

For a general overview of these Standards and Guidelines, including purpose and disclaimer, see Section A

F CLINICAL BIOCHEMICAL GENETICS

F1 **General**

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 prenatal diagnosis, 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 (ABMG)-certified clinical biochemical geneticist, 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. Detailed specimen collection and handling instructions are given below for selected categories of testing.

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 C3](#).

F6 **Quality Control/Assurance/Improvement**

See [Section C4](#)

F7 **General Considerations**

F7.1 Analytical Methods: 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: 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 in cases of prenatal diagnosis or 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 so as to permit consideration of, and plans for, alternative testing.
- F7.3 Method Validation: 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 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.
- F7.4 Reference Ranges: 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 outside review.
- F7.5 Quantitative Amino Acid Analysis (*Updated December 2006*)
- F7.5.1 Introduction: Amino acid analysis is performed for the biochemical screening, diagnosis and monitoring of patients with inborn errors of amino acid metabolism or transport. Amino acid abnormalities are also seen in a wide variety of other metabolic disorders including organic acidemias and disorders of ureagenesis. The clinical significance of amino acid profiling stems from their role in literally all metabolic and cellular functions. Amino acids serve as protein building blocks, as metabolic intermediates, and as substrates for energy production. By definition, amino acids contain an amino group and a carboxyl group, and may contain another functional group (e.g., sulfhydryl, hydroxyl, or secondary amino- or carboxyl-group). Amino acids are typically measured in the following broad clinical settings:

Evaluation of symptomatic patients
Evaluation of previously diagnosed patients for purposes of treatment monitoring
Evaluation of asymptomatic or presymptomatic (at-risk) family members of known patients
Follow-up testing following an abnormal newborn screening result

The most common analytic method to evaluate amino acid concentrations is based on cation-exchange chromatography using post-column derivatization with ninhydrin and spectrophotometric detection. Other high-performance liquid chromatography (HPLC) methods also exist. Amino acids can also be analyzed using tandem mass spectrometry (MS/MS). This method is well suited for newborn screening, but because it does not resolve isomers or compounds of identical molecular weight (e.g., leucine and isoleucine) it is generally considered inadequate for the general screening of symptomatic patients unless chromatographic separation of amino acids prior to MS/MS analysis can be achieved. Finally, qualitative screening methods, including thin-layer chromatography and paper chromatography, do not allow for the positive identification of all amino acids

of clinical interest and can lead to diagnostic errors. Laboratories that use such methods should make the limitations of the methodology clear and indicate the appropriate follow-up testing in the patient report (see section 7.5.6).

F7.5.2 Background

F7.5.2.1 **General Description of Disease (Amino Acidemias and Acidurias):** Amino acid defects are clinically and biochemically heterogeneous. They are characterized biochemically by the accumulation of pathological amounts of normal metabolites, or by the accumulation of metabolites that are not present under physiological conditions but are produced from the activation of alternative pathways in response to the loss of function of a specific gene product (enzyme). Primary disorders of amino acids include defects in the catabolic pathways of aromatic amino acids (e.g., phenylketonuria, tyrosinemia), sulfur-containing amino acids (classic homocystinuria), branched-chain amino acids (maple syrup urine disease), urea cycle intermediates (including ornithine transcarbamoylase deficiency, citrullinemia, and argininosuccinic aciduria) and others. The broad category of amino acid disorders also includes transport defects such as lysinuric protein intolerance, citrullinemia type II and Hartnup syndrome. Clinical findings are diverse and often disease-specific, and include overwhelming neonatal illness (e.g., hyperammonemia, hypoglycemia, metabolic acidosis or respiratory alkalosis), neurologic abnormalities (seizures, hypotonia, lethargy, coma, developmental delay or unexplained mental retardation), and failure to thrive. Depending on the particular disorder, other abnormalities of the liver, eyes, heart, skin, muscle, kidneys, or other organs, can also be seen.

F7.5.2.2 **Prevalence:** The birth of a child with an amino acid disorder is not rare. The estimated incidence of phenylketonuria (PKU) alone is 1:12,000, with the combined incidence for all amino acidopathies estimated at 1:6,000. This estimate does not include other inborn errors of metabolism (e.g., organic acidemias and congenital lactic acidemias) that may require amino acid analysis for diagnosis and monitoring of patient treatment.

F7.5.2.3 **Mode of Inheritance:** The majority of disorders of amino acid metabolism or transport are inherited as autosomal recessive traits, while rare exceptions are X-linked (e.g., ornithine transcarbamoylase deficiency) or result from dominant-negative mutations.

F7.5.3 Preanalytical Requirements

F7.5.3.1 **Specimen Requirements:** Plasma is the preferred specimen for the evaluation of most primary disorders of amino acid metabolism. Serum can also be used, but the added time for clotting can lead to artifacts from deamination, conversion of arginine to ornithine by red blood cell arginase, and release of oligopeptides. Testing can also be performed on amino acids eluted from dried blood spots (DBS). Urine is the appropriate specimen for the identification of generalized or specific disorders of renal amino acid transport (e.g., renal Fanconi syndrome, cystinuria), but is less reliable in first-tier investigations for primary amino acid disorders due to renal influences and interference from medications. When urine is analyzed, it should be collected without preservatives or fecal contamination. Cerebral spinal fluid (CSF) is useful in the diagnosis of several disorders, most notably glycine encephalopathy (nonketotic hyperglycinemia). In this case, CSF should be collected and analyzed along with a simultaneously collected plasma sample, so that the ratio of CSF glycine to plasma glycine can be calculated.

F7.5.3.2 **Conditions of Sample Shipping, Handling and Storage:** Plasma or serum samples should be centrifuged as soon as possible after collection in order to reduce the influence of other blood constituents on the free amino acids. Following centrifugation, samples must be kept frozen (-20°C) until the time of analysis. CSF and urine specimens must be frozen as soon as possible after collection and stored frozen prior to analysis. Samples being sent to the laboratory from outside referral sources should be shipped on dry ice via overnight courier.

F7.5.3.3 **Preanalytical Variables:** Factors of sample collection and handling associated with artifactual results include excessive hemolysis, leading to elevations of certain amino acids (e.g., taurine, glutamate and aspartate), and prolonged storage of whole blood at room temperature, leading to losses of cysteine and homocysteine due to protein binding. Interference from co-eluting, ninhydrin-positive compounds occurs with certain antibiotics

(e.g., ampicillin), contrast dyes and other medications. Amino acid levels can also be influenced by anticonvulsants (e.g., hyperglycinemia with valproate use), nutritional status, clinical status (including fever, infections, and liver or kidney dysfunction) and bacterial contamination. These factors may affect results and should be taken into account in the interpretation of results.

F7.5.3.4 Clinical Indications for Testing: Clinical presentations of different disorders of amino acid metabolism are variable and often non-specific. 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, including:

- Lethargy, coma, seizures, or vomiting in a neonate
- Hyperammonemia
- Failure to thrive
- Electrolyte abnormalities, including metabolic acidosis or respiratory alkalosis
- Lactic acidemia
- Unexplained mental retardation or developmental delay
- Abnormal amino acid results by newborn screening
- Previous sibling with similar clinical presentation
- Clinical presentation suggestive of a specific amino acid disorder

The laboratory should be made aware of the clinical indication for testing, so that results can be most appropriately interpreted and additional testing can be recommended when indicated. 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 as part of a comprehensive metabolic workup. Ideally, these tests should all be performed by the same laboratory, and results integrated into an overall interpretation when appropriate.

F7.5.4 Method Validation

F7.5.4.1 Calibration and Quantitation: Quantitation of amino acid concentrations must be performed in relation to a known reference or calibration standard. 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 mixed with the commercial standards to form a complete set, if desired. Performance of calibration material should be verified either with weighed standards, or with amino acid calibrators obtained from an independent commercial source.

Instrument calibration should be verified, and/or the instrument should be recalibrated, at regular intervals established by the laboratory and as required by CLIA. This should also occur with the introduction of new reagent lots, or following any major service to the instrument or its components.

Quantitation should be performed using at least one internal standard. The choice of standard varies from laboratory to laboratory, but it should elute at a unique position in the chromatogram and not interfere with the analysis of other compounds. Quantitative results should be calculated using an internal standard method that adjusts for the amount of internal standard in the patient sample relative to that in the calibration mixture (Slocum, 1991).

The laboratory should establish protocols to determine and periodically validate the linear range, analytical measurement range and lower limit of detection for all reported amino acids. Procedures should be in place for reporting any amino acid values that fall outside of these performance limits.

F7.5.4.2 Reference Ranges: Reference ranges for all reported amino acids should be established and periodically validated. When literature-based ranges are used, they must also be periodically verified. Because amino acid levels vary significantly with age, amino acid reference ranges must be age-specific

F7.5.4.3 Testing Personnel: Appropriate training procedures and ongoing competency requirements for laboratory

personnel performing amino acid analysis must be established and documented.

F7.5.5 Analysis of Amino Acids

F7.5.5.1 Specimen Preparation: In order to analyze free amino acids in physiologic specimens, deproteinization of the sample is necessary. This is commonly achieved by acid precipitation of proteins using sulfosalicylic acid (SSA) or trichloroacetic acid (TCA), followed by centrifugation and/or filtration. Alternatively, low molecular weight cut-off filtration can also be used. Supernatants or filtrates are then diluted with buffer of appropriate pH prior to analysis. Sample preparation should also include the addition of at least one internal standard to control for run-to-run variations in sample injections. For urine, creatinine concentration is determined prior to sample preparation, and amino acid concentrations are compared to a fixed creatinine amount (e.g., $\mu\text{mol/g}$ creatinine). Urine amino acid levels can also be reported from a 24-hour specimen collection (e.g., $\mu\text{mol}/24$ h).

F7.5.5.2 Analytical Methods: Ion-exchange chromatography is the most commonly used method of amino acid analysis. Other methods that result in the positive identification of amino acids, such as gas chromatography/mass spectrometry or tandem mass spectrometry, may also be used. Tandem mass spectrometry typically is used to measure specific amino acids for newborn screening, and to monitor specific analytes (e.g., phenylalanine) in treated patients with specific disorders. A number of different instrument configurations are commercially available for amino acid analysis; these instruments have published methodologies that must be validated in the individual laboratory.

Ion-exchange chromatography requires derivatization of compounds 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 is the method currently used by the vast majority of laboratories. In this method, compounds are simultaneously detected at wavelengths of 570 nm and at 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 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 quantitating roughly 40 amino acid peaks in a typical patient run (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 must be visually inspected for run performance, as well as for atypical peaks not normally included in peak identification tables including Δ -1-pyrroline-5-carboxylate, homocitrulline, argininosuccinic acid and alloisoleucine. 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 440 nm to that at 570 nm. The 440/570 ratio can be established for all standard compounds, and compared to patient values to confirm peaks where co-eluting interferents are suspected. In determining these ratios, peak baselines must be carefully examined such that potential artifacts from baseline fluctuations are eliminated. Quantitation should be based on the recovery of the internal standard in each specimen compared to the recovery of the internal standard in the calibration mixture to which that specimen is being evaluated (see F7.5.5.3).

F7.5.5.3 Quality Control: Quality control (QC) material should be evaluated at two different concentration levels for all reported amino acids, and should be derived from a different source than the solution used for instrument calibration. QC material should be analyzed along with each patient batch. Thresholds for acceptance or rejection of a QC run, 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. Thresholds for appropriate internal standard response should be established, and internal standard responses for each specimen should be monitored as

another level of quality control. The use of Westgard rules for clinical specimen analysis further controls the parameters for quality patient diagnosis and reporting (Westgard and Klee, 1999).

F7.5.5.4 Proficiency Testing: An ongoing proficiency testing (PT) program for amino acid analysis must be implemented and documented. A bi-annual PT program that evaluates both analytical and interpretive/diagnostic proficiency is offered by the College of American Pathologists (CAP) and supervised by the joint CAP/ACMG Genetic Biochemical and Molecular Genetic Resource Committee. An excellent program 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>), in which analytic proficiency is monitored through the regular testing and reporting of synthetic amino acid mixtures

F7.5.6 Test Interpretation and Reporting

F7.5.6.1 Interpretation: All results should be interpreted by an ABMG-certified clinical biochemical geneticist. Because normal amino acid concentrations vary with age, quantitative results should be compared to a properly defined 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 abnormal levels alone. Amino acid abnormalities or overall profiles should also be considered in the context of clinical findings and/or additional test results.

F7.5.6.2 Reporting: Patient and specimen information, as contained in Sections C2.4, C2.4.1, and C2.4.2 of these *Guidelines*, must be included on each report. Identification of all relevant amino acids must be listed and the quantity may be listed. When abnormal results are detected, a detailed interpretation should include an overview of the results and their significance, a correlation to available clinical information, elements of a differential diagnosis, recommendations for additional testing, including *in vitro* enzyme assay (blood cells, cultured cells, tissue biopsy) or molecular analysis as appropriate and contact information for the reporting laboratory.

Laboratories should be encouraged to develop methods by which quantitative data can be electronically transferred from the analytical instrument into a laboratory information system directly interfaced with the patient report, thereby eliminating the occurrence of data entry errors.

F7.6 Qualitative Amino Acid Analysis (*Updated December 2006*)

Amino acid analysis by thin layer chromatography (TLC), paper chromatography, or other less discriminating separation methods are suitable only for the detection of gross abnormalities, but may miss cases with more subtle changes. For this reason, these methods are not appropriate for the purpose of evaluating acutely-presenting and/or otherwise high-risk patients. Qualitative amino acid analysis must reliably detect conditions in which there are either gross or modest elevations of specific amino acids in blood and/or urine.

F7.6.1 Reports should indicate the qualitative method used. If TLC or paper chromatography is employed, a statement should be added indicating that these methods are suitable only for detection of gross abnormalities, and that quantitative analysis of plasma, urine, or CSF using a more sensitive method is recommended for the diagnosis and monitoring of treatment of conditions characterized by abnormal amino acids in blood and/or urine.

F7.7 Organic Acid Analysis (*Updated December 2006*)

F7.7.1 Introduction: Organic acids are analyzed in patients suspected of having a broad range of metabolic disorders including inborn errors of organic acid, amino acid, fatty acid, carbohydrate, and purine and pyrimidine metabolism. Organic acids are non-amino containing water-soluble compounds with one or more carboxyl groups as well as other functional groups (-keto, -hydroxyl), and are metabolic intermediates of a large number of biochemical pathways. Organic acid analysis is generally performed to detect abnormal accumulations of one or more compounds, resulting from an enzymatic deficiency at a particular metabolic step.

Organic acids are efficiently excreted by the kidneys, and the most appropriate sample for analysis is therefore urine. Organic acids are extracted from urine using organic solvents or ion-exchange methods, and measured by gas chromatography/mass spectrometry (GC/MS). Both qualitative and quantitative analyses are suitable for the detection of patients with organic acidemias, although quantitative testing is more useful in the follow up of previously diagnosed patients. Specific organic acids (e.g., methylmalonic acid, glutaric acid, succinylacetone) can be monitored in blood or urine from known patients using other techniques such as tandem mass spectrometry (MS/MS) and stable isotope dilution, although these targeted analyses but should not replace a more comprehensive organic acid screen during an initial workup.

7.7.2 Background

F7.7.2.1 **General Description of Disease (Organic Acidurias):** Organic acidurias (also known as organic acidemias) are a biochemically heterogeneous group of inborn errors of metabolism. They are characterized biochemically by the accumulation of metabolites which are not present under physiological conditions, produced from the activation of alternative pathways in response to a specific metabolic defect, or by the accumulation of pathological amounts of normal metabolites. Disorders associated with abnormal organic acid patterns include inborn errors of branched-chain amino acids (e.g., isovaleric acidemia, methylmalonic acidemia, maple syrup urine disease), fatty acid oxidation disorders (medium-chain acyl-CoA dehydrogenase deficiency, short-chain acyl-CoA dehydrogenase deficiency), disorders of carbohydrate metabolism (primary lactic acidosis, fructose-1,6-bisphosphatase deficiency), and others (glutaric acidemia type I, ethylmalonic encephalopathy, Canavan disease, glutathione synthetase deficiency). Clinical findings are diverse and often nonspecific, and include overwhelming neonatal illness (e.g., hyperammonemia, hypoglycemia, metabolic acidosis), neurologic abnormalities (seizures, hypotonia, lethargy, coma, developmental delay or unexplained mental retardation), and failure to thrive. Depending on the particular disorder, other abnormalities including unusual odors, macrocephaly, or liver failure may also be seen.

F7.7.2.2 **Prevalence:** The incidence of individual inborn errors of organic acid metabolism varies from 1 in 10,000 to >1 in 1,000,000 live births. Collectively, their incidences approximate 1 in 3,000 live births. 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 and monitoring also require organic acid analysis. All possible disease entities included, the incidence of conditions where informative organic acid profiles could be detected in urine is likely to approach 1 in 1,000 live births. It is appreciated that as a group, these defects are under-diagnosed.

F7.7.2.3 **Mode of Inheritance:** The majority of organic acidemias are inherited as autosomal recessive traits; a few are X-linked.

F7.7.3 Preanalytical Requirements

F7.7.3.1 **Specimen Requirements:** Organic acids are analyzed in urine. In rare instances and/or for follow-up or prenatal diagnostic purposes, quantitation of specific metabolites in other physiological fluid specimens is appropriate. Urine specimens require creatinine measurement for standardization of sample preparation (i.e., extraction of a fixed creatinine equivalent) and for determination of relative concentration.

F7.7.3.2 **Conditions of Sample Shipping, Handling and Storage:** Urine is collected from either a random void or ideally a first-morning specimen, in a clean container without preservatives. Samples should be frozen as soon as possible after collection and stored frozen prior to analysis. Samples being sent to the laboratory from outside referral sources should be shipped on dry ice via overnight courier.

In specific situations such as the monitoring of patients with a known diagnosis (e.g., methylmalonic acidemia), targeted analytes are analyzed in plasma collected from heparanized whole blood. Separate plasma by centrifugation and store frozen prior to analysis.

F7.7.3.3 **Preanalytical Variables:** A number of dietary factors may influence organic acid results, including total

parenteral nutrition (leading to elevated n-acetylytyrosine), medium-chain triglyceride supplementation (dicarboxylic aciduria) and ketogenic diets. Similarly, medications such as valproate, aspirin, levetiracetam and many others can be the source of a wide variety of artifactual peaks. These variables should not hinder analysis as long as the laboratory considers the role of diet and medications in the interpretation of results. Compounds of bacterial origin can be enhanced with prolonged storage of the sample at room temperature.

Ideally, specimens should be collected during the time of acute illness as abnormal metabolite levels often decrease, sometimes to near-normal concentrations, when the patient is well.

F7.7.3.4 Clinical Indications for Testing: Urine organic acid analysis should be undertaken in patients suspected of having a wide range of disorders of amino acid, organic acid, fatty acid, and carbohydrate metabolism. Clinical symptoms are variable and often non-specific, and can occur for the first time at any age from the neonatal period to adulthood. Common clinical indications for organic acid analysis include:

Overwhelming neonatal illness, including altered mental status, tachypnea, vomiting, metabolic acidosis (including lactic acidosis and ketosis), hypoglycemia and hyperammonemia

History of episodic metabolic decompensation

Neurological abnormalities presenting at any age, including seizures, hypotonia, lethargy, coma, developmental delay or unexplained mental retardation

Clinical findings associated with particular disorders, such as macrocephaly in Canavan disease and glutaric acidemia type I, and neonatal liver dysfunction in tyrosinemia type I

Failure to thrive

History of a previously affected sibling

Abnormal newborn screening results suggestive of an inborn error of fatty acid or organic acid metabolism

The laboratory should be made aware of the clinical indication for testing, as well as medication and diet history. Depending on the clinical situation, organic acid analysis is often ordered together with plasma amino acids, acylcarnitine profile and carnitine (free and total) as part of a comprehensive metabolic workup. Ideally, these tests should all be performed by the same laboratory, and results integrated into an overall interpretation when appropriate.

F7.7.4 Method Validation

F7.7.4.1 Calibration and Quantitation: The laboratory should establish protocols to determine and periodically validate the linear range, analytical measurement range and lower limit of detection for all reported compounds. Procedures should be in place for reporting any values that fall outside of these performance limits. Initial validation should also include analysis of the precision and recoveries for each analyte.

Quantitation of organic acid concentrations must be performed in relation to a known reference or calibration standard whenever possible. Quantitation should be based on the performance and regular updating of calibration curves covering the normal range and expected pathological values. To generate or update calibration curves, a mixture of as many known standard compounds as possible should be carried through the entire specimen process. The use of solvent standards (i.e., standards mixed directly in organic solvent and not carried through the full extraction process) is not appropriate as these do not adequately control for the different extraction efficiencies of the various acids.

Instrument calibration should be verified, and/or the instrument should be recalibrated, at regular intervals established by the laboratory and as required by CLIA. This should also occur following any major service to the instrument or its components.

Quantitation should be performed using at least one internal standard. The choice of standard varies from laboratory to laboratory, but it should be uniquely identifiable and not interfere with the analysis of other

compounds. Quantitative results should be calculated using an internal standard method that adjusts for the amount of internal standard in the patient sample relative to that in the calibration mixture.

When appropriate for measurement of specific analytes such as n-acetylaspartic acid in amniotic fluid or methylmalonic acid in plasma, stable-isotope dilution, selected ion monitoring (SIM) gas chromatography-mass spectrometry should be used for more accurate analyte measurement.

Quantitation without the use of a reference standard should be discouraged. However, for compounds where quantitative data is desired but no reference standard is available, assessment of a peak response relative to an external standard added to the sample, such as tetracosane, may be used. In these cases, this should be clearly indicated on the patient report.

F7.7.4.2 Reference Ranges: Reference ranges for all reported compounds should be established and periodically validated. When literature-based ranges are used, they must also be periodically verified. Age-specific ranges should be determined for compounds where concentration is determined to vary with age.

F7.7.4.3 Testing Personnel: Appropriate training procedures and ongoing competency requirements for laboratory personnel performing organic acid analysis must be established and documented.

F7.7.5 Analysis of Organic Acids

F7.7.5.1 Extraction of organic acids: In a typical analysis, the creatinine concentration of a urine sample is determined, and the urine is diluted with water to a fixed creatinine concentration. One or more internal standards are added to the specimen, followed by the addition of an acid such as HCl. Extraction of organic acids from acidified urine is performed using organic solvents or ion-exchange methods. An oximation step with the addition of hydroxylamine can be performed to stabilize α -keto acids such as pyruvate, succinylacetone, and certain intermediates of branched-chain amino acid metabolism. Oximation may be performed routinely, or as a reflex step under specific circumstances such as the clinical suspicion of maple syrup urine disease or tyrosinemia type I (See F9 for Chalmers and Lawson, 1982). Organic extracts are then evaporated to dryness under nitrogen, and derivatized as described below.

F7.7.5.2 Derivatization: Trimethylsilyl (TMS) derivatives of organic acids in dried urine extracts are formed by addition of N_5O_5 -bis-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/TCMS) or similar commercial reagents. In a typical derivatization procedure, dried extracts are mixed with BSTFA/TCMS and incubated at 80°C for 20-30 minutes. Alkane standards of known carbon chain length may be added for the monitoring of chromatographic separation.

F7.7.5.3 Gas Chromatography/Mass Spectrometry Analysis: Several instrument configurations are commercially available that allow for the separation of compounds and their 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. Mass spectral data is typically collected in scan mode, but methods using selected ion monitoring (SIM) can also be developed for the targeted analysis of specific compounds.

F7.7.5.4 Chromatogram Analysis: Compound identification is critical to the diagnosis of genetic disorders associated with abnormal organic acid excretion. Identification of organic acids relies primarily on evaluation of their mass spectra. The use of a computer library of mass spectra for comparison and visualization of the printed spectra is vital for definitive identification and interpretation of each patient specimen. A limited number of commercial libraries containing mass spectra of TMS-derivatives of physiologic and drug-related compounds are available for the identification of compounds. Laboratories are encouraged to develop in-house libraries on their own equipment, using known organic acid standards and/or patient-derived peaks (once positive identification of a compound has been established). The methods and criteria by which peaks are identified must be documented in the laboratory.

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 structurally similar compounds. Particular attention should be paid to regions of the chromatogram in which complete or partial overlap of clinically relevant peaks could hinder interpretation (e.g., 2-hydroxyisocaproic acid, 2-hydroxybutyric acid and urea; 2-hydroxyglutaric acid and 3-hydroxyglutaric acid; hexanoylglycine and p-hydroxyphenylacetic acid; orotic acid and cis-aconitic acid; sebacic acid and p-hydroxyphenyllactic acid). The examination of extracted ion chromatograms and/or subtracted spectra is often useful clarifying the composition of apparently complex peaks. Care must also be taken to examine each chromatogram for the presence of peaks which, when present even in small amounts, could indicate a clinically significant abnormality (e.g., hexanoylglycine, succinylacetone, orotic acid).

- F7.7.5.5 **Quality Control:** The mass spectrometer should be tuned prior to each batch of patient samples analyzed, as described by the instrument manufacturer. 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. Thresholds for acceptance or rejection of a QC run, 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 controls the parameters for quality patient diagnosis and reporting (Westgard and Klee, 1999).
- F7.7.5.6 **Proficiency Testing:** An ongoing proficiency testing (PT) program for organic acid analysis must be implemented and documented. A bi-annual PT program that evaluates both analytical and interpretive/diagnostic proficiency is offered by the College of American Pathologists (CAP) and supervised by the joint CAP/ACMG Genetic Biochemical and Molecular Genetic Resource Committee. 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>).
- F7.7.6 **Test Interpretation and Reporting**
- F7.7.6.1 **Interpretation:** Clinically meaningful interpretation of organic acid results should be based on the overall pattern of metabolites present in abnormal quantities, rather than on individual abnormal values. The diagnostic specificity of organic acid analysis under acute vs. asymptomatic conditions may vary considerably. Informative profiles may not always be detected in disorders where 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. In some cases, methods of higher specificity and sensitivity based on the use of stable-isotope labeled internal standards, selected ion monitoring and chemical ionization can effectively overcome the limitations of standard organic acid analysis for the investigation of non-acutely ill patients. An abnormal organic acid analysis is not sufficient to conclusively establish a diagnosis of a particular disorder. Confirmation by an independent method is recommended whenever practical, typically by *in vitro* enzyme assay (blood cells, cultured cells, tissue biopsy) or molecular analysis.
- F7.7.6.2 **Reporting:** Patient reports must contain appropriate patient and specimen information as contained in Section C 2.4, 2.4.1, and 2.4.2 of these guidelines. Identification of all relevant compounds must be provided on the report, and quantity may be listed (as determined). Quantitative reports must include comparisons to appropriate reference ranges (see F7.7.4.3). When no clinically significant abnormalities are detected, an organic acid analysis could be reported and interpreted in qualitative terms only. When abnormal results are detected, a detailed interpretation should include an overview of the results and their potential significance, a correlation to available clinical information, elements of a differential diagnosis, recommendations for additional biochemical testing and/or other confirmatory studies (e.g., enzyme assay, molecular analysis), and contact information for the reporting laboratory.

- F7.8 Methods used to screen for mucopolysaccharide (MPS) (glycosaminoglycan) analysis must reliably detect excessive urine MPS (glycosaminoglycan) excretion.
- F7.8.1 Qualitative methods for MPS (glycosaminoglycan) analysis must distinguish between increases in heparan sulfate, keratan sulfate, dermatan sulfate and chondroitin sulfates.
- F7.8.2 Diagnoses based on urine mucopolysaccharides should be confirmed by enzyme assays on appropriate tissue(s).
- F7.9 Methods used to analyze very long chain fatty acids should reliably detect adrenoleukodystrophy, adrenomyeloneuropathy and other disorders of peroxisomes.
- F7.10 Diagnostic enzyme assays must reliably distinguish between patients with specific diseases and control subjects. When assays are used to distinguish between heterozygous carriers and noncarriers, data must be available to justify cut-off points. When appropriate, methods should be available to distinguish between enzyme deficiency and pseudodeficiency.

F8 **Reports**

- F8.1 See [E8](#).
- F8.2 **The report must include an interpretation of the results.** When appropriate, this may extend to discussion of the significance of the results and recommendations for further diagnostic procedures. The terms used must be such that the implications of the results are clear to a non-geneticist professional.

F9 **References**

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