Chapter 11 Recommendations for the measurement of urine organic acids

Mike Bennett

Background:

The measurement of urine organic acids is an important component of the investigation of inherited metabolic disease. If utilized appropriately, this one assay is capable of identifying abnormal metabolic profiles that occur in approximately 150 distinct genetic disorders. A significant number of metabolic diseases can only be identified using this procedure. Early diagnosis before repeated episodes of metabolic decompensation occur is likely to result in better patient outcome for a number of disorders. For other, presently untreatable conditions early diagnosis enables genetic counseling to be provided before multiple affected siblings are delivered.

We recommend that urine organic acid analysis using the procedures identified below be made readily available to all patients (children and adults) in whom a metabolic disease is suspected.

Pre analytical concerns:

Time of sample collection: Many disorders of organic acid metabolism present with abnormal metabolite profiles at all stages of clinical severity. These disorders should be readily identifiable in affected patients irrespective of time of sample collection. However, some disorders of energy metabolism only present with abnormal organic acid profiles during periods of metabolic decompensation. Samples collected after the acute illness may not demonstrate significant abnormalities for these patients and the diagnosis may be missed. Frequently, samples of urine are collected in the emergency room for infection and toxicology investigations from patients with metabolic decompensation.

Concurrent therapies: Certain therapeutic modalities can produce urine organic acid profiles that may mask underlying metabolic disease. Examples of therapeutic interference include seizure treatment with valproic acid and caloric supplementation with medium-chain triglycerides. If an acceptable infectious or toxicological etiology for the acute presentation is identified metabolic studies including urine organic acid analysis may not be necessary. **Therefore, we recommend that whenever possible urine for organic acid analysis should be collected from patients at the same time.**

Sample storage: Urine organic acids are stable for long periods of time (several years) if stored at minus 70°C and for several months at minus 20°C.

We recommend that samples be stored at minus 20°C prior to analysis unless analysis is immediate when freezing is not necessary.

Analytical concerns: The only acceptable method of analysis for urine organic acids is by capillary gas chromatography-mass spectrometry.

Sample preparation: A volume of thawed, thoroughly mixed urine equivalent to a constant amount of creatinine is aliquoted for extraction. This is typically the equivalent volume containing around 1-2 μmol (0.1-0.2 mg) of creatinine. For most samples this results in between 0.5-3.0mL of urine to be extracted.

For extremes of concentration we recommend that the minimum volume to be extracted is 0.5mL and the maximum is 3.0mL.
To this volume of urine a fixed volume of internal standard is added. It is also acceptable to aliquot a fixed amount of urine and add to it a variable amount of internal standard to achieve the same ratio of the two components. The internal standard chosen should not be a metabolite that might be detected in normal or pathological urine, nor should it co chromatograph with significant metabolites. Typical internal standards include heptadecanoic acid, 2-phenylbuyric acid, and dimethylmalonic acid. The final concentration of internal standard should be chosen to generate a peak on the total ion chromatogram that is similar in height to the highest detected organic acids.

Oximation: The addition of an oximating regent such as ethoxylamine hydrochloride serves to preserve ketoacids that are present in urine. Important ketoacids include the 2-ketoisocaproic, 2-keto-3-methylvaleric and 2-ketoisovaleric acids present in maple syrup urine disease. In the absence of oximation, a significant proportion of ketoacids is converted to the corresponding 2-hydroxyacid. 2-hydroxyisovaleric acid is an important indicator of maple syrup urine disease, which is readily identified in non-oximated urine samples.

Method of sample extraction: Urine plus internal standard should be acidified to pH 1-2 and extracted into an equal volume of an organic solvent. Ethyl acetate extraction is most commonly employed. The sample may be extracted up to three times for greatest efficiency. The addition of saturating amounts of sodium chloride prior to the extraction process may reduce the extraction efficiency of urea, which can interfere with the identification of other organic acids. Solid phase extraction using silicic acid mini columns has also been employed successfully for sample extraction.
We recommend that information regarding all concurrent therapies be provided with the patient order for urine organic acid analysis.

Method of sample derivatization: Most data bases for organic acid spectra are based upon spectra generated from trimethylsilyl (TMS)-derivatives.

Gas Chromatography-mass spectrometry:

We recommend that TMS derivatives of extracted urinary organic acids be prepared for GC-MS analysis.

Instrument tuning: It is critical for mass assignment to ensure that the analyzer is tuned on a regular basis. Most bench top GC-MS systems have an auto tune capability.

We recommend that an auto tune is performed daily and that analysis only proceeds if the tune falls within the specifications provided by the instrument manufacturer.

Choice of column: A variety of capillary GC columns are used for separation of organic acids with equivalent efficiency of separation. Columns are typically 25-30meters in length, 0.2-0.5mm internal diameter and coated with a 0.1-1.0 μm layer of an OV1, OV5 or OV17 comparable liquid coating. Each manufacturer has a proprietary brand. Overloading the column can cause difficulty in peak identification.
We recommend that sample injection onto the column is in the split mode with a 1-2 μL injection and a split ratio of at least 1:15 to prevent column overload.

Running conditions: A temperature ramp is important to elute organic acids with low volatility. Typical and recommended GC temperatures are Injection port 240-250°C, Initial oven temperature 70-100°C, temperature ramp 3-8°C per minute, final oven temperature 270-295°C.
We recommend that the temperature of the mass spectrometer interface be equal to or greater than the highest column temperature The initial oven temperature, rate of temperature ramp and highest temperature will determine the total run time, which is typically between 30-60min.

Data acquisition: Data acquisition in the mass spectrometer should not begin until the solvent front has returned to the baseline. Data should then be acquired in scan mode with a full scale scan every 0.5seconds. Depending upon the mass range of the mass spectrometer we recommend that the range of ions scanned be from m/z 50 to m/z 500-650. This data should be presented as a total ion chromatogram

Peak identification: Peaks should be identified both by retention time and by spectral match in an appropriate library of TMS-derivative spectra. Spectral match should be greater than 80% in the presence of a known co -chromatographing peak to provide positive identification. Several commercial libraries are available for purchase but we recommend that centers measuring urinary organic acids also build their own in-house library based upon experience and availability of samples from patients with organic acidurias.

Calibration: The analytical system should be calibrated using a solution of multiple organic acids of known concentration that elute at various points during the chromatographic run. We recommend that 10-15 analytes be used in this calibrator mix and that they consist of significant compounds of diagnostic interest.

Data Interpretation:

Quantitative versus qualitative data analysis: Some laboratories provide extensive quantitative reports whilst others generate a qualitative interpretation. There is no consensus as to which format is most favorable.
For quantitative reporting, most analytes are quantified as a unique ion ratio for that compound to an ion specific to the internal standard.
We recommend that for this purpose standard curves encompassing the reportable range for an analyte be generated at frequent intervals.

For concentrations of organic acids less than 100mmol/mol creatinine:
We recommend that quantitation is by isotope ratio mass spectrometry utilizing stable isotope labeled internal standards.
Data collection for this purpose should be in the selected ion mode using at least two ions for both internal standard and native compound. Experience in interpreting both quantitative and qualitative reports is essential. The rarity of some organic acidurias means that very few laboratories have a great depth of experience.
We recommend that laboratories measuring urine organic acids participate in CAP activities and in addition, also exchange abnormal samples to extend their experience.

Identification of minor pathological components: We recognize that there are some urine organic acid components that have critical diagnostic value but are only present in small amounts, often hidden in the background noise. These components may be identified in a total

ion chromatogram if selected ions are investigated. Compounds that should be sought in all organic acid chromatograms include:

1. n-Hexanoylglycine, an important marker of medium-chain acyl CoA dehydrogenase deficiency.

Ethylmalonate, a marker for multiple disorders which frequently co-chromatographs with phosphate a quantitatively more significant compound.

Orotic acid, a marker for a number of urea cycle disorders, which frequently co-chromatographs with aconitate.

4-Hydroxybutyrate (gamma hydroxybutyrate) a marker for succinic semialdehyde dehydrogenase deficiency.

3-Hydroxyglutarate a marker for glutaric acidemia type 1.

Acknowledgments: The following have shared their procedures to help create this document. Donald Chace PhD, *Neo Gen Screening, Bridgeville PA.* David Millington PhD, *Duke University Medical Center, Raleigh NC*. Steve Goodman MD, *University of Colorado Health Science Center, Denver CO.* Rodney Pollitt PhD, *Sheffield Children's Hospital, UK.* Kevin Carpenter PhD, *New South Wales Biochemical Genetics Service, Westmead, Australia.* Mike Gibson PhD *Oregon Health Science University, Portland OR.*

Piero Rinaldo MD, PhD, Mayo Clinic, Rochester MN. Larry Sweetman PhD Baylor Research Institute, Dallas TX.

References:

Newborn Screening Manual. South Carolina Department of Health and Environmental Control. July 2000.

C-reactive Protein, interleukin-6, and Procalcitonin in the immediate postnatal period . Influence of illness severity, risk status, antenatal and perinatal complications and infection. Chiesa, C.; Pellegrinin, G; Panero, A,; Osborn, J.; Signore, F; Assumma, M; and Pacifico, L. Clin. Chem. 49:1. pp 60 – 68. 2003.

Immunoglobulin infusion for isoimmune haemolytic jaundice in neonates. Internet Review article Alcock GS, Liley H. Dr H Liley, Kevin Ryan Centre, Mater Mothers Hospital, South Brisbane , Queensland, AUSTRALIA

American Academy of Pediatrics. Newborn Screening Fact Sheet. (RE9362) 1996.

Griffith CH, Crit Care Medicine, 25:704-709 1997)

[1]Weiss IK et al. Pediatrics, 103:440-45 1999;

[2]Widness JA et al, Pediatrics, 106:497-504 2000.

6. Bihutani VK et al, Pediatrics, 106:e2-17 2000.

8. Garg SK et al, Diabetes Care , 22:1708-1714 1999

The Utility of Serial C-Reactive Proteins in the Newborn as an indicator for Neonatal Sepsis contributed by Sharon Geaghan

---

[i] Kaiser Medical Group Berkeley, Kaiser Medical Group Los Angeles, Western Clinical Laboratory, Allied Clinical Laboratory,

[ii] Why mothers die 1997-1999. The confidential enquiries into maternal deaths in the UK. London:RCOG Press. 2001.

[iii] E. Rey, J. LeLorier, E. Burgess, I. R. Lange, and L. Leduc. Report of the Canadian Hypertension Society Consensus Conference: 3. Pharmacologic treatment of hypertensive disorders in pregnancy. *CMAJ.* 157 (9):1245-1254, 1997.

[iv] Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am.J.Obstet.Gynecol.* 183 (1):S1-S22, 2000.

[v] M. A. Brown, W. M. Hague, J. Higgins, S. Lowe, L. McCowan, J. Oats, M. J. Peek, J. A. Rowan, and B. N. Walters. The detection, investigation and management of hypertension in pregnancy: executive summary. *Aust.N.Z.J.Obstet.Gynaecol.* 40 (2):133-138, 2000.

[vi] K. A. Douglas and C. W. G. Redman. Eclampsia in the United-Kingdom - Reply. *British Medical Journal* 310 (6987):1138, 1995.

[vii] S. Caritis, B. Sibai, J. Hauth, M. D. Lindheimer, M. Klebanoff, E. Thom, P. VanDorsten, M. Landon, R. Paul, M. Miodovnik, P. Meis, and G. Thurnau. Low-dose aspirin to prevent preeclampsia in women at high risk. *New England Journal of Medicine* 338 (11):701-705, 1998.

[viii] A. P. Mackay, C. J. Berg, and H. K. Atrash. Pregnancy-related mortality from preeclampsia and eclampsia. *Obstetrics and Gynecology* 97 (4):533-538, 2001

[ix] D. K. Stevenson, L. L. Wright, J. A. Lemons, W. Oh, S. B. Korones, L. A. Papile, C. R. Bauer, B. J. Stoll, J. E. Tyson, S. Shankaran, A. A. Fanaroff, E. F. Donovan, R. A. Ehrenkranz, and J. Verter. Very low birth weight outcomes of the National Institute of Child Health and Human Development Neonatal Research Network, January 1993 through December 1994.

[x] P. von Dadelszen, L. A. Magee, S. K. Lee, S. D. Stewart, C. Simone, G. Koren, K. R. Walley, and J. A. Russell. Activated protein C in normal human pregnancy and pregnancies complicated by severe preeclampsia: A therapeutic opportunity? *Critical Care Medicine* 30 (8):1883-1892, 2002.

[xi] P. J. Saudan, M. A. Brown, T. Farrell, and L. Shaw. Improved methods of assessing proteinuria in hypertensive pregnancy. *British Journal of Obstetrics and Gynaecology* 104 (10):1159-1164, 1997.

[xii] P. Saudan, M. Brown, and T. Farrell. Spot urine protein-to-creatinine ratio for assessing proteinuria in hypertensive pregnancies. *Kidney International* 51 (4):1306, 1997.

[xiii] J. Waugh, S. C. Bell, M. Kilby, P. Lambert, A. Shennan, and A. Halligan. Effect of concentration and biochemical assay on the accuracy of urine dipsticks in hypertensive pregnancies. *Hypertension in Pregnancy* 20 (2):205-217, 2001.

[xiv] Caetano M, Ornstein M, von Dadelszen P, Hannah ME, Logan AG, Gruslin A, Willan A, Magee LA. A survey of Canadian practitioners regarding the diagnosis and evaluation of the hypertensive disorders of pregnancy. *Hypertens Preg* (MS#2002-70, in press)

[xv] H. U. Irgens, L. Reisaeter, L. M. Irgens, and R. T. Lie. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. *British Medical Journal* 323 (7323):1213-1216, 2001.

[xvi] I. A. Greer. Thrombosis in pregnancy: maternal and fetal issues. *Lancet* 353 (9160):1258-1265, 1999.

[xvii] N. S. Macklon and I. A. Greer. Venous thromboembolic disease in obstetrics and gynaecology: The Scottish experience. *Scottish Medical Journal* 41 (3):83-86, 1996.

[xviii] Report on the RCOG Working Party on Prophylaxis Against Thromboembolism in Gynaecology and Obstetrics. London: Royal College of Obstetricians and Gynaecologists 1995.

[xix] M. Laffan and E. Tuddenham. Science, medicine, and the future - Assessing thrombotic risk. *British Medical Journal* 317 (7157):520-523, 1998.

[xx] P. Brill-Edwards, J. S. Ginsberg, M. Gent, J. Hirsh, R. Burrows, C. Kearon, W. Geerts, M. Kovacs, J. I. Weitz, K. S. Robinson, R. Whittom, and G. Couture. Safety of withholding heparin in pregnant women with a history of venous thromboembolism. *New England Journal of Medicine* 343 (20):1439-1444, 2000.

[xxi] R. Rai, H. Cohen, M. Dave, and L. Regan. Randomised controlled trial of aspirin and aspirin plus heparin in pregnant women with recurrent miscarriage associated with phospholipid antibodies (or antiphospholipid antibodies). *British Medical Journal* 314 (7076):253-257, 1997.