control

Quality control (QC) material should be evaluated at two different concentrations for all reported amino acids, and should be derived with the same matrix as patient specimens. QC material should be analyzed along with each patient batch. In situations where samples are run continuously (e.g., adding samples as they arrive to an ongoing queue, rather than in discrete batches), QC samples should be run daily (every 24 hours) per College of American Pathologists (CAP, Inspector Checklist CBG 12800). Thresholds for acceptance or rejection of a QC sample result, and remedial actions in the event of a QC failure, should be established and documented by the laboratory. QC data should be regularly monitored for trends that may affect test performance, and problems should be documented and remediated as appropriate. Thresholds for appropriate IS response should be established, and IS responses for each specimen should be monitored as another level of quality control. It is recommended to consider basic statistical QC analyses for clinical testing to enhance the performance of laboratory methods[61] (see also CLSI document EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline—Third Edition).

Proficiency testing

Participation in biannual proficiency testing (PT) activities is an important element of any laboratory quality assurance program. A PT program that evaluates both analytical and interpretive/diagnostic proficiency of amino acid analysis is offered by CAP and supervised by the CAP/ACMG Genetic Biochemical and Molecular Genetic Resource Committee. Another excellent program is offered by the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM; http://www.erndimqa.nl), in which analytical and interpretive (or clinical) proficiency is monitored through the regular distribution of PT.

TEST INTERPRETATION AND REPORTING

Interpretation

Clinical amino acid analyses should be interpreted by an American Board of Medical Genetics and Genomics (ABMGG)-certified clinical biochemical geneticist. Because normal amino acid concentrations vary with age, quantitative results should be compared with a properly defined reference age group. Interpretations of amino acid results are based upon relative amino acid levels, pattern recognition, and correlation of positive and negative findings, rather than on individual amino acids levels alone. Amino acid abnormalities

or overall profiles should also be considered in the context of clinical findings and/or additional test results.

Reporting

Reports should contain appropriate patient and specimen information whenever available as described in the American College of Medical Genetics Standards and Guidelines for Clinical Genetics Laboratories, Sections 2.4, 2.41, and 2.42 (https://www.acmg.net/acmg/Publications/Standards_Guidelines/General_Policies.aspx) and as specified by CLIA '88. Written reports should provide units of measure, age-dependent, laboratory-specific reference ranges, and an interpretation of findings. When abnormal results are detected, the interpretation should include an overview of significant results, correlation to any available clinical information, elements of differential diagnosis, recommendations for additional testing or confirmatory studies (e.g., enzyme assay, molecular analysis), and a phone number to reach the reporting laboratory for any additional questions. Recommendations for follow-up evaluation, including referral to a metabolic specialist, should also be included when appropriate.

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DISCLOSURE

The following authors direct laboratories that perform amino acid analysis as a fee-for service: J.D.S., I.D.B., D.M., S.Y., M.J.B., and A.A.T.

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Appendix 2: Laboratory analysis of organic acids, 2018 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

ACMG TECHNICAL STANDARD | Genetics inMedicine

Laboratory analysis of organic acids, 2018 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

Renata C. Gallagher MD, PhD[1], Laura Pollard, PhD[2], Anna I. Scott, PhD[3,4], Suzette Huguenin, PhD[5], Stephen Goodman, MD[6], Qin Sun, PhD[7]; on behalf of the ACMG Biochemical Genetics Subcommittee of the Laboratory Quality Assurance Committee

Disclaimer: This laboratory standard is designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to this standard is voluntary and does not necessarily assure a successful medical outcome. This standard should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her own professional judgment to the specific circumstances presented by the individual patient or specimen.

Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with this standard. They also are advised to take notice of the date any articular standard was adopted, and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Organic acid analysis detects accumulation of organic acids in urine and other body fluids and is a crucial first-tier laboratory test for a broad spectrum of inborn errors of metabolism. It is also frequently ordered as follow-up for a positive newborn screen result, as recommended by American College of Medical Genetics and Genomics newborn screening ACTion sheets and algorithms. The typical assay is performed by gas chromatography–mass spectrometry. These technical standards were developed to provide guidance for laboratory practices in organic acid analysis, interpretation, and reporting. In addition, new diagnostic biomarkers for recently discovered organic acidurias have been added.

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Key Words: clinical genetic testing; gas chromatography–mass spectrometry; organic acid; organic aciduria; technical standards

1. INTRODUCTION

Organic acids are analyzed in patients suspected of having a broad range of metabolic disorders including inborn errors of amino acid, fatty acid, carbohydrate, neurotransmitter, vitamin, sterol, mitochondrial energy, and purine and pyrimidine metabolism. Organic acids are non-amine-containing, water-soluble compounds with one or more carboxyl groups as well as other functional groups (-keto, -hydroxyl), and are intermediates of a large number of biochemical pathways. Organic acid analysis is performed to identify abnormal accumulation of one or more compounds resulting from an enzymatic or transporter deficiency. Exogenous compounds are also detected, and these may be relevant to the clinical presentation. The kidneys efficiently excrete organic acids, both endogenous and exogenous, therefore urine is the preferred specimen type.

Organic acids are extracted from urine using organic solvents or ion exchange methods, and their presence and abundance are determined by gas chromatography–mass spectrometry (GC-MS). Over 500 organic acids have been identified in urine.[1] Both qualitative and quantitative analyses are appropriate for the detection of organic acidurias, with careful quality assessment and control. Diagnostic compounds are often significantly elevated in classic organic acidurias, so a diagnosis is possible without quantitative methods. It is important to note that for some disorders diagnostic compounds may be normal or near-normal in clinically well patients.[1,2] Care must be taken to examine the

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chromatogram for clinically significant compounds that may be present in small amounts or obscured by coelution with other compounds; proficiency testing programs have reported missed diagnoses from poor detection of low-abundance pathognomonic compounds.[3] Quantitative analyses are particularly useful for therapeutic monitoring of previously diagnosed patients.[1–5]

Specific organic acids (e.g., methylmalonic acid, glutaric acid, 3-hydroxyglutaric acid, orotic acid, and succinylacetone) can be determined in blood or urine using other techniques that utilize stable isotope dilution and GC-MS or liquid chromatography–tandem mass spectrometry. These targeted analyses should not replace a more comprehensive organic acid screen in the initial laboratory evaluation of undiagnosed cases. This technical standard focuses on untargeted urine organic acid analyses by GC-MS.

2. METHODS

The laboratory technical standard was informed by a review of the literature, including any current guidelines, and expert opinion. Resources consulted included PubMed (search terms: organic acidemias, organic acid analysis methods, organic acid extraction methods), the American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories, Clinical and Laboratory Standards Institute guidelines, CLIA regulations, and the Centers for Disease Control and Prevention *Morbidity and Mortality Weekly Report* on Good Laboratory Practices for Biochemical Genetics Testing and Newborn Screening for Inherited Metabolic Disorders. When the literature provided conflicting evidence about a topic or when there was insufficient evidence, the authors used expert opinion to inform the recommendations. Expert opinion included the coauthors of the document, members of the Biochemical Genetics Subcommittee of the Lab Quality Assurance Committee, as well as any experts consulted outside the workgroup and acknowledged in this document. Any conflicts of interest for workgroup members or consultants are listed. The ACMG Laboratory Quality Assurance Committee reviewed the document providing further input on the content, and a final draft was delivered to the ACMG Board of Directors for review and approval to send out for member comment. The final draft of the document was posted on the ACMG website and an e-mail link was sent to ACMG members inviting all to provide comment. All members' comments were assessed by the authors and additional evidence was included and our recommendations were amended as deemed appropriate. Member comments and author responses were reviewed by a representative of the Laboratory Quality Assurance Committee and the ACMG Board. The final document was approved by the ACMG Board of Directors. This updated technical standard replaces the previous version in Section F: Clinical Biochemical Genetics, American College of Medical Genetics and Genomics Standards and Guidelines for Clinical Genetics Laboratories (2008 Edition, Revised February 2007), section F7.7.[6]

3. BACKGROUND

3.1 Overview of diseases identified through urine organic acid analysis

Organic acidurias (also known as organic acidemias) are a biochemically heterogeneous group of inborn errors of metabolism. They are characterized by the accumulation of metabolites that are not present, or are only present in small amounts, under normal physiological conditions. Abnormalities detected by organic acid analysis include pathologically elevated quantities of normal metabolites, and compounds produced by alternative biochemical pathways (e.g., succinylacetone). The possible, or likely, diagnosis of a specific organic aciduria, or other disorder, relies on pattern recognition and the determination of the presence, or absence, of expected compounds. An identified defect in organic acid metabolism can be due to an enzyme deficiency; a disorder of cofactor biosynthesis, recycling, or modification; a transporter defect; or nutritional deficiency. Disorders that may be identified or suggested through abnormal urine organic acid analysis include the classic organic acidemias due to inborn errors of amino acid metabolism (isovaleric acidemia [MIM 243500], methylmalonic acidemia[s], propionic acidemia [MIM 606054], glutaric acidemia type I [MIM 231670]), as well as other amino acid disorders (phenylketonuria [MIM 261600], tyrosinemia type I [MIM 276700], alkaptonuria [MIM 203500], 3-methylglutaconic aciduria type I [MIM 250950], maple syrup urine disease [MIM 248600]). Abnormal urine organic acids may also be consistent with disorders of the urea cycle (suggested by elevated orotic acid), fatty acid oxidation (short-chain acyl-CoA dehydrogenase deficiency [MIM 201470], medium-chain acyl-CoA dehydrogenase deficiency [MIM 201450], multiple acyl-CoA dehydrogenase deficiency [MIM 231680]), energy metabolism (pyruvate dehydrogenase deficiency, fumarase deficiency [MIM 606812], SUCLA2 deficiency [MIM 603921]), purine and pyrimidine metabolism (uridine monophosphate synthetase deficiency [MIM 613891], dihydropyrimidine dehydrogenase deficiency [MIM 274270]), neurotransmission (aromatic L-amino acid decarboxylase deficiency [MIM 608643]), and others (ethylmalonic encephalopathy pMIM 602473), Canavan disease [MIM 271900], glutathione synthetase deficiency [MIM 266130], glycerol kinase deficiency [MIM 307030], primary hyperoxaluria type I [MIM 259900] and type II [MIM 260000]).[7–9] Clinical indications for this testing are, therefore, diverse and include neonatal or late-onset acute illness associated with hyperammonemia, hypoglycemia, and/or ketolactic acidosis; neurologic abnormalities including seizures, ataxia, hypotonia, lethargy, coma, developmental delay, or unexplained intellectual disability; failure to thrive; pancreatitis; unexplained metabolic acidosis; unusual odor; macrocephaly; and liver failure.[10,11] Some symptoms, including lethargy and acidosis, can be due to exogenous intoxication, and organic acid analysis may aid in diagnosis

(e.g., ethylene glycol poisoning, ibuprofen overdose, and γ-hydroxybutyric acid intoxication as distinct from succinic semialdehyde dehydrogenase deficiency [MIM 271980]).

3.2 Incidence

The incidence of individual classical inborn errors of organic acid metabolism varies from 1 in 10,000 to 1 in 1,000,000 live births. Collectively, their incidence has been suggested to be approximately 1 in 10,000–35,000 live births.[8,12,13] This estimate, however, does not include other inborn errors of metabolism (i.e., amino acid disorders, urea cycle disorders, congenital lactic acidemias) for which diagnosis may also be suggested by organic acid analysis. All possible disease entities included, the incidence of conditions for which informative organic acid profiles can be detected in urine is likely to be higher than recognized. As a group, these defects are likely underdiagnosed.

3.3 Mode of inheritance

Most organic acidemias, and other inborn errors of metabolism identified through organic acid analysis, are inherited as autosomal recessive traits. X-linked disorders that may be identified by urine organic acid analysis include the amino acid and mitochondrial energy metabolism disorder, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (MIM 300438); the urea cycle disorder, ornithine transcarbamylase deficiency (MIM 311250); the energy metabolism disorder, pyruvate dehydrogenase E1-α deficiency (MIM 312170); and glycerol kinase deficiency (MIM 307030). The identification of an affected individual has important implications for other family members, who may also require urine organic acid analysis and additional genetic testing.

4. PREANALYTICAL REQUIREMENTS

4.1 Specimen requirements

Organic acids are preferably analyzed in urine. Urine specimens are normalized to a fixed equivalent of creatinine to allow determination of relative concentration. Sample volume required for testing depends on the patient's hydration status. The minimal sample volume will also depend on specifics of the organic extraction; typically 1–5 ml of specimen is recommended.

4.2 Conditions of sample collection, shipping, handling, and storage

Urine is ideally collected at the time of acute illness. If the patient is decompensated the urine should be obtained at the time of decompensation, as diagnostic metabolites are likely to be highest in this state.[1,14] If the patient is not ill, a random urine sample is appropriate.[14,15] Urine should be collected in a clean container without preservatives, frozen as soon as possible, and stored frozen until analyzed. Samples sent to the laboratory from outside referral sources should be shipped on dry ice via overnight courier. Urine samples are stable for most organic acids for several years if stored at −20 °C or lower.[1]

4.3 Exogenous variables

Diet, medication, and clinical state may contribute to an abnormal organic acid profile.[1] Dietary factors include total parenteral nutrition (isolated *N*-acetyltyrosine), gelatin (isolated adipic acid), medium-chain triglyceride oil supplementation (dicarboxylic aciduria), infant formula (dicarboxylic aciduria due to medium-chain triglyceride and other artifacts), and the ketogenic diet (ketoaciduria). Medications such as valproate, levetiracetam, aspirin, benzoic acid, ibuprofen, acetaminophen, and many others can be the source of a wide variety of exogenous peaks.[16] Treatment with certain medications may also be associated with increased production and excretion of organic acids with diagnostic relevance. For example, valproate treatment inhibits 3-methylcrotonyl-CoA carboxylase and results in elevated urinary excretion of 3-hydroxyisovaleric acid,[17] a marker of biotinidase deficiency and other disorders. Pyroglutamic acid (5-oxoproline) elevation, a marker of glutathione synthetase deficiency, may also occur secondary to treatment with acetaminophen, vigabatrin, and other medications.[18,19] Clinical information may be required to determine if these are relevant to the clinical presentation (e.g., ibuprofen overdose). Sample contamination can also lead to abnormal findings. For example, lubricants or soaps can result in elevations of glycerol (also elevated in glycerol kinase deficiency), palmitic acid, and stearic acid. Mild elevations of methylmalonic acid can be secondary to intestinal bacteria metabolism or nutritional status (low infant or maternal B12 or folate). Compounds of bacterial origin can be enhanced with prolonged sample storage at ambient temperature.[20] Clinical history, diet, and medication should be considered to aid the interpretation of the chromatographic findings.

4.4 Clinical indications for testing

Urine organic acid analysis should be performed for patients suspected of having a wide range of disorders based on clinical symptoms and/or other laboratory abnormalities. Clinical symptoms are varied and often nonspecific, as described above in section 3.1, and can occur for the first time at any age from the neonatal period to adulthood. Certain clinical findings may be associated with particular disorders, such as macrocephaly in Canavan disease (MIM 271900) and glutaric acidemia type I (MIM 231670), and neonatal liver disease in tyrosinemia type I (MIM 276700). A family history of a previously affected sibling or an abnormal newborn screen result may suggest a specific inborn error of metabolism.

Organic acid analysis is required as part of newborn screening confirmatory testing when the differential diagnosis includes an organic aciduria(s) as well as other disorders. Follow-up newborn screening recommended testing algorithms and ACTion (ACT) sheets are available at the ACMG website.[21,22] Newborn screening platforms, analytes, analyte cutoffs, protocols, and algorithms differ widely. A "normal" newborn screen, one in which none of the screened analytes were outside of the established high and low cutoffs, should

not be considered diagnostic testing for any disorder screened by that program; diagnostic testing, including organic acid analysis and other testing, should be ordered if there is clinical suspicion for a particular disorder(s). There is worldwide interest in continued assessment and optimization of newborn screening, due to its benefit in early diagnosis of organic acidurias and other conditions and programs may change platforms and adjust cutoffs over time.[23–27]

For optimal interpretation, the laboratory should be made aware of the clinical indication for testing, the clinical state of the patient at the time of the testing, as well as concurrent medications and diet. The laboratory should have contact information for the ordering physician to aid interpretation, if needed, and to communicate urgent and emergent results. Depending on the clinical situation, and in a diagnostic evaluation, organic acid analysis should be ordered with analyses of plasma amino acids, acylcarnitine profile, and carnitine (free and total). Ideally, these tests should all be performed by the same laboratory, and the results integrated into an overall interpretation.

5. METHOD ESTABLISHMENT AND VALIDATION

5.1 Qualitative versus quantitative analysis

Urine organic analysis can be performed as a qualitative or quantitative test and both methods are effective approaches for establishing a diagnosis. Qualitative urine organic acid analysis reports contain written descriptions of the observed pattern with an overall interpretation. Quantitative urine organic analyses typically determine concentrations of selected metabolites through the use of numerous calibrators and internal standards. Qualitative and quantitative analyses share multiple assay parameters. The volume of urine analyzed generally contains a fixed amount of creatinine and, therefore, varies with patient hydration status. Abnormal metabolites are identified by both retention time[28] and fragmentation pattern in the mass spectrometer. Typically, each laboratory develops a custom library of compounds that identify retention time and relative ion abundance based on instrument parameters such as flow rate, column type, column length, and elution ramp temperatures. Given the challenges of manual organic acid extraction, internal standards are necessary to ensure proper sample preparation (see section 5.2).

Quantitative analysis offers certain benefits and caveats. Some clinicians find quantitative reports helpful for monitoring known patients. In this case, clinicians should be aware that, in the absence of stable isotope dilution, or another robust quantitative method, quantitative results may not be portable between laboratories so therapy monitoring should be performed in one laboratory.[1] For metabolites excreted in low levels among clinically normal individuals, quantitative methods, along with reference values, may be helpful to determine whether an analytical level is indicative of an inborn error of metabolism. However, pathological levels of certain metabolites, such as mevalonic acid, may be minimal, and below the limit of detection of some methods, and thus, more sensitive targeted assays are necessary with stable isotope dilution using selected ion monitoring.[29,30]

5.2 Calibration and quantitation

Urine organic acid analysis should be performed using at least one internal standard. The choice of internal standard varies between laboratories but it should be uniquely identifiable, not present in human specimens, and resolvable from other compounds by gas chromatography. The internal standard controls for extraction efficiency, and allows the direct comparison of metabolite concentrations in patient specimens to calibration standards. Commonly used internal standards include heptadecanoic acid, 2-phenylbutyric acid,[31] tropic acid, pentadecanoic acid,[15] 2-ketocaproic acid,[32] p-chlorobenzoic acid, and dimethylmalonic acid.[33] A second internal standard added immediately before derivatization may help assess instrument function; tetracosane is commonly used as a second standard.

Quantification of organic acid concentrations must be performed by comparison with a known reference or external calibration standard(s) whenever possible. A calibration curve is prepared for each analyte quantified during the procedure, covering the normal range and expected pathological values. The calibration curve should include several dilutions of the standard mix containing the organic acids quantified. To generate and update calibration curves, calibrators should be carried through the entire specimen preparation process. Usually, only a subset of organic acids is quantified, and most other organic acids are assessed qualitatively. Quantification could be performed using a single quantifying ion for each compound. The peak area of the compound quantifying ion is normalized to the peak area of the internal standard ion and sample volume, and then quantitation is obtained using the calibration curve slope.[32,34] The internal standards used are typically nonisotopic compounds unless specific organic acids are targeted. The use of solvent standards (i.e., standards mixed directly in organic solvent and not carried through the full extraction process) is inappropriate because these do not adequately control for different extraction efficiencies.[20] Quantitation without the use of a specific reference standard is discouraged. However, for compounds for which quantitative data is desired but no reference standard is available, the laboratory may compare peak response with that of a known quantity of internal standard to derive a relative concentration. In these cases, the metric should be clearly indicated on the report.

5.3 Reference ranges

Reference ranges for all reported quantified compounds should be established and periodically validated. Reference ranges should be normalized to the creatinine concentration. When literature-based ranges are used they must also be periodically verified. Age-specific ranges should be determined for compounds for which the concentration is known to vary with age.[7,9,15] Optimally, each laboratory should establish its own reference ranges based on its method.

5.4 Validation

Validation of qualitative organic acid analysis utilizes urine samples from unaffected controls and patients with previously confirmed inborn errors of metabolism. The proper identification of expected abnormal metabolites in abnormal control specimens (and absence in unaffected control specimens) should be documented for as many inborn errors of metabolism as is reasonably possible. Proficiency testing programs are one source of confirmed abnormal samples. Specimen exchange between testing laboratories is also an effective means of increasing exposure to rare diseases, and of validating a new method or instrument.

For quantitative analysis, the laboratory should establish protocols to determine the linear range, analytical measurement range, and lower limit of quantification for all quantified compounds. Procedures should be in place for addressing any values that fall outside of these performance limits. Initial validation should include analysis of the precision and recoveries for each quantified analyte. Performance characteristics (e.g., linearity, analytical measurement range, accuracy, and precision) should be determined for all quantified analytes and verified at appropriate, regular intervals.[14] Instrument calibration should be validated and verified at regular intervals established by the laboratory and as required by CLIA. Calibration verification should also be performed following any major service to the instrument, or before the use of a new lot of reagents.

5.5 Testing personnel

Appropriate training procedures and ongoing competency requirements for laboratory personnel performing organic acid analysis must be established and documented. Organic acid analysis is considered high-complexity testing, so personnel should, at minimum, satisfy CLIA requirements. Specific training in peak identification and interpretation may be necessary.

6. ANALYSIS OF ORGANIC ACIDS

6.1 Extraction of organic acids

The volume of urine required for organic acid analysis is dependent on its creatinine concentration. The sample volume should be adjusted to reach a set creatinine amount, and dilution of concentrated samples performed as necessary. A volume of urine equivalent to 0.25 mg (2.2 μmol) of creatinine is typically used; various protocols call for 0.1 to 0.4 mg of creatinine. One or more internal standards is added to the specimen (see section 5.2), followed by the addition of an acid such as hydrochloric acid. Addition of sodium chloride or urease prior to extraction can reduce the amount of urea, which may interfere with other compounds.[3,35] Urease treatment nonextraction methods are reported to increase detection of glycerol and glycerol-3-phosphate, aiding in the diagnosis of fructose-1,6-bisphosphatase deficiency, and are capable of screening carbohydrates and amino acids in addition to organic acids.[35,36]

Extraction of organic acids from acidified urine (pH 1–2) is performed using organic solvents or ion-exchange solid-phase extraction. Traditional protocols use diethyl ether and/or ethyl acetate as the preferred organic solvents.[15,33] Alternatively, tetrahydrofuran shows higher sensitivities for polar compounds than ethyl acetate.[37] Solid phase extraction with strong anion exchange cartridges is sometimes favored to improve extraction of more polar compounds without urine acidification, though it can have reduced extraction efficiency of less polar, pathologically relevant metabolites.[38,39] Less polar compounds such as orotic acid, succinylacetone, and uracil may be poorly extracted depending on the specific method used. A targeted quantitative analysis may be warranted to query these specific metabolites. Alternatively, prior to acidification and extraction, an oximation step can stabilize and improve detection of keto acids such as pyruvic acid, α-ketoglutaric acid, succinylacetone, and certain intermediates of branched-chain amino acid metabolism: 2-ketoisocaproic acid, 2-keto-3-methylvaleric acid, and 2-ketoisovaleric acid. Oximation may be performed routinely or under select circumstances, such as the clinical suspicion of maple syrup urine disease (MIM 248600) or tyrosinemia type I (MIM 276700).

6.2 Derivatization

Following extraction, an additional standard, often tetracosane, can be added to the specimen. Organic extracts are then evaporated to dryness under nitrogen and derivatized as described below. Incomplete dryness can reduce derivatization efficiency.

Trimethylsilyl derivatives of organic acids in dried urine extracts are formed by the addition of *N,O*-bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/TMCS) or similar commercial reagents. In a typical derivatization procedure, dried extracts are mixed with BSTFA/TMCS and incubated at 65–90 °C for 10–30 minutes. Alkane standards of known carbon chain length may be added to monitor chromatographic separation.

6.3 Gas chromatography–mass spectrometry instrumentation

Several instrument configurations are commercially available that separate compounds and enable identification by chromatographic retention time and mass spectra. Separation is most often carried out on a capillary column, and mass spectra obtained from either a quadrupole filter or ion trap mass spectrometer using electron impact ionization. Fixed electron impact energy is applied to generate reproducible and portable spectra. A small amount of sample (1–3 μl) is typically injected. Application of a split inlet mode helps to avoid column overload with a split ratio of 1:15–70. Mass spectral data are typically collected in full scan mode from m/z 50 to 550 to generate total ion current chromatograms. Selected ion monitoring may be used by methods that target specific compounds.

6.4 Chromatogram analysis

Total ion current chromatograms are analyzed by manual interrogation of the spectral data. Compound identification is critical to the diagnosis of genetic disorders associated with abnormal organic acid excretion and relies on the evaluation of both their mass spectra and retention time. A computer library of mass spectra of known compounds is typically used to confirm the identity of metabolite peaks. A limited number of commercial libraries (e.g., the National Institute of Standards and Technology and Wiley) containing mass spectra of trimethylsilyl derivatives of physiologic and drug-related compounds are available for the identification of compounds and are compatible with most GC-MS manufacturer software packages. These commercial reference libraries may not contain all compounds of interest to biochemical genetics laboratories. The common, nonsystematic chemical names may not be available in these reference libraries. Laboratories are encouraged to modify commercial reference libraries and develop in-house libraries on their own equipment, using pure organic acid standards and/or patient-derived peaks (once positive identification of a compound has been established). A minimal library in a biochemical genetics laboratory should contain all of the compounds in the expanded newborn screening follow-up testing guideline by National Academy of Clinical Biochemistry and organic acid master list in the College of American Pathologists (CAP) Biochemical Genetics Survey.[1,28] It is good laboratory practice to continuously update the analytical library when new diagnostic compounds are reported in the literature.

The methods and criteria by which peaks are identified must be documented in the laboratory. Although instrument software can generate analyte match reports according to preset criteria, manual review of peaks and spectra is necessary to achieve accurate peak identification.[1] Examination of chromatographic retention times and/or relative retention times to an internal standard is also necessary, particularly for the correct identification of isomers or spectrally similar compounds.[9] Particular attention should be paid to regions of the chromatogram in which complete or partial overlap of a clinically relevant peak with that of another compound could hinder identification. Possible critical organic acid overlaps include 3-hydroxybutyric acid/3-hydroxyisobutyric acid, urea/4-hydroxybutyric acid, hexanoylglycine/4-hydroxyphenylacetic acid, ethylmalonic acid/phosphoric acid, 3-methylglutaconic acid/3-hydroxyadipic lactones, tiglylglycine/3-methylcrotonylglycine, orotic acid/*cis*-aconitic acid, and others.[1,15] Modification of analytical system parameters such as column type, elution temperature ramp, and run time may resolve some of these coeluting compounds. Examinations of postrun extraction ion chromatograms and subtracted spectra are often helpful to clarify the composition of apparently complex peaks. Care must be taken to examine each chromatogram for the presence of peaks that, when present even in small amounts, could be clinically significant (e.g., hexanoylglycine, suberylglycine, succinylacetone, 2-methylcitric acid, and orotic acid).

6.5 Quality control

The mass spectrometer should be tuned prior to each batch of patient samples analyzed, as described by the instrument manufacturer and required by the CAP. Procedures should be developed and documented for the routine evaluation of system performance, as well as the detection and evaluation of potential carryover effects. A quality control (QC) program based on the analysis of normal and abnormal controls should be implemented and performed with every batch of patient specimens. Peak areas of internal standards should be monitored for extraction and derivatization efficiency in all patient and QC specimens. Thresholds for acceptance or rejection of QC data, and remedial actions in the event of a QC failure, should be established and documented by the laboratory. QC data should be regularly monitored for overall trends that may affect test performance, and problems should be documented and remediated as appropriate. The use of Westgard rules for clinical specimen analysis further specifies the parameters for quality patient diagnosis and reporting.[40]

6.6 Proficiency testing

An ongoing proficiency testing program for organic acid analysis must be implemented and documented. A biannual proficiency testing program that evaluates both analytical and interpretive/diagnostic proficiency is offered by the CAP and supervised by the joint CAP/ACMG Genetic Biochemical and Molecular Genetic Resource Committee.[28] An excellent program for both qualitative and quantitative organic acid analysis is also offered by the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM; http://www.erndimqa.nl).[2,5,41] In addition to the official CAP and ERNDIM testing, sample exchange between laboratories and sharing of unusual data are valuable educational resources.

7. TEST INTERPRETATION AND REPORTING

7.1 Interpretation

Urine organic acid analysis may be the most complex test performed in many biochemical genetic laboratories. Interpretation of urine organic acid results should be performed by an American Board of Medical Genetics and Genomics or other relevant medical board-certified laboratory director. Clinically meaningful interpretation of organic acid results should be based on the overall pattern of metabolites present in abnormal quantities, rather than on individual abnormalities. The diagnostic sensitivity of organic acid analysis under acute and asymptomatic clinical states may vary considerably. Although certain diagnoses can be confidently suggested based on an organic acid profile, there are limitations of this test. Informative profiles may not always be detected in disorders in which the excretion of diagnostic metabolites is a reflection of the residual activity of the defective enzyme, the dietary load of precursors, and the anabolic/catabolic status of the patient. For example, some individuals with glutaric acidemia type I (MIM 231670) are considered to be "low

Table 1 Recently described inborn errors of metabolism with abnormalities in urine organic acid analysis

Disorder	Elevated metabolite(s)
Aminoacylase 1 deficiency, MIM 609924 (ref. 44)	*N*-acetyl amino acids
Aromatic L-amino acid decarboxylase deficiency (primary or due to abnormal B6 level or metabolism), MIM 608643 (refs. 45,46)	Vanilpyruvic acid, 3-*O*-methyldopa (3-methoxytyrosine), *N*-acetylvanilalanine, vanillactic acid
Combined malonic and methylmalonic aciduria, MIM 614265 (ref. 47)	Methylmalonic acid (larger amount), malonic acid
3-OH-Isobutyryl-CoA hydrolase deficiency, MIM 250620 (ref. 48)	2,3-Dihydroxy-2-methylbutyric acid[a]
3-OH-3-Methylglutaryl-CoA synthase 2 deficiency, MIM 605911 (refs. 49,50)	3,5-Dihydroxyhexanoic 1,5 lactone, trans-5-hedroxyhex-2-enoate, 4-hydroxy-6-methyl-2-pyrone, 5-hydroxy-3-ketohexanoate, 3,5-dihydroxyhexanoate
Methylmalonyl-CoA epimerase deficiency, MIM 251120 (ref. 51)	Ketones, methylmalonic acid, 3-hydroxypropionic acid, methylcitric acid, propionylglycine
Combined D-2 and L-2-hydroxyglutaric aciduria, MIM 615182 (ref. 52)	D- and L-2-hydroxyglutaric acid
Cytosolic phosphoenolpyruvate carboxykinase deficiency, MIM 261680 (ref. 53)	Tricarboxylic acid cycle metabolites: fumaric acid, succinic acid, malic acid, α-ketoglutaric acid, adipic acid, 3-hydroxysebacic acid, and 3-hydroxydodecanedioic acid
Riboflavin transporter defect, MIM 211530 and MIM 211500 (ref. 54)	Ethylmalonic acid, adipic acid, 2-hydroxyglutaric acid, suberic acid, hexanoylglycine, and suberylglycine
Short/Branched-chain acyl-CoA dehydrogenase deficiency, MIM 610006 (refs. 55,56)	2-Ethylhydracrylic acid, 2-methylbutyrylglycine
Short-chain enoyl-CoA hydratase deficiency, MIM 616277 (ref. 48)	2,3-Dihydroxy-2-methylbutyric acid[a]
Succinate-CoA ligase (SUCLA2 and SUCLG1) deficiencies, MIM 612073 and MIM 612224 (refs. 57,58)	Methylmalonic acid, methylcitric acid, and others
Succinic semialdehyde dehydrogenase deficiency, MIM 271980 (ref. 59)	4-hydroxybutyric acid, 3,4-dihydroxybutryric acid, dicarboxylic Aciduria, 4,5-dihydroxyhexanoic acid, and lactones (not present in γ-hydroxybutyric acid intoxication)
Xanthurenic aciduria, MIM 236800 (ref. 60)	Xanthurenic acid

[a]Follow-up testing is indicated to look for urine acrylate conjugates, and molecular studies are recommended.[48]

excretors" in that urinary glutaric acid and 3-hydroxyglutaric acid levels may not demonstrate significant abnormalities.[42] Communication with the ordering physician to better understand the clinical, medication, and nutritional history may help the laboratory director accurately interpret a complex chromatogram. Comprehensive tables are available in published literature and online to assist in interpretation of organic acid findings.[7,9,19,43] A brief table of recently identified organic acidurias and their associated abnormal organic acids is provided here (**Table 1**).

A characteristic abnormal organic acid profile may not be sufficient to establish a definitive diagnosis. Other methods of higher specificity and sensitivity based on stable isotope labeled internal standards, selected ion monitoring, or chemical ionization can overcome the limitations of standard organic acid analysis for the investigation of nonacutely ill or low excretor patients (e.g., targeted methods for the determination of methylmalonic acid, 3-hydroxyglutaric acid, or succinylacetone). In some instances targeted quantification of specific metabolites in other physiological fluid (cerebrospinal fluid, amniotic fluid, plasma, or serum) may be appropriate to clarify an ambiguous urine finding. Confirmation by an independent method is recommended whenever practical, such as molecular analysis, associated analyte determination, or in vitro enzyme assay (blood cells, cultured cells, or tissue biopsy). Such follow-up testing is particularly helpful to clarify nonspecific organic acid elevations such as tricarboxylic aciduria suggestive of mitochondrial dysfunction, vanillactic acid suggestive of a neurotransmitter synthesis defect, or hypoglycemic ketonuria.

7.2 Reporting

According to a 10-year review by ERNDIM, report quality and content varied greatly upon ambiguous findings in qualitative organic acid assay.[5] Reports should be clear to a nonspecialist. Patient reports must contain appropriate patient and specimen information as contained in sections C2.4, 2.4.1, and 2.4.2 of the American College of Medical Genetics Standards and Guidelines for Clinical Genetics Laboratories (2008 Edition).[6] Identification of all relevant compounds must be provided on the report, and quantity may be listed (as determined). Quantitative reports must include appropriate reference ranges (see section 5.3). The report may include a brief description of the method and a list of known analytes that are not well determined. When no clinically significant abnormalities are detected, an organic acid analysis can be reported and interpreted in qualitative terms only. When abnormal results are detected, the detailed interpretation should include an overview of the results and their potential significance, correlation with available clinical information, a differential diagnosis, recommendations for additional confirmatory or other follow-up studies, and

contact information for the reporting laboratory. In the case of a new diagnosis the laboratory should attempt to reach the ordering physician to ensure the recommendations are communicated directly. If appropriate, referral of the patient to a metabolic center for further evaluation and management should also be recommended, as this is likely to optimize the patient outcome.[5]

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DISCLOSURE

The following authors direct laboratories that perform urine organic acid analysis as a fee-for-service: L.P., A.I.S., S.H., S.G., and Q.S. The other authors declare no conflict of interest.

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Appendix 3: Laboratory analysis of acylcarnitines, 2020 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

ACMG TECHNICAL STANDARD | Genetics inMedicine

Laboratory analysis of acylcarnitines, 2020 update: a technical standard of the American College of Medical Genetics and Genomics (ACMG)

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Disclaimer: This technical standard is designed primarily as an educational resource for clinical laboratory geneticists to help them provide quality clinical laboratory genetic services. Adherence to this technical standard is voluntary and does not necessarily assure a successful medical outcome. This technical standard should not be considered inclusive of all proper procedures and tests or exclusive of other procedures and tests that are reasonably directed to obtaining the same results. In determining the propriety of any specific procedure or test, the clinical laboratory geneticist should apply his or her professional judgment to the specific circumstances presented by the individual patient or specimen.

Clinical laboratory geneticists are encouraged to document in the patient's record the rationale for the use of a particular procedure or test, whether or not it is in conformance with this technical standard. They also are advised to take notice of the date any particular technical standard was adopted, and to consider other relevant medical and scientific information that becomes available after that date. It also would be prudent to consider whether intellectual property interests may restrict the performance of certain tests and other procedures.

Acylcarnitine analysis is a useful test for identifying patients with inborn errors of mitochondrial fatty acid β-oxidation and certain organic acidemias. Plasma is routinely used in the diagnostic workup of symptomatic patients. Urine analysis of targeted acylcarnitine species may be helpful in the diagnosis of glutaric acidemia type I and other disorders in which polar acylcarnitine species accumulate. For newborn screening applications, dried blood spot acylcarnitine analysis can be performed as a multiplex assay with other analytes, including amino acids, succinylacetone, guanidinoacetate, creatine, and lysophosphatidylcholines. Tandem mass spectrometric methodology, established more than 30 years ago, remains a valid approach for acylcarnitine analysis. The method involves flow-injection analysis of esterified or underivatized acylcarnitines species and detection using a precursor-ion scan. Alternative methods utilize liquid chromatographic separation of isomeric and isobaric species and/or detection by selected reaction monitoring. These technical standards were developed as a resource for diagnostic laboratory practices in acylcarnitine analysis, interpretation, and reporting.

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Keywords: clinical genetic testing; fatty acid oxidation disorders; organic acidemias; acylcarnitine; tandem mass spectrometry

BACKGROUND

Acylcarnitine profile (ACP) analysis is performed for the biochemical detection of disorders of mitochondrial fatty acid β-oxidation (FAO) and organic acid metabolism.[1,2] ACP results can be diagnostic for some disorders, such as medium-chain acyl-CoA dehydrogenase (MCAD) deficiency; however, additional testing may be needed in other cases to obtain a precise diagnosis.[1–3] The conditions revealed by acylcarnitine analysis have in common the accumulation of short, medium, and/or long-chain acyl-CoA species, which are substrates for one of several carnitine acyl-CoA transferases expressed in different intracellular compartments.[4,5] The resulting acylcarnitine species are measured in the following clinical situations: (1) evaluation of symptomatic patients, (2) evaluation of asymptomatic (at-risk) siblings of known patients, (3) newborn screening and follow-up testing, (4) prenatal diagnosis, (5) postmortem evaluation.

Laboratories providing this analysis as a clinical service most often do so using flow-injection analysis–tandem mass spectrometry (FIA-MS/MS) as the analytical platform.[6,7] Newer MS/MS methods that include a liquid chromatographic (LC) step to separate isobars and isomers are also used.[8–12] Other reported methodologies include gas chromatography–mass spectrometry (GC-MS),[13] radio high-performance LC,[14,15] and capillary electrophoresis.[16] As is the case for all complex metabolic profiles, appropriate

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acylcarnitine analysis requires both analytical proficiency and in-depth interpretation of results with informative reporting.

CLINICAL DESCRIPTION OF DISORDERS IDENTIFIED THROUGH ACYLCARNITINE ANALYSIS

ACP analysis detects disorders of mitochondrial FAO and organic acid metabolism. Examples of disorders that may be detected by acylcarnitine analysis are listed in Tables 1 and 2. Inborn errors of mitochondrial FAO disorders may present at any age, from birth to adulthood. They are associated with life-threatening episodes of metabolic decompensation after a period of inadequate caloric intake and/or intercurrent illness. Typical manifestations may include hypoketotic hypoglycemia, liver disease, skeletal myopathy and cardiomyopathy, and sudden unexpected death.[17] ACP abnormalities have also been described in peroxisomal disorders associated with impaired very long–chain fatty acid (VLCFA) β-oxidation.[18,19] However, this latter group of disorders are better diagnosed by plasma VLCFA and branched-chain fatty acid analysis.[20]

Organic acidemias are a heterogeneous group of inborn errors of metabolism (IEM).[21] Classic organic acidemias typically present with recurrent episodes of acute life-threatening illness, hypo- or hypertonia, failure to thrive, and developmental delay. Common acute manifestations include vomiting, lethargy, coma, and seizures.

With rare exceptions, the diagnosis of these conditions is almost exclusively a laboratory process, of which acylcarnitine analysis is a key component. A comprehensive metabolic evaluation may also include the analysis of plasma amino acids, urine organic acids, and plasma carnitine (free and total). Depending on the results of these studies, additional testing may be warranted.

Table 1 Inborn errors of metabolism detected by acylcarnitine profile analysis.

Fatty acid oxidation disorders	OMIM[a]	Gene
Carnitine uptake defect	212140	*SLC22A5*
Carnitine palmitoyltransferase I (CPT I) deficiency	255120	*CPT1A*
Carnitine-acylcarnitine translocase (CACT) deficiency	212138	*SLC25A20*
Carnitine palmitoyltransferase II (CPT II) deficiency	608836, 600649, 255110	*CPT2*
Short-chain acyl-CoA dehydrogenase (SCAD) deficiency	201470	*ACADS*
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	201450	*ACADM*
Very long–chain acyl-CoA dehydrogenase (VLCAD) deficiency	201475	*ACADVL*
Long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency	609016	*HADHA*
Trifunctional protein (TFP) deficiency	609015	*HADHA*, *HADHB*
Multiple acyl-CoA dehydrogenase (MAD) deficiency (glutaric acidemia type II):	231680	
i α-ETF		*ETFA*
ii β-ETF		*ETFB*
iii ETF-ubiquinone oxidoreductase		*ETFDH*
3-Hydroxyacyl-CoA dehydrogenase (HADH) deficiency	231530	*HADH*
Dienoyl-CoA reductase deficiency caused by mitochondrial NAD kinase 2 deficiency	616034	*NADK2*

Medium-chain 3-ketoacyl-CoA thiolase (MCKAT; MIM 602199) deficiency has been reported in one patient[61] and it is not known whether this disorder can be detected by acylcarnitine analysis.
[a]Phenotype MIM number.

Table 2 Inborn errors of metabolism detected by acylcarnitine profile analysis.

Organic acid disorders	OMIM[a]	Gene
Ethylmalonic encephalopathy	602473	*ETHE1*
Glutaryl-CoA dehydrogenase deficiency (glutaric acidemia type I)	231670	*GCDH*
Glutamate formiminotransferase deficiency (formiminoglutamic aciduria)	229100	*FTCD*
3-Hydroxyisobutyryl-CoA hydrolase deficiency	250620	*HIBCH*
3-Hydroxy-3-methylglutaryl-CoA lyase (HMG-CoA lyase) deficiency	246450	*HMGCL*
Isobutyryl-CoA dehydrogenase deficiency	611283	*ACAD8*
Isovaleryl-CoA dehydrogenase deficiency (isovaleric acidemia)	243500	*IVD*
β-Ketothiolase (2-methylacetoacetyl-CoA thiolase, or 3-oxothiolase)	203750	*ACAT1*
Malonyl-CoA decarboxylase deficiency	248360	*MLYCD*
2-Methylbutyryl-CoA dehydrogenase (short/branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency)	610006	*ACADSB*
3-Methylcrotonyl-CoA carboxylase (3-MCC) deficiency	210200, 210210	*MCCC1*, *MCCC2*
3-Methylglutaconyl-CoA hydratase deficiency	250950	*AUH*
2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency (HSD10 mitochondrial disease)	300438	*HSD17B10*
Methylmalonic acidemia (MMA):		
i Methylmalonyl-CoA mutase deficiency	251000	*MUT*
ii Methylmalonyl-CoA racemase deficiency	251120	*MCEE*
iii Cobalamin metabolism disorders		
cblA	251100	*MMAA*
cblB	251110	*MMAB*
cblC	277400	*MMACHC*, *PRDX1*
cblD	277410	*MMADHC*
cblF	277380	*LMBRD1*
cblJ	614857	*ABCD4*
cblX	309541	*HCFC1*
Multiple carboxylase deficiency caused by deficiency of:		
i Holocarboxylase synthetase	253270	*HLCS*
ii Biotinidase	253260	*BTD*
Propionyl-CoA carboxylase deficiency (propionic acidemia)	606054	*PCCA*, *PCCB*
Succinyl-CoA ligase deficiency		
i Mitochondrial DNA depletion syndrome 5	612073	*SUCLA2*
ii Mitochondrial DNA depletion syndrome 9	245400	*SUCLG1*

[a]Phenotype MIM number.

PREVALENCE

The combined incidence of these disorders is estimated to be between 1:5,000 and 1:10,000 live births.[22]

MODE OF INHERITANCE

A majority of IEM detectable by acylcarnitine analysis are inherited as autosomal recessive traits. Two X-linked disorders are potentially detected by acylcarnitine analysis. The first is HSD10 mitochondrial disease (MIM 300438), which is caused by a deficiency of a mitochondrial ribonuclease P subunit, encoded by *HSD17B10* (MIM 300256). This multifunctional protein has dehydrogenase activity toward a range of substrates, including 2-methyl-3-hydroxybutyryl-CoA.[23] The second is cobalamin disorder type X (MIM 309541), caused by a deficiency of a global transcriptional coregulator encoded by *HCFC1*.[24,25] It should be noted that acylcarnitine analysis may have low sensitivity for detecting HSD10 mitochondrial disease,[23] and the sensitivity of acylcarnitine analysis to detect cobalamin disorder type IX has not been fully evaluated.

METHODS

This laboratory technical standard was informed by a review of the literature, including any current guidelines, and expert opinion. Resources consulted included PubMed (search terms: acylcarnitine quantitation, acylcarnitine analysis, separation, UPLC, peroxisomal, prenatal, urine), Clinical and Laboratory Standards Institute (CLSI) guidelines[26–28] and CLIA regulations, and the Centers for Disease Control and Prevention (CDC) *Morbidity and Mortality Weekly Report* on Good Laboratory Practices for Biochemical Genetics Testing and Newborn Screening for Inherited Metabolic Disorders.[29] When the literature provided conflicting evidence about a topic or when there was insufficient evidence, the authors used expert opinion to inform the recommendations. Expert opinion included the coauthors of the document, members of the Biochemical Genetics Subcommittee of the Lab QA Committee, as well as the experts consulted outside the workgroup and acknowledged in this document. Any conflicts of interest for workgroup members or consultants are listed. The American College of Medical Genetics and Genomics (ACMG) Laboratory Quality Assurance Committee reviewed the document providing further input on the content, and a final draft was delivered to the ACMG Board of Directors for review and approval to send out for member comment. The final draft of the document was posted on the ACMG website and an email link was sent to ACMG members inviting all to provide comment. All members' comments were assessed by the authors; additional evidence was also included, and our recommendations were amended as deemed appropriate. Member comments and author responses were reviewed by a representative of the Laboratory Quality Assurance Committee and the ACMG Board. The final document was approved by the ACMG Board of Directors. This updated standard replaces the previous document "Acylcarnitine profile analysis" by Rinaldo et al.[30]

PREANALYTICAL REQUIREMENTS

Specimen requirements

Carnitine and its esters are found in virtually all biological fluids. The clinical utility of an acceptable biological fluid will depend upon the purpose of the analysis. Concentrations of acylcarnitine species vary between sample types and plasma concentrations may not be reflective of tissue concentrations.[10] Typical volumes of different sample types are shown in Table 3.

Plasma/serum

Heparinized plasma is the preferred sample type for the diagnostic evaluation of symptomatic patients and asymptomatic at-risk individuals. EDTA plasma or serum may also be accepted by testing laboratories as alternative sample types.

Dried blood spot

Dried blood spots (DBS) are used for newborn screening, and have utility in the clinical diagnostic setting, providing an alternative to plasma. In comparison with plasma or serum, blood spots have higher concentrations of long-chain acylcarnitines and certain other species such as C2, C3, C4-DC, and C5-OH, and lower free carnitine (C0).[31] DBS may therefore have a greater sensitivity for detecting carnitine palmitoyltransferase (CPT) IA deficiency (MIM 255120), a disorder characterized by reduced concentrations of long-chain acylcarnitines and elevated free carnitine.[31] In contrast, plasma may have greater sensitivity for diagnosing disorders associated with an accumulation of long-chain acylcarnitines, such as CPT II deficiency (MIM 600649, 608836, 255110) and mitochondrial trifunctional protein deficiency (MIM 609015).[31,32]

Bile or bile spot

Postmortem screening can be performed in bile as a first-level evaluation for sudden or unexpected death.[33,34] Bile can be

Table 3 Typical sample testing volumes for acylcarnitine profile analysis.

Sample type	Typical testing volume
Amniotic fluid (cell-free supernatant)	20 µL
Amniocyte culture medium	50 µL[a]
Bile (liquid specimen)	2 µL[b]
Bile dried spot (postmortem)	one 1/8-inch punch[b]
Blood dried spot (neonatal, postmortem)	one 1/8-inch or 3/16-inch punch
Cell culture medium	50 µL[a]
Plasma/serum	20 µL
Urine	Variable; standardized to creatinine

[a]Spotted on filter paper.
[b]May require dilution if profile is abnormal.

spotted onto filter paper and analyzed in the same manner as DBS.

Urine

Urinary acylcarnitine analysis can be useful in the workup of organic acidemias and FAO disorders[35] associated with accumulations of short- or medium-chain acylcarnitine species,[36–39] which are efficiently excreted in the urine. This is most often applied in the case of glutaric acidemia type I, in which an elevation of glutarylcarnitine may be more readily discernible in urine compared with DBS or plasma.[39] Long-chain acylcarnitines have low solubility and hence low concentrations in urine; urinary acylcarnitine analysis is not helpful for the diagnosis of long-chain mitochondrial FAO disorders. However, utility for diagnosing patients with certain peroxisomal disorders has been suggested.[19] In patients with peroxisomal biogenesis disorders and D-bifunctional protein deficiency, an increase was reported in urinary long-chain dicarboxylic acid acylcarnitines, and to a lesser extent in very long–chain monocarboxylic acid species.[19]

Cultured fibroblasts

ACP performed in cultured fibroblasts incubated with fatty acid substrates and carnitine can be useful for the diagnosis of FAO disorders.[2,40,41]

Amniotic fluid

Acylcarnitine analysis in amniotic fluid has been used for the prenatal diagnosis of certain IEM.[42,43] This testing may be useful when there is a family history of IEM and molecular studies were not obtained or were uninformative in the proband, and when performed in combination with one or more additional independent tests.[1]

Conditions of sample collection, shipping, handling, and storage

Plasma, serum, whole blood, and urine samples collected at the time of acute illness have the greatest sensitivity for detecting fatty acid oxidation disorders and organic acidemias. At ambient temperature, plasma acylcarnitines are stable for less than two days and in DBS for less than two weeks. At colder temperatures, specimens may be preserved for longer. Short-chain species are more unstable compared with long-chain species. Performing laboratories should define appropriate handling, shipping, and storage guidelines for their specific application.

Exogenous variables

Dietary factors such as medium-chain triglyceride (MCT)–containing formulas, fasting, ketogenic diet, carnitine supplementation, and total parenteral nutrition may influence acylcarnitine profiles. Carnitine insufficiency lowers the concentrations of acylcarnitines in body fluids and may decrease the sensitivity of ACP testing. Conversely, carnitine supplementation enhances blood concentrations and urinary excretion of acylcarnitines,[38] and may complicate interpretation of profiles. Renal impairment may cause an increase in short and medium-chain dicarboxylic acid acylcarnitine species (S. Young, unpublished observations, DUHS Biochemical Genetics Laboratory), whereas liver dysfunction may cause an increase in long-chain dicarboxylic acid acylcarnitine species.[6]

Clinical indications for testing

There are a variety of clinical indications for ACP analysis. Most commonly, ACP analysis is obtained following an abnormal newborn screen. ACP analysis can be helpful when an organic acidemia is suspected; findings often include metabolic acidosis, poor feeding, and lethargy or encephalopathy. Note that for an organic acidemia, urine organic acid analysis is the preferred method for diagnosis.[44] For disorders of fatty acid oxidation, typical clinical indications include hypoglycemia (in the absence of ketonuria), rhabdomyolysis, and cardiomyopathy. ACP analysis should also be considered in the setting of low free carnitine, to evaluate for the possibility of secondary carnitine deficiency, as well as in cases where the acyl to free carnitine ratio is elevated.

Due to an increased risk of long-chain 3-hydroxyacyl-CoA dehydrogenase or trifunctional deficiency, and possibly other fatty acid oxidation disorders, ACP analysis may be obtained in neonates with maternal complications of acute fatty liver of pregnancy and/or hemolysis, elevated liver enzymes, and low platelet count (HELLP syndrome).[45,46] Both CPT II deficiency and multiple acyl-CoA dehydrogenase deficiency (MADD) can present in the neonatal period with cystic kidneys and other malformations;[47] as these early presentations are often fatal, it is imperative to obtain the correct diagnosis for genetic counseling.

ACP analysis should be considered in cases of sudden infant death or other unexpected death at any age, particularly if there was fasting or vomiting in the preceding hours, a history of Reye syndrome, or if fatty infiltration of the liver is noted on autopsy. ACP analysis may be obtained in first-degree relatives following the diagnosis of an FAO disorder or organic acidemia in the proband. Finally, ACP analysis may be followed over time in patients with known disorders of fatty acid oxidation or organic acidemias for monitoring the disease and compliance with treatment.

ANALYSIS OF ACYLCARNITINES

Sample preparation

Acylcarnitine analysis may be performed as a dedicated standalone diagnostic test, or as a component of a multiplexed method for newborn screening that includes analysis of other analytes such as amino acids, succinylacetone, guanidinoacetate, creatine, and very long–chain lysophospholipids.[7,48,49] Acylcarnitines are most often analyzed as butyl-esters,[2] methyl-esters,[32] or without derivatization.[49] Esterification of the carboxylic acid groups results in a fixed quaternary positive charge on the carnitine moiety of acylcarnitine species. This increases the sensitivity of the method in

positive-ion mode, especially for dicarboxylic acids. However, this approach requires specimen heating under acidic conditions, resulting in hydrolysis of acylcarnitine ester bonds, reducing the levels of acylcarnitines and increasing the pool of free carnitine.[50] Therefore, the derivatization conditions must be carefully controlled to minimize loss of acylcarnitines. The use of stable-isotope internal standards is also important to compensate for any losses that do occur.

Plasma specimens are diluted with a mixture of deuterium-labeled internal standards dissolved in aqueous organic solvent mixtures, with or without organic acid modifiers (e.g., formic acid). Commonly used internal standards include [$^2H_3$]-labeled C2, C3, C4, C8, C14, C16; [$^2H_9$]-labeled C5 and C14; and [$^2H_6$]-C5-DC. Other internal standards can be used, but care must be taken not to interfere with the signal of a clinically relevant species (e.g., the butyl-ester of d_3-C6 at m/z 319 is only 1 Da from m/z 318, the molecular ion of hydroxyisovalerylcarnitine). Samples are centrifuged to remove protein precipitate and the supernatant containing extracted acylcarnitines is transferred to another tube and dried under nitrogen. Acylcarnitines are derivatized to their butyl-esters with 3 M HCl in *n*-butanol and heating at 65 °C for 15 minutes. Samples are again dried under nitrogen and reconstituted with a solvent matrix. DBS are similarly prepared; acylcarnitines are extracted from one or more punches from the spot using methanol or acetonitrile containing internal standards.

Analytical methods

Flow-injection analysis–tandem mass spectrometry with precursor-ion scan

Acylcarnitine analysis by FIA positive-ion electrospray–tandem mass spectrometry (MS/MS) utilizes a precursor-ion scan to separate mono- and dicarboxylic acid species, 2 to 18 carbons in length. Butylated and underivatized acylcarnitines are detected by precursors of m/z 85 with a scan from approximately m/z 200 to 500. Methylated acylcarnitines are detected using a precursor-ion scan of m/z 99. This mode of detection confers sufficient sensitivity and specificity to the method without the need for chromatographic separation, allowing for a rapid analysis. FIA combined with a precursor-ion scan has several advantages over methods that utilize a selected reaction monitoring (SRM) mode of detection, with or without chromatography. The whole profile of acylcarnitine species with masses within the scan range can be evaluated. Additionally, common drug artifacts, interfering compounds, and evidence of poor derivatization can be more readily detected. For example, the parent-product ion pair for butylated C4-acylcarnitine species (m/z 288 > 85) can have a contribution from the [M + 1] isotope of butylated formiminoglutamate, which has a $[M+H]^+$ of m/z 287.[51] A contribution from glutamate to the m/z 260 > 85 transition can result in an inaccurate quantification of acetylcarnitine (C2) by the butylation method.[52] The use of a more specific transition (m/z 260 > 141) can be used to more accurately quantify butylated acetylcarnitine.[53] Flow-injection methods do not allow separation of isomeric and isobaric species, which limits the diagnostic specificity of profiles generated by these methods. However, when performed in combination with other tests, such as urine organic acid or acylglycine analysis, the differential diagnosis suggested by flow-injection acylcarnitine profiles can usually be discerned. The isobaric pairs differ depending on the derivatization method (Supplemental Table 1). For example, C3-DC and C4-OH are isobaric by the underivatized method, C3-DC and C5-OH by the methylation method, and C5-DC and C10-OH by the butylation method. With flow-injection methods, when there is an elevated signal for an isobaric pair, a reflex test using an alternative derivative can be used to identify which acylcarnitine species are elevated. Acylcarnitine species and the corresponding mass of the derivatized (butylated or methylated) or nonderivatized molecular ions are listed in Supplemental Table 1. Tolerance limits for the identification by mass must be defined by the laboratory.

LC-MS/MS methods

Liquid chromatography methods are used to enable more sensitive and specific acylcarnitine identification. In this approach, specimens are subjected to LC prior to MS/MS identification with the goal of separating isomeric or isobaric acylcarnitines and interferents. These methods can be performed on underivatized specimens, simplifying sample preparation and allowing concurrent quantification of free carnitine. Reversed phase LC generates the highest chromatographic resolution of acylcarnitines,[8,9] but hydrophilic interaction liquid chromatography (HILIC) methods have also been described that provide relatively rapid analysis times with simple sample preparations.[10,11] LC-MS/MS acylcarnitine chromatograms are highly complex in comparison with flow-injection profiles. For example, C8-carnitine alone has 26 different hypothetical structural isomers, each potentially having unique chromatographic properties.[12] Numerous other acylcarnitines have one or more chiral centers, including all hydroxylated acylcarnitines. These enantiomeric compounds generate multiple peaks by some LC-MS/MS methods.[9] Pure analytic standards are not available for the majority of these compounds, challenging the confident assignment and quantification of the full range of acylcarnitines potentially detectable by LC-MS/MS methods. Even fewer isotopically labeled acylcarnitine standards exist, making it difficult to account for matrix effects. Considering these challenges, chromatographic methods are best used in the setting of focused reflex testing or research studies.

METHOD VALIDATION

Each laboratory must validate the performance characteristics of their specific analytical protocol and periodically verify its performance in accordance with local regulations. In the United States, this includes CLIA and the College of American Pathologists (CAP) requirements. Examples of published method validation approaches are provided by

CLSI in several different documents (e.g., C24-A3: Statistical Quality Control for Quantitative Measurement Procedures: Principles and Definitions, 4th edition[27] and C26-A: Liquid Chromatography–Mass Spectrometry Methods; Approved Guidelines[28]) and ACMG technical standards, such as this one. Laboratories should also devise procedures to address analytical values outside of their established criteria for performance and for clinical specimens with ambiguous stability.

Calibration and quantitation

Stable-isotope internal standards are available from a variety of commercial sources. These standards must be validated by determining their recoveries from weighed, nonisotopic acylcarnitine standards for as many analytes as possible. Internal standards must be periodically revalidated at a regular frequency to account for natural decay. Reference standards are not available for all acylcarnitine species detected by a profile analysis, such as the hydroxylated acylcarnitine species. Quantitation of these compounds should be extrapolated by application of the calibration for the nearest species of similar structure and m/z. The laboratory should determine their method's linear range, analytical measurement range, and lower limit of detection for all clinically informative acylcarnitines, when possible. These performance characteristics should be verified on a regular basis as specified by CLIA.

Reference intervals

Laboratory-specific, age-appropriate reference intervals for all reported acylcarnitines should be established and periodically validated per applicable regulations (e.g., CLSI EP28-A3c: Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory, Approved Guideline, 3rd edition).[26] When literature-based intervals are used, they must be verified by the laboratory before implementation. For all applications, reference intervals should be established on the appropriate tissue, collection tube, and matrix. For instance, the comparison of a pediatric result to a blood spot, plasma, or serum normative interval derived from an adult population is inappropriate. Free carnitine and short-chain acylcarnitine species are increased in postmortem blood samples[34] and reference intervals for this sample type should be generated using postmortem control samples.

Instrument quality cross-check

When more than one mass spectrometric instrument is used to complete quantitative analyses for acylcarnitines, instrument comparisons should be routinely performed, and compatibility confirmed at regular intervals. This comparison should include instruments implemented as a backup for emergency analyses. The process to confirm acceptable performance can include quality control (QC) comparisons, proficiency testing (PT) evaluations, or interlaboratory comparisons at the laboratory's discretion.

Testing personnel

Appropriate training and ongoing competency requirements for laboratory personnel performing ACP analysis must be established and documented. MS/MS-based applications, instrument optimization, method validation, and clinical testing must be performed by personnel with specialized training in the operation of MS/MS.

Quality control

A positive and negative control should be prepared and analyzed with every batch of patient samples tested. The internal standard mix should also be analyzed separately with each batch.

A QC program based on the quantitative analysis of normal and abnormal control specimens should be implemented on a regular basis. Target ranges for QCs should be established for each metabolite and used to accept or reject a given run. The internal standards in each specimen serve as a QC check for each specimen. The use of Westgard rules for clinical specimen analysis further controls the parameters for quality patient diagnosis and reporting.[54] A laboratory director or designee should perform a monthly review and approval of control values.

Proficiency testing

Laboratories should participate in a PT program at time intervals as required by regulating agencies. PT performance should be monitored and insufficiencies requiring further investigation identified. Remedial action for laboratories uncovered by PT performance should be documented. The CAP provides a commercially available, external proficiency testing service for quantitative plasma acylcarnitine analysis. The European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Disorders of Metabolism (ERNDIM) also has a PT scheme for plasma and DBS acylcarnitines. The CDC provides a PT scheme for DBS. Scheduled interlaboratory comparison (alternative PT) can be used when external proficiency testing is unavailable for certain tissues and matrices.

TEST INTERPETATION AND REPORTING

Interpretation

Results should be reviewed and interpreted by an ABMGG-certified biochemical geneticist or other qualified individual. Informative results are usually characterized by a pattern of one or more elevated acylcarnitine species in comparison with age-matched reference ranges. Interpretation of results is based on pattern recognition, rather than on individual abnormal values (see Fig. 1). The differential diagnosis of abnormal results of species potentially representing multiple isomers (C4, C5, C5-OH) is critical and cannot be overstated.[2,3] Basic acylcarnitine patterns associated with various disease states are listed in Fig. 1. In addition to the primary markers listed, calculation of ratios is useful in the interpretation of abnormal results (Fig. 1). Examples of

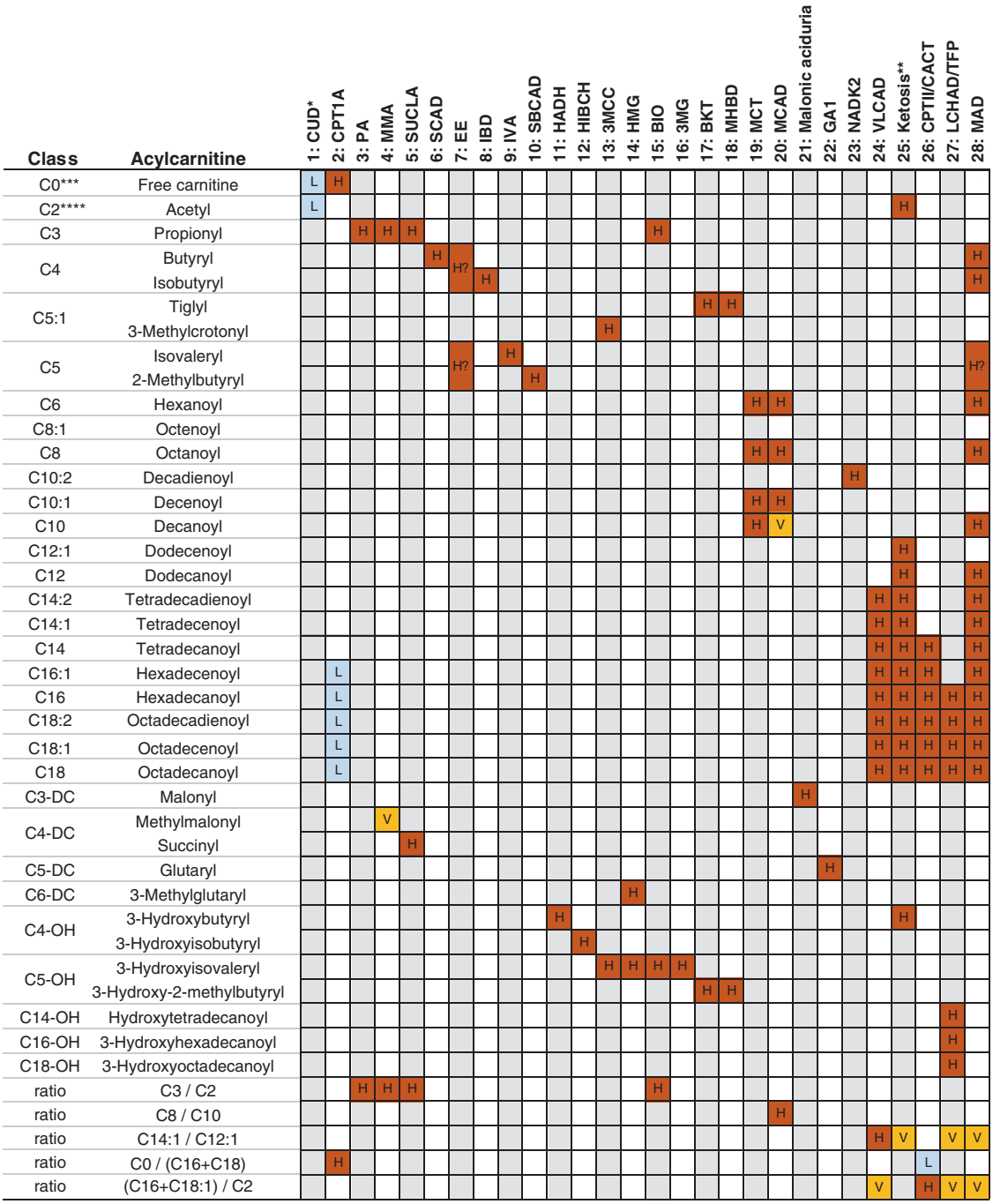

Fig. 1 Common acylcarnitine patterns associated with various disease states. *H* high, *H?* high but specific isomeric species are not clearly defined for this disorder, *L* low, *V* level varies from high-normal to elevated, *In CUD other acylcarnitine species are generally low. **C14:1/C12:1 is typically in the high-normal range or slightly elevated (< 3). ***For many listed disorders, C0 can be low due to secondary carnitine depletion. ****Low C2 may also be observed in other fatty acid oxidation and amino acid metabolism disorders, secondary to factors such as low flux through the β-oxidation pathway, and/or carnitine depletion. Note, in SUCLA2 deficiency, C4-DC elevations were shown to be succinylcarnitine, whereas methylmalonylcarnitine was within normal reference limits.[60] 1: *CUD* carnitine uptake deficiency, 2: *CPT1A* carnitine palmitoyltransferase I deficiency, 3: *PA* propionic acidemia, 4: *MMA* methylmalonic acidemia, 5: *SUCLA* succinyl-CoA ligase deficiency, 6: *SCAD* short-chain acyl-CoA dehydrogenase, 7: *EE* ethylmalonic encephalopathy, 8: *IBD* isobutyryl-CoA dehydrogenase deficiency, 9: *IVA* isovaleryl-CoA dehydrogenase deficiency, 10: *SBCAD* short/branched-chain acyl-CoA dehydrogenase deficiency, 11: *HADH* 3-hydroxyacyl-CoA dehydrogenase, 12: *HIBCH* 3-hydroxyisobutyryl-CoA hydrolase deficiency, 13: *3-MCC* 3-methylcrotonyl-CoA carboxylase deficiency, 14: *HMG* HMG-CoA lyase deficiency, 15: *BIO* biotinidase or holocarboxylase synthetase deficiency, 16: *3MG* 3-methylglutaconyl-CoA hydratase deficiency (AUH), 17: *BKT* β-ketothiolase deficiency, 18: *MHBD* 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency, 19: *MCT* supplementation with medium-chain triglycerides, 20: *MCAD* medium-chain acyl-CoA dehydrogenase deficiency, 21: *Malonic aciduria* malonyl-CoA decarboxylase deficiency, 22: *GA1* glutaric aciduria type 1, 23: *NADK2* dienoyl-CoA reductase deficiency caused by mitochondrial NAD kinase 2 deficiency, 24: *VLCAD* very long–chain acyl-CoA dehydrogenase deficiency, 26: *CPT II/CACT* carnitine palmitoyltransferase II/carnitine-acylcarnitine translocase deficiency, 27: *LCHAD/TFP* long-chain L-3-hydroxyacyl-CoA dehydrogenase/trifunctional protein deficiency, 28: *MAD* multiple acyl-CoA dehydrogenase deficiency.

commonly used ratios include C3/C2, which is elevated in propionic and methylmalonic acidemia and related disorders; C8/C10, which is elevated in MCAD deficiency; and C14:1/C12:1, which is elevated in very long–chain acyl-CoA dehydrogenase (VLCAD) deficiency, although mild elevations of this ratio may also be observed in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and trifunctional protein (TFP) deficiencies, and in ketosis. Informative profiles may not always be detected in disorders where the accumulation of diagnostic species is a reflection of the residual activity of the defective enzyme, the dietary load of carnitine and precursors, and the anabolic or catabolic status of the patient. Modest abnormalities should be correlated to the patient's carnitine levels as a status of carnitine insufficiency could affect the outcome of this analysis. It is important to correlate ACP results with other laboratory findings, such as the urine organic acid profile and routine chemistries, and the demographic and clinical status of the patient. For instance, in premature infants, elevations of short and medium-chain dicarboxylic acid species may be observed, presumably secondary to renal immaturity. In adults with late-onset CPT II or VLCAD deficiency that presents as a myopathic phenotype, elevations of long-chain species can be subtle.

Several drugs may induce or mimic abnormal levels of one or more known acylcarnitine species;[55,56] these include pivalic acid (a C5 isomer), valproic acid (a C8 isomer), cefotaxime (a C16:1-OH isobar by the butyl-ester method). Other exogenous compounds could result in the appearance of atypical species, some of them at m/z values in close proximity to or even overlapping acylcarnitines of diagnostic significance. Examples include 2-ethylhexanoic acid from plasticizers used in tubing used for extracorporeal membrane oxygenation and other procedures,[57] and intravenous fluids containing dextrose.[58] Dietary artifacts are also possible, usually related to food intake enriched with fatty acids (MCT oil, ketogenic diet).[59]

Reporting

Important elements to include in patient reports are appropriate patient and specimen information, analyte values reported against reference limits, and interpretive comments. As discussed above, interpretations should integrate other lab findings (e.g., organic acids, free and total carnitine, and/or amino acids), and take into consideration the clinical and dietary history, when applicable and available. When abnormal results are detected, the interpretation should include an overview of the results and their significance, quantitative results with age-appropriate reference ranges, a correlation to available clinical information, elements of differential diagnosis, recommendations for additional biochemical testing and in vitro confirmatory studies (e.g., enzymatic analysis, molecular analysis), and a phone number to reach the reporting laboratory with additional questions.

SUPPLEMENTARY INFORMATION

The online version of this article (https://doi.org/10.1038/s41436-020-00990-1) contains supplementary material, which is available to authorized users.

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DISCLOSURE

All authors (M.J.M., D.O., K.C-O., and S.Y.) work for clinical laboratories that offer acylcarnitine analysis as a clinical service.

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