UK Metabolic Biochemistry Network Recommendations for the Analysis of Urinary Organic Acids by Gas Chromatography Mass Spectrometry

November 2018

RS Carling[1], R Barski[2], SL Hogg[3], K Witek[1] and SJ Moat[4]

1Biochemical Sciences, Viapath, Guys & St Thomas' NHSFT, London
[2] Biochemical Genetics, Leeds teaching Hospitals NHS, Leeds
[3] Biochemical Genetics, Cambridge University Hospitals NHSFT, Cambridge
[4]Medical Biochemistry, University Hospital Wales, Cardiff

Corresponding authour:Rachel.carling@viapath.co.uk

Expert review by N Dalton[5], C Langhans[6] and G Lynes[4].

[4] Biochemical Sciences, Viapath, Guys & St Thomas' NHSFT, London
[5] Well Child Laboratory, Evelina London Children's Hospital, Guys & St Thomas' NHSFT, London
[6] Centre for Metabolic Disease, University Children's Hospital, Heidelberg

Table of Contents

1. Introduction

Organic acid disorders are a heterogeneous group of disorders. They represent the largest single group of inborn errors of metabolism. Individually they are rare, but collectively the incidence is between 1 in 10,000 and 1 in 30,000 live births. Approximately 150 disorders give rise to a characteristic urine organic acid profile. In simple terms, profiles can be diagnostic either because there are excess amounts of abnormal organic acids present, for example pathognomonic metabolites such as succinylacetone in Tyrosinaemia Type 1, or because one or more normally occurring organic acids are present in excess. For example, 2-hydroxyglutarate in 2-hydroxyglutaric aciduria.

Organic acids are water soluble compounds containing one or more carboxyl groups. They are usually weak acids and tend to be of low molecular weight (< 300). They are the end products of metabolism of a wide range of compounds including amino acids, sugars, steroids, lipids and drugs. As such there are a large number of organic acids and a human urine sample can contain more than 250 different organic acids. The most common organic acids are the carboxylic acids but organic acid analysis also detects a class of compounds known as the acylglycines, which are formed by conjugation of acylCoA species with glycine. In addition, drug metabolites, feeds, intestinal bacterial metabolites, exogenous compounds (soaps, creams, plasticisers etc) and artefacts due to sample storage can also give rise to detectable compounds.

Metabolic disorders can result in the urinary excretion of a wide range of compounds, many of which are potentially significant in establishing a diagnosis. Organic acid profiling by Gas Chromatography Mass Spectrometry (GCMS) represents one of the more sophisticated screening methods currently available as a large number of metabolic disorders may be detected either directly or indirectly by this technique. The compounds that need to be detected comprise mainly acidic (but some neutral) metabolites, some with additional hydroxyl, oxo, and conjugated amino functional groups present. Many metabolites also contain phenolic or heterocyclic rings.

Although organic acids are detectable in blood, plasma, serum, amniotic fluid and CSF, urine is widely considered the most suitable biological matrix for analysis; it contains very little protein which simplifies sample preparation and organic acids are concentrated in the urine by the kidneys.

Organic acid analysis is a complex process which is prone to many limitations and pitfalls (1). It is important that laboratories understand the limitations of the methodology so that they can provide a robust and safe organic acid service.

1.1 Clinical Indications for Organic Acid Analysis

The clinical presentation of metabolic disorders may be variable, non-specific and can occur at any age. Therefore, urine organic acid analysis should be considered if any of the following are present:

1. Lethargy, coma, seizures or vomiting in a neonate
2. Hyperammonaemia
3. Hypoglycaemia
4. Unexplained metabolic acidosis or lactic acidaemia
5. Unexplained alkalosis
6. Metabolic decompensation
7. Unexplained global developmental delay or developmental regression
8. Unexplained liver dysfunction
9. Unexplained neurological signs/symptoms
10. Abnormal results on newborn screening programme
11. Sibling with similar clinical presentation

Clinical information and a drug/medication history should be supplied when requesting metabolic investigations.

1.2 Gas Chromatography Mass Spectrometry (GCMS)

GCMS is a powerful analytical technique. Separation of organic acids is achieved by virtue of the differences in their boiling points as well as their affinity for the stationary phase in the analytical column. A typical GC system utilises a carrier gas (usually helium) to transport the analytes from the injection port, through the column and after separation, into the mass spectrometer. While the injection port is held at a constant temperature (above the highest boiling point of all compounds in the sample) the column temperature, after a suitable delay, is gradually increased. As each organic acid reaches its boiling point it vaporises, enabling the carrier gas to carry the analyte through the column where it is differentially retarded by interactions with the stationary phase (to a greater or lesser extent) determined by its structure, number and type of functional groups and the chemical composition of the stationary phase itself. Sophisticated

temperature programs enable the separation of closely eluting metabolites to be controlled.

For organic acid analysis, the GC is usually linked to single quadrupole mass spectrometer or ion trap operating in positive ion mode. As the organic acids elute from the GC column, the eluent enters the ion source where a metallic filament discharges electrons into its path. When these electrons hit the compounds present, energy is transferred causing the compounds to split into fragments, the composition and abundance of which will be compound specific. These fragments are accelerated through an electromagnetic field which separates the ions on the basis of their mass/charge (m/z) ratio.

Data acquisition: a full scan is typically collected between 50 and 550 amu with a scan speed of <1 second. Each spectrum is stored along with a summation of all ions detected in that scan, which allows the data to be reviewed as a total ion chromatogram. Selective ion monitoring may also be used to monitor specific ions, allowing greater sensitivity for quantitation of specific organic acids.

2. Pre-Analytical Considerations

Sample Type: Organic acid analysis should be performed on a random urine sample collected into a sterile, preservative free container. Samples collected in boric acid are unsuitable.

Sample storage: The urine should be stored frozen prior to analysis. In the authors' experience, samples are stable for several years if stored at -20°C, however the evidence base for this is acknowledged to be weak and one report relating to 'total titratable organic acids' demonstrated a significant decrease in 10 samples stored at -20°C (2). It is therefore recommended that for long term storage samples are kept at -70°C and freeze/thaw cycles are kept to the minimum.

Sample volume: At least 5mL of urine should be collected. The minimum volume required is approximately 2mL, but this depends on the creatinine concentration; the more dilute the urine sample, the greater the volume that must be extracted. Very dilute urine samples (creatinine concentration < 1mmol/L) may not produce valid results.

Timing of sample collection: Samples collected during an acute episode are likely to be more informative than those collected during the recovery phase, whilst the patient is receiving I.V. fluids or whilst the patient is well.

Timed urine collections are unnecessary and should be avoided as the diagnostic metabolites may not be detectable even after partial recovery or if the patient is receiving I.V. fluids.

Sample pH: Prior to analysis, it is useful to check the pH of each urine sample. A pH >8.5 is indicative of bacterial contamination which makes the sample unsuitable for analysis. However, it is recommended that such samples are analysed regardless to ensure there are no obvious diagnostic markers present but then reported as "unsuitable for analysis, repeat required".

Clinical details: It is important that requests for organic acid analysis are accompanied by clinical information as this can alert the laboratory to look for specific compounds. Clinical signs and symptoms, the presence of any unusual odours, and information on family history, medication and choice of feeds may be pertinent and should be documented. Although this in practice is not the case and highlights the need for close liaison with clinical teams.

3. Analytical Considerations

3.1 Overview

The interpretation and, where required, quantitation of urine organic acids requires close attention to the detail of all analytical aspects of the process. In particular:

- The concentration of the urine (creatinine concentration) must be corrected by extraction of varying volumes of urine to allow for 'standardisation' of the amount of organic acids extracted enabling consistent interpretation and quantitation of organic acids (where required).
- Appropriate internal standards must be added to account for systematic losses during extraction and derivatisation, to normalise the chromatogram and allow quantitation of organic acids (where required). They also enable the sensitivity of the GC-MS to be monitored. Wherever possible, stable isotope internal standards are recommended as these are fundamental to the quality control process.
- Organic acids must be efficiently extracted from aqueous urine into an organic phase to allow GC-MS analysis.
- Organic acids must be converted to volatile derivatives so that they are thermally stable and chemically inert allowing GC-MS analysis.

- The GC must use an appropriate column, injection volume, oven temperature, ramp and timing to achieve adequate separation of organic acids over a suitable run time.
- The mass spectrometer should be operated in full scan mode, typically scanning between 50 and 550 amu. If oximation is performed with pentafluorobenzyloxylamine (PFBO), succinylacetone produces derivatives with m/z 620 hence the scan range would need to be increased accordingly. This enables the data acquired to be reviewed as a total ion chromatogram (TIC) and a full profile of organic acids to be detected.
- The mass spectrometer must be regularly and frequently maintained to ensure optimum performance.

3.2 Qualitative Versus Quantitative Analysis

There is currently no consensus opinion on whether organic acid analysis should be qualitative or quantitative. In the absence of evidence to support a specific approach, individual laboratories need to be aware of the limitations of each approach. Care should be applied to the term 'quantitative' organic acid analysis.

When an MS is acquiring data in full scan mode, organic acid analysis is deemed qualitative. Qualitative organic acid analysis has the advantages of simplicity and reduced cost whilst still enabling the diagnosis of most organic acidurias. Quantitation from full scan data is not recommended.

Laboratories may choose to quantify a number of key compounds by introducing additional data acquisition in Selective Ion Monitoring (SIM) mode. Previously this would have necessitated one injection in full scan mode and another in SIM, with increased run times accordingly. However, modern instruments have the capability to acquire scan and SIM data simultaneously, negating the need for a second injection. It is important that laboratories take advantage of such changes in technology and if quantitation is desired, it is recommended that methods are updated accordingly. The advantage of quantitation is the ability to provide additional information which may aid diagnosis and/or be useful for monitoring treatment e.g. quantification of methylmalonic acid in B12 responsive Methylmalonic Aciduria.

However, it is also important to understand the limitations as quantitative measurements are often made under less than ideal circumstances. It is not practical to establish calibration curves for every organic acid which might be

detected. Daily calibrations can be onerous and as such many laboratories choose to calibrate periodically. Whilst this may be a legitimate approach provided assay performance is closely monitored and analysts are aware of the importance of internal standard consistency, it probably reflects historic practice with less sophisticated instruments. Since the introduction of GCMS into the clinical laboratory, quantitative methods have suffered from the limited availability of stable isotope internal standards with most analytes being linked to a compound on the basis of availability rather than structural similarity. As the cost of stable isotopes reduces and availability increases, laboratories that wish to quantitate are strongly encouraged to purchase appropriate stable isotopes and review and update these historic methods. Where stable isotopes are used for quantitation it is recommended that a stable isotope that is at least three mass units higher than the native compound is used.

Likewise, the ideal quantitative GCMS method would monitor a qualifier ion as well as a quantifier, an approach rarely utilised in organic acid analysis.

3.3 Sample Preparation

3.3.1 Sample Volume

Generally laboratories perform the extraction on approximately 1mL of urine, for a sample with a creatinine concentration of 1 mmol/L. The urine creatinine concentration is used to determine the volume of urine to be extracted; effectively normalising the results to a creatinine of 1 mmol/L. For example, if urine creatinine = 1.239 mmol/L then the nominal volume of urine extracted would be 1/1.239 = 0.807 mL. Where less than the minimum volume is required, deionised water should be used to make it up to the desired volume.

In recent years, as instrument sensitivity has improved, a number of laboratories have chosen to reduce the volume of urine extracted to a nominal volume of 0.5mL, for a sample with a creatinine concentration of 1 mmol/L. In this case, for a sample with a urine creatinine = 1.239 mmol/L, the nominal volume of urine extracted would be 0.5/1.239 = 0.404mL.

3.3.2 Internal Standards

Addition of an internal standard must be the first step in sample preparation to account for any losses in subsequent steps. Given the number and variety of compounds present in urine, no single internal standard is ideal, therefore a combination of internal standards is recommended. The local laboratory should determine which combination to use, bearing in mind the following

recommendations as a minimum. If quantitation is to be performed a stable isotope internal standard is recommended.

Internal standards may be broadly categorised into three groups: exogenous compounds not seen in urine (often halogenated), analogues of compound groups seen in urine that are not themselves present (margaric acid, heptanoylglycine) and stable isotope labelled analogues of compounds that may be seen in urine.

Exogenous compounds are often so chemically different from normal urine constituents that their use as internal standards is questionable. Stable isotopes are the ideal internal standard, but may be expensive, in some cases prohibitively so. Deuterium labelled compounds are most common albeit at the expense of a slight retention time shift which increases with the number of ^{2}H present. ^{13}C and ^{15}N do not show this shift and consquently are preferred but the costs are much higher. The main advantage of stable isotope internal standards is that they provide an additional quality control measure: urine is a complex matrix from which a wide range of chemical structures is extracted and ultimately introduced into a high temperature, reactive environment (the liner). The addition of stable isotope internal standards to every patient sample enables the analyst to monitor any sporadic losses which may occur. We recommend the following internal standards as a minimum:

Exogenous compound.
Rationale: Many laboratories choose to include an exogenous compound against which to normalise the chromatogram, at a concentration which produces a peak (on total ion chromatogram) that is considered acceptable in abundance. An exogenous compound that does not co-elute with other compounds can help achieve consistent normalisation of the chromatogram for interpretation. It is important that the chosen compound is stable and reproducible.
Examples: 2-phenylbutrate, d3-methylmalonate, d4-4-nitrophenol and 2-oxocaproate are commonly used. Margaric acid (C17) and chlorobenzoic acid have also been used but due to the presence of an interfering ion and limited solubility in aqueous phase respectively, are not recommended.

Stable isotope of orotic acid.
Rationale: assurance that orotic acid has not been lost during analysis of any individual sample. The exact mechanism for this loss is not known, but recent evidence from the author's laboratory suggests that orotic acid is extracted from

the urine sample successfully but becomes unstable once injected into the GC, potentially decomposing in the injection port/liner of the GC under specific reaction conditions (3). We would recommend the concentration of the orotic acid stable isotope to be at the upper limit of the reference range (5 μmol/mmol).
Example: ^{15}N2-orotic acid is readily available and should be utilised to monitor loss of orotic acid however, it is of limited value for quantitation of orotic acid, because the standard curve is non-linear.

Glycine conjugate.
Rationale: assessment of sensitivity of key diagnostic compounds which may be excreted at low concentration. Ideally the glycine conjugate should be a stable isotope, alternatively an exogenous one.
Example: heptanoylglycine or d3-hexanoylglycine. We would recommend the concentration of the glycine conjugate stable isotope to be 2 μmol/mmol creatinine.

A short chain acid.
Rationale: assurance that short chain acids and ketoacids have not been lost during the drying down process
Example: stable isotope of lactic acid or 3-hydroxyisovalerate

Ideally stable isotopes of both hydrophilic and hydrophobic compounds should be used, for example citrate and phenylbutyrate, respectively. Additionally, if oximation is used, a stable isotope of an oxo acid (e.g. 2-oxocaproate) should be included to assess completion of oximation.

3.3.3 Sample Extraction

The various steps in the extraction process ensure optimal extraction of organic acids into the organic phase. It is recommended that high purity chromatography grade solvents are used for analysis.

Oximation: The first step in sample preparation may be oximation of the urine sample and a variety of reagents have been used for this purpose, hydroxylamine, methoxylamine and pentafluorobenzyloxylamine being most common. Oximation stabilises the oxo form over the enol form by producing oximes which are chemically more stable. For hydroxylamine and methoxylamine this is typically achieved by adjusting the pH of the sample to pH 14 with 5M NaOH, adding aqueous oximating reagent and heating at 60°C for 30 minutes.

For pentafluorobenzyloxylamine the reaction can proceed at pH<2 so the reagent may be added at the same time as the internal standards and HCl. Although oximation necessitates an extra step in the sample preparation process, in addition to stabilising the oxo acids it simplifies the chromatogram as fewer peaks are present for several compounds eg 2-oxoglutarate. The selective conversion of oxo acids will alter their retention time relative to other compounds present and this may also be a consideration when selecting which reagent to use. There is little published evidence supporting the requirement for oximation. Although a survey by ERNDIM in 2008 (4) concluded that there was no correlation between proficiency scheme score and use of oximation, this was overly simplistic and was at best an indirect assessment of the efficacy of oximation as many other factors contribute, not least the matrix of the sample. At the time approximately 2/3 of ERNDIM participants used a method including oximation. In the authors' experience, the detection of succinyl acetone is improved by oximation hence oximation is the recommended approach (5).

Urease: Urease can be added during the sample preparation process to remove urea (incubation at 56°C for 60 minutes). This is an advantage because urea may co-elute with other compounds such as methylmalonic and 4-hydroxybutyric acids. This must be balanced against the need for an extra step in the process.

Addition of excess salt: improves the extraction of organic acids because large amounts of inorganic salts "bind" water, thus decreasing the solubility of organic metabolites in aqueous solution. Commonly referred to as 'salting out'.

Acidification with HCl: aids extraction of acids in the subsequent liquid-liquid extraction step by converting them to their neutral (protonated) state.

Liquid-liquid extraction: two different organic solvents are recommended since different acids extract preferentially, with hydroxy acids and low molecular weight acids being preferentially extracted into ethyl acetate (6). For example, mevalonic acid, which extracts poorly into diethylether or a mixture of ethylacetate/diethylether, extracts preferentially into ethylacetate alone. Sequential extractions with ethylacetate and diethylether are recommended to maximise recovery across the range of analytes present. Following the addition of the organic solvent the mixture should be shaken vigorously for 30 seconds, centrifuged briefly to break any emulsion that has formed, and the top, organic

layer removed to a fresh sample tube then the process is repeated a second time (with the same or different solvent) combining the extracts into one tube.

Drying of the combined organic layers:
Although solvents such as ethylacetate and diethyl ether are considered immiscible with water, water will dissolve in them to some extent which has the potential to hinder the subsequent derivatisation reaction. It is therefore common at this stage to dry the combined extracts, typically by standing over anhydrous sodium sulphate with occasional mixing for 30 minutes. After brief centrifugation, the organic layer is carefully removed to a clean tube. The rationale is that any residual water present during derivatisation will hinder the derivatisation reaction, however, this step is not always performed without apparent problems. This may be due to the fact that both ethylacetate and diethyl ether form azeotropic mixtures with water making its removal easier than anticipated.

Evaporation to dryness: once the solvent extracts have been combined (and dried) they should be evaporated to dryness. We would recommend the use of a centrifugal evaporator to ensure drying is gentle and consistent. In the event that this is not possible, solvent extracts should be dried under nitrogen at ambient temperature. Although it is common practice in many laboratories to use heat at this stage (40°C), we do not recommend this approach because of the likelihood that volatile short chain acids will be lost if insufficient attention is paid to this step (5).

Derivatisation: It is recommended that reagents of the purest grades are used for derivatisation to avoid extra peaks in the chromatogram. Trimethylsilyl (TMS) derivatisation is commonly used and is recommended because it is quick and effective. The TMS groups react with exchangeable hydrogen atoms (e.g. hydroxy groups on carboxylic acids and alcohols, and amine groups) on the organic acids. The hydrogen atom(s) is replaced with a TMS group (molecular weight 73 amu) and resulting in an increase in the organic acid molecular weight of 72 amu (addition of TMS and a loss of one hydrogen) per TMS group added. TMS derivatisation is achieved by treating the solvent extract with pyridine and N,O,-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethyl-chlorosilane (TMCS) and heating to approximately 75°C for between 15 and 30 minutes. It is important that the reaction conditions are kept constant between batches so that the number of TMS derivatives formed is constant for annotation and interpretative purposes. In addition higher temperatures for longer time

periods may increase the number of less stable derivatives formed. For example; of the mono and di TMS derivatives of acylglycines formed, the di TMS derivatives are less stable and have a tendency to breakdown during GC analysis if the system is not clean.

3.4 Gas Chromatography Mass Spectrometry (GCMS)

It is difficult to be prescriptive of the GC conditions as they will relate to the specific GC instrument and the column being used. The GC system may be operated at constant pressure or constant flow. Each laboratory will therefore need to optimise their instrument and validate their method.

Injection: the injection volume should be of the order of 1μL with a split ratio of at least 1:15 to prevent overloading of the column. Lower split volumes may result in a loss of sensitivity.

Run times: short run times may lead to overlapping peaks and make annotation more difficult.

Table 3.4.a Typical Gas Chromatograph (GC) settings.

Oven Ramp	Rate (°C/min)	Next Temp (°C)	Hold Time (mins)	Run Time (mins)
Initial		60.0	1.90	1.90
Ramp 1	25.0	80.0	1.00	3.70
Ramp 2	7.5	280.0	5.00	35.37

Choice of column: Organic acid analysis is usually performed on a non-polar GC column - 100% methyl or 95% methyl, 5% phenyl. For example, HP-5 ms (methylsiloxane 5% phenyl), 25m x 0.20mm x 0.25μm fused silica capillary column or an Agilent J&W ULTRA2 (methylsioxane 5% phenyl) 25m x 0.20mm x 0.33μ, fused silica capillary columns. Columns with smaller internal diameter can afford better resolution but at the expense of lower loading capacity for sample.
Injection volume: 1-2μL
Carrier gas 1mL/min (constant flow)
Split ratio 1:20
Front inlet: 280°C
Transfer Line temperature: 280°C

3.4.1 Maintenance of the GCMS

Regular maintenance of the GCMS is recommended. While this document is not intended to provide an exhaustive troubleshooting guide, the following should be considered in conjunction with the manufacturer's user guide. For further

information on troubleshooting, the reader is referred to 'GC Troubleshoooting' (7).

It may be useful to record and monitor certain parameters on the GC-MS system to help identify when performance is deteriorating and when maintenance is required. Examples include:

- Abundance of the key ions during the autotune
- Repeller voltage
- EM voltage
- Intensity of internal standards key ions including mono and di derivatives of glycine conjugates

Many systems will now perform an autotune evaluation which provides automatic guidance on when performance is suboptimal. It is essential that the instrument is regularly tuned to ensure optimal performance of the MS. This should be undertaken as a minimum weekly and after any maintenance or engineer visit. A tuning gas such as perfluorotributylamine (PTFBA) is used because it produces volatile but chemically stable fragments across the mass range of interest and contains ^{13}C and ^{15}N isotopes. A range of parameters (number of peaks, peak width at half height, peak resolution and abundance of key ions) should be recorded and monitored against locally agreed acceptable limits.

Repeller voltage: the repeller voltage provides an excellent indication of the cleanliness of the ion source; as the ion source becomes dirty, the potential required by the repeller to achieve a particular target ion abundance for a tune mass, will be higher. This effect generally becomes evident in ions with a higher m/z first because a higher potential is required to eject them from the ion source.

Electron multiplier voltage: the EM voltage should be recorded and monitored to allow assessment of the need to replace it. The voltage will naturally increase with age, once it begins to increase more rapidly; this is a sign that it should be replaced.

Internal standard peak area: Peak area of the internal standard starts to decrease typically after 500-600 injections, requiring system maintenance (ion source, column inlet liner etc).

4 Post Analytical Considerations

Organic acid interpretation is complex and should be performed by staff with sufficient experience and competency. Overall interpretation of organic acid

chromatograms is based on pattern recognition. There are many good references describing the significance of particular compounds/patterns (8) (9) (10). Participation in interpretative EQA schemes (see below) is essential. The following procedures should take place when reviewing a batch of organic acids:

1) Check integrity of the batch:
   A process blank should be run with each batch and checked for signs of analytical contamination. For example, plasticisers, lubricants, resins, column bleed, septa bleed.

2) Check internal quality control (IQC) samples (see later):
   Appropriate action should be taken if locally defined standards are not met. It is important to remember that IQC samples are of limited value as they will not account for matrix effects of individual samples.

3) For each individual chromatogram:
   a) The TIC abundance of all internal standards must be monitored. If abundance is too low or internal standards are not present, the sample should be re-extracted and re-analysed to ensure correct procedure has been followed and there have been no problems with sample extraction and/or matrix effects.
   b) The chromatogram should be methodically interrogated and the mass spectrum of each peak on the chromatogram should be reviewed and compared to an appropriate compound library. It is often useful to break down the chromatogram into smaller discrete segments to ensure all peaks are identified. Some co-eluting peaks may make identification difficult and more sophisticated peak interrogation techniques may be helpful where software allows (e.g. mass subtraction, peak purity). Software providing auto peak recognition/annotation are available from most major instrument manufacturers. These can be used to speed up the annotation/interpretation process but should not be completely relied upon. However, it is important that the specifics of a given autoannotation process and its limitations are understood by the user. Manual interrogation of peaks should be performed to check accuracy of auto annotation software where necessary. This is particularly important for peaks which are known to run at the same retention time and where the presence of compounds in small quantities is of significant clinical importance. NB Beware spectral libraries developed in house which may

contain 'impure' spectra that can lead to confusion. It is recommended that NIST is included in any automatic library search. Modern software allows extracted ion scans to be quickly performed and these should be utilised to check for key compounds in every patient sample. We recommend performing extracted ion scans of the following analytes (if they are not quantitated separately):

- 4-hydroxybutyrate
- butyrylglycine
- isovalerylglycine
- tiglylglycine
- hexanoyl glycine
- suberyl glycine
- 3-hydroxyglutarate
- orotic acid
- methyl citrate
- succinyl acetone
- malonic acid
- methylmalonic acid
- It may be prudent to include additional compounds dependent upon individual laboratory experience and/or ERNDIM performance.

4) Perform/check quantitation of any metabolites that are analysed quantitatively (if any).

5) Reporting of results - qualitative reports should be as clear and concise as possible. Overly complicated and long reports risk confusing users who may potentially miss key information. As a general guideline reports should:
   - State key abnormal peaks present (NB in certain circumstances it may not be helpful to state all abnormal peaks).
   - Suggest possible causes for abnormal peaks (e.g. possible diagnoses, dietary factors).
   - Guidance on what follow up is required (including further testing/referral to relevant clinical teams).
   - If a specific condition has been queried, its exclusion should be mentioned.
   - If clinical details suggest a specific condition associated with diagnostic difficulty users may choose to comment on this (e.g. 3-hydroxyglutarate in a patient with non-accidental injury, mevalonate in asymptomatic patients.

- Quantitative results should be reported clearly with appropriate units and reference intervals. Quantitative data should be used to enhance interpretive analysis and comments.
- It is important to note that organic acid analysis will often not provide sole confirmation of an exact diagnosis. Results of other key analyses (e.g. acylcarnitine and amino acid analysis) must be taken into account and reports should recommend any required confirmatory (e.g. enzyme or molecular) analysis.
- In many circumstances urgent communication of results may be necessary. Details of whom results have been communicated to and when should be added to the report.

5. Uncertainty of Measurement

Tables 5.1 to 5.3 summarise the critical steps in the analysis of urinary organic acids. It can be useful to highlight those stages where a significant degree of control is necessary and ensure the analyst has considered what stated reference is adequate to ensure the final analytical result obtained and reported is fit for its intended clinical purpose. In Table 3, the degree of control required is indicated by (G), (A) and (R) standing for green, amber and red respectively. Green steps require only a minimal or basic degree of control. Amber steps are those in which a significant degree of control is required but can be achieved by the use of properly maintained and calibrated equipment for common measurements e.g. mass, temperature, volume, instrument response etc. Red steps indicate that a significant degree of control is required and that the analyst must select a special stated reference. The reader is referred elsewhere for further information on this topic (11).

Table 5.1 Pre-Analytical Factors

Step	Measurement Uncertainty	Examples of Control Measures
Sample collection (patient history)	Certain dietary factors/medications may cause abnormal profiles	Training and competency in reporting Clinical details Lab handbook/website
Sample collection (timing)	Samples collected when a patient is well may have no/very subtle abnormalities	Collect samples during an acute episode if possible Lab handbook/website Training and competency in reporting
Sample collection (contamination)	Introduction of compounds that may cause diagnostic confusion	Training in sample collection. Repeat analysis to confirm findings before diagnosis confirmed
Sample storage	Bacterial degradation of organic acids	Temperature monitoring Urine pH check User information Training and competency in reporting

Table 5.2 Post Analytical Factors

Step	Measurement Uncertainty	Examples of Control Measures
Interpretation of chromatogram/reporting of result	Potential for subjective/inconsistent/ incorrect interpretation of results	Consistent staff training and competency (for interpretation) Results should be assessed by >1 person (e.g. annotated/reported by one competent member of staff before authorisation by a second) Where possible, results should be reported in conjunction with other relevant metabolic investigations and not in isolation Method-specific reference ranges in use (if quantitative results produced) Participation in interpretative EQA scheme(s)

Table 5.3 Analytical Factors

Critical step	MU	Control measure	Degree of control (R,A,G)	Traceability
Determining concentration of stable isotope internal standard	Molecular weight used to calculate concentration of stock internal standard. Accuracy of the assay relates directly to the concentration of internal standard.	MW taken from appropriate resource material.	R	MW taken from Chemspider database (N.B. ensuring correct salt)
Preparation of stock internal standard solution	Accuracy of patient result for quantitative analysis	Regular use of check weight/ used immediately prior to weighing IS Solution prepared in Grade A volumetric flask	R	Annual calibration of balance by UKAS accredited company Tolerance of 100 mL flask is ± 0.02% at 20°C
Preparation of working internal standard solution	Accuracy of patient result for quantitative analysis	Calibrated Gilson pipette used to deliver working IS solution Regular pipette verification of precision	R	Certificate of pipette calibration by UKAS accredited company
Measurement of urine creatinine concentration	Volume of urine extracted depends on creatinine concentration	Creatinine measured by UKAS accredited method	R	Traceability of method EQA scheme participation
Acidification (pH<2) of sample with HCl	Poor extraction of acids	Excess acid added Check pH with indicator paper	G	

Table 5.3 cont. Analytical Factors

Critical step	MU	Control measure	Degree of control (R,A,G)	Traceability
Solvent extraction	Complete extraction of organic acids from urine sample	Solvents present in excess	G	
	Different organic acids have different extraction efficiencies	Two different solvents used and extracts pooled. Presence of IS to correct for recovery	G	
Evaporation of solvent extract	Potential loss of volatile compounds resulting in a missed diagnosis	Oximation	G	
		Use of centrifugal evaporator	G	
Derivatisation	Incomplete derivatisation	BSTFA and pyridine present in excess	G	
	Derivatisation reaction time	Electronic timer for 30 minutes	A	
	Derivatisation reaction temperature	Hot block temp calibrated. Temperature is ok up to ± 10°C	A	
Chromatographic separation	Separation of analytes	Visual check of chromatogram and retention times Profile of QC checked to ensure adequate resolution and expected peaks are detected	A	
Detection of specific ions by MS	Mass calibration of MS detector	Weekly autotune performed (includes tests for relative abundance, m/z, peak width etc of target ions)	R	Mass calibration and electron multiplier tune performed by manufacturer at annual PM
Identification of spectra	Interpretation of patient results	User defined libraries AMDIS deconvolution software	R	Competency Spectral library searching using NIST database

6. Performance Characteristics

Several EQA schemes exist for urinary organic acids but no single scheme is fully comprehensive. The European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) provides the Quantitative Urine Organic Acid scheme which includes 24 organic acids. In 2017, 131 laboratories participated in this scheme. Review of the intra laboratory CVs for given analytes provides a crude measure of performance and highlights the difficulties associated with measuring organic acids. For example, for 3-hydroxyisovaleric acid (HIVA) the average intra-laboratory CV was 22% and the inter-laboratory CV was 51%. Likewise, for 3-hydroxyglutaric acid the average intra-laboratory CV was 30% and the inter-laboratory CV was 181%.

The lack of certified reference materials, availability/choice of stable isotope internal standard and variation in sample preparation and extraction efficiency are likely contributing factors. The utility of the scheme is also limited by the small number of samples distributed annually (n=8) (12).

6.1 Reference Intervals

Due to the inherent limitations associated with the quantitation of organic acids, it is recommended that any laboratory reporting quantitative results on a given compound has established in house reference ranges.

6.2 Traceability

For most UK laboratories, with the exception of a small number of analytes which are reported quantitatively, organic acid analysis is essentially a qualitative procedure. More than a hundred different organic acids can be detected by this method and in almost all cases, identification and detection is a qualitative process based on retention time and the presence of key ions in the corresponding mass spectrum. Annotation of the chromatogram is performed using spectral library matching although considerable operator experience is also required. Although quantitation of key compounds may be performed routinely, clinical interpretation is based on assessment of the complete urine organic acid profile, not on the basis of an individual organic acid result. Interpretation should also take into account clinical details and other available test results.

For those compounds which are quantitated, evaluation of traceability is likely to identify 4 property values requiring a high degree of control (a property value is one of the critical steps that directly relates to the final analytical result and hence one which should be traceable, see Table 5.3) (11). The final analytical result is derived from the following calculation:

Conc (µmol/L) = [(analyte response) / (IS response)] x M (µM)

where M is concentration of the internal standard added to each sample. The volume of urine extracted is calculated on the basis of the creatinine concentration, allowing results to be expressed as µmol/mmol.

7. Performance Monitoring

7.1 Internal Quality Control

It is recommended that two levels of matrix matched IQC are analysed with each batch of patient samples. It is also recommended that the IQC material should contain any analytes which are being reported quantitatively. It is also good practice to include a range of compounds which are only detected qualitatively. Particular weight might be placed on those which are clinically important and/or known to be associated with analytical issues (e.g. orotic acid, hexanoyl glycine, short chain oxo acids).

It is important to reiterate that inclusion of orotic acid in an IQC sample does **not** negate the need for a stable isotope of orotic acid to be added to every sample because the loss of orotic acid is sample specific.

It is important to carefully consider which compounds are present in the IQC material. The method is capable of detecting >150 different compounds and it is not practical to include all of these in the IQC material. Labs should make an informed decision based on their individual requirements.

Commercial IQC material is available which contains a range of useful compounds and target concentrations. For example, SKML https://www.skml.nl/en/home list

7.2 External Quality Assessment (EQA)

All labs performing organic acid analysis should be enrolled in an appropriate EQA scheme. It is recommended that all users should be enrolled in an interpretative

scheme and for labs providing quantitative results, appropriate quantitative schemes. Relevant EQA schemes include:

ERNDIM Qualitative Organic Acids (3 X 3 samples annually)
ERNDIM Diagnostic Proficiency scheme (2 X 3 samples annually)
ERNDIM Quantitative Organic acid analysis (8 samples annually)
ERNDIM special assays urine (8 samples annually)

7.3 Reagent Acceptance Testing

To ensure compliance with UKAS standards, laboratories must be able to demonstrate reagent acceptance testing.

Tables 7.3.1 to 7.3.4 outline such a process as an example. It should be noted that individual IQC samples, even those which are human urine, are of limited utility due to sample specific matrix effects. IQC samples will demonstrate that the system is working but not the effect of an individual sample matrix. Where small numbers of individual patient samples are used for comparison, the percentage difference between the previous result and the new result should be less than the critical difference for the assay. Critical difference at 95% confidence limit may be calculated using the formula; critical difference = 2.77 × √(CVa2 + CVi2) where CVa = analytical coefficient of variation and CVi = CV of within-subject biological variation. Since within-subject biological variation is not relevant as the same sample is being analysed, this may be calculated by multiplying the long-term IQC coefficient of variation by 2.77. (The coefficient of 2.77 is derived from √2 × 1.96; where 1.96 is the probability factor for 95% probability of a true change in either direction, and multiplication by √2 accounts for the fact that the critical difference includes variation in two measurements.)

Table 7.3.1 Assay Reagents

Method of testing	Details of testing required	Acceptance criteria
Sample comparison	All IQC samples associated with the assay	Within current ranges
	Three previously analysed patient samples	Within critical difference for assay

Table 7.3.2 In-house Calibration Standards*

Method of testing	Details of testing required	Acceptance criteria
Run standards	Run 3 standard curves on 3 batches against current calibration.	Calculate standards against current calibration curve. Within 10% of expected target concentration and R^2>0.95
Sample comparison	Previous three (minimum) EQA samples	+/-2SD all laboratory mean depending on EQA scheme
	All IQC samples associated with the assay	Within current ranges
	Three batches of patient samples	Slope between 0.9 to 1.1, $R^2 \geq 0.95$ and <10% average bias on Bland-Altman plots

*For analytes where a Certified Reference Material exists, e.g. methylmalonic acid, the recommended approach would be to cross reference the new calibrator with the CRM and reduce the associated testing accordingly.

Table 7.3.3 Internal Standards (used with external calibration)

Method of testing	Details of testing required	Acceptance criteria
Sample comparison	Analyse standards and all associated IQC samples with new internal standard	Within current ranges for calibration acceptance and IQC
	Three previously analysed patient samples	Within critical difference for assay

Table 7.3.4 GC Column Acceptance Testing

Method of testing	Details of testing required	Acceptance criteria
Assess for column bleed	Inject a sample and monitor TIC Check XIC for ions of interest	Stable baseline (compared to previous column) Absence of any m/z associated with column bleed
Retention time lock	Run standard containing lock compound	
Analyse calibrators	Run standard curve on single batch	Calibrators within current ranges
Sample comparison	IQC samples	Within current ranges
	Assess retention times for glycine conjugates & internal standards compared to previous column	No criteria – all annotators to be informed of any retention time differences

8. Pitfalls and Limitations

Organic acid analysis is a complex process which is prone to many limitations and pitfalls (10). It is important that laboratories understand the limitations of the methodology so that they are able to provide a robust and safe organic acid service. The detection rate of IMDs is lower when the diagnostic compound is prone to analytical issues. Key points to be aware of are listed below.

Solvent extraction:

The efficiency with which organic acids are extracted from the urine sample varies. Some acids extract relatively well and others less so. It is recommended that two different solvents are used to maximise the extraction efficiency.

Generally speaking free acids can be extracted into organic solvents from an acidic solution, acid salts will extract into water and neutral substances will extract at any pH. Because of the difference in extraction efficiency, peak size does not correlate with concentration. For example, methylcitrate extracts poorly from the urine sample so a relatively small peak can correspond to a high concentration. Likewise, glycerol, a small neutral molecule, is also poorly extracted.

The solvents are present in excess during the extraction step so the precision with which the solvent is added is not critical. However, the inherent differences in extraction efficiency mean it is important to perform this step consistently. It is recommended that extraction efficiency is monitored by recording the relative intensities of key compounds in an IQC material.

If the separation of the solvent layer from the aqueous layer is performed poorly, water soluble compounds are carried over and will be detectable on the chromatogram. Common examples are urea and phosphate. In addition, when the organic solvents are evaporated the water-soluble salts may precipitate out of solution.

Evaporation of solvent extract:

Care is required during the drying down step. If samples are over dried at this stage the shorter chain, volatile acids and oxo acids may be lost. These include but are not limited to, lactate, 2-hydroxybutyrate, 3-hydroxybutyrate, 4-hydroxybutyrate, 2-oxo-isocaproate, acetoacetate, 3-hydroxyisovalerate some of which are diagnostic.

Derivatisation:

The time and temperature of the derivatisation reaction is important. The derivatisation step must be done consistently to ensure the products formed are consistent. Residual moisture (caused by aqueous phase being carried over from solvent extraction) will result in poor derivatisation of samples. If this is apparent (samples will be difficult to dry down fully) then they should be re-extracted and re-analysed.

Co-eluting peaks:

There are many examples of co-eluting compounds and this can lead to difficulties in correctly identifying the spectra and/or missed peaks. With the increasing use of sophisticated software this is becoming less of a problem but analysts should be aware of key compounds that co-elute on their local system. Common examples include 3-hydroxyisobutyrate and 3-hydroxy-N-butyrate, 2-hydroxyglutarate, isopropylmalate and 3-hydroxyglutarate, orotic acid and aconitate, and 2-methylcrotonyl/3-methylcrotonylglycine.

Chiral compounds:

D and L isomers will not be separated by standard columns used in organic acid analysis. This can be of clinical significance (for example to identify the different forms of 2-OH-glutaric aciduria, identification of D-lactate) and separate analysis with a chiral column must be performed in such circumstances.

Interfering compounds with spectra containing the same ions as a compound being quantitated e.g. isopropylmalate and 3-hydroxyglutarate (m/z 185).

Sporadic loss of key compounds:

The potential to 'lose' certain compounds, for example orotic acid, while recovery of other compounds is unaffected is a known limitation of analysis. Addition of a stable isotope of orotic acid, to **every** patient sample, is vital to enable the sporadic loss of orotic acid to be monitored. The mechanism of this loss has yet to be ascertained but is hypothesised to be a chemical effect influenced by specific reaction conditions created in the injection port/liner of the GC, not failure to extract the orotic acid. The nature of the loss is sample specific hence analysis of a quality control sample containing orotic acid will not detect this problem in patient samples. Irrespective of the cause, addition of stable isotope orotic acid is vital to prevent a missed diagnosis. In the author's experience other compounds,

for example uracil, are also prone to sporadic losses and the only way to counteract this is the use of stable isotopes.

General background contamination:
Contaminants such as column bleed, septa bleed, hydrocarbons, and phthalate plasticizers can result in a large TIC signal during the analytical scan. This decreases the sensitivity and can prevent the detection of key compounds. Table 8 summarises some common contaminant mass ions which can alert the user to a problem.

Table 8 Common Contaminant Ions in GCMS.

Mass Ions	Compounds	Source of contamination
18, 28, 32, 44	H2O, N2, O2, CO2	Air leak
28, 44	CO, CO2	Hydrocarbon fragments
31	Methanol	Lens cleaning solvent
43, 58	Acetone	Cleaning solvent
69	Fore pump fluid	Saturated trap pellets
69, 131, 219, 254, 414, 502	PFTBA	Calibration gas leak
73, 207, 281, 327	Polysiloxanes	Column bleed
73, 207, 281, 149	Polysiloxanes	Septum bleed
73, 147, 207, 221, 295, 355, 429	Dimethylpolysiloxanes	Septum breakdown
77	Benzene/xylene	Cleaning solvent
77, 94, 115, 141, 168, 170, 262, 354, 446	Diffusion pump oil	Improper shut down of pump heater
91, 92	Toluene/xylene	Cleaning solvent
105, 106	Cleaning solvent - xylene	Cleaning solvent
151, 153	Cleaning solvent - trichloroethane	Cleaning solvent
149	Plasticisers (phthalates)	Vacuum seal damage
14 amu spaced peaks	Hydrocarbons	Saturated trap pellets, fingerprints, pump fluid

Drugs and exogenous compounds:

Abnormal peaks may be present due to factors unrelated to metabolic disease. These are usually due to contamination of the urine with exogenous substances or metabolites of specific dietary components/drugs. Care must be taken with interpretation as certain metabolic conditions could wrongly be attributed to presence of such compounds and similarly a diagnosis could be wrongly assigned to an unaffected patient (9).

Care should be taken if drug metabolites are to be reported; organic acid methods are not set up to specifically measure these compounds and it is not clear how such metabolites relate to plasma concentrations. There may be certain circumstances, however, where reporting of such metabolites maybe useful (e.g. paracetamol/antibiotic metabolites in patients with significant pyroglutamic aciduria (aka 5-oxoprolinuria); presence of paracetamol metabolites in a patient with acute liver failure where a paracetamol overdose has not been excluded.

Unknown compounds:

It is not uncommon to detect 'unknown' peaks in a urine organic acids chromatogram. Any compound containing one TMS group will show a significant ion at mass 73, if more than one TMS group mass 73 and 147 will be present. It is important to check all relevant/available libraries (e.g. in-house, NIST etc) and to look for the presence of key ions. Be aware that different nomenclature maybe used for the same compound in different libraries. If no match for a compound can be found it is often useful to make a note of key ions and retention times for future reference (as new biomarkers or drug peaks may later become apparent). If spectral matches are < 90%, subtracting the baseline spectra may improve the spectral match factor. When the spectral match is below 50%, baseline subtract is unlikely to help.

Linearity:

If specific analytes are being reported quantitatively it is important to determine the linearity. Whilst this is true for any analytical method, capillary GC columns are particularly prone to overloading and without diluting the sample appropriately, quantitative results on compounds such as methylmalonic acid, will not just be inaccurate but can be misleading (potentially by orders of magnitude).

References

(1) Blau N, Duran M, Gibson K editors. Laboratory Guide to the Methods in Biochemical Genetics, Heidelberg: Springer-Verlag; 2008.

(2) Remer T, Montenegro-Bethancourt G, Shi L.
Long-term urine biobanking: storage stability of clinical chemical parameters under moderate freezing conditions without use of preservatives. Clin Biochem 2014;47(18):307-311.

(3) Witek K, Turner C, Carling R. Orotic Acid: Lost without trace. British Inherited Metabolic Disease Annual Symposium: Trainees Meeting 2017.

(4) Peters V, Garbade S, Langhans C, Hoffmann G, Pollitt R, Downing M, et al. Qualitative urinary organic acid analysis: Methodological approaches and performance. . J Inherit Metab Dis 2008;31:690-696.

(5) Witek K, Carling R. Personal Communication. 2018.

(6) Tanaka K, Hine D, West-Dull A, Nad Lynn T. Gas Chromatographic method of analysis for urinary organic acids. Retention indices of 155 metabolically important compounds. Clin Chem 1980;26:1839-1846.

(7) Phenomonex. GC Troubleshooting Guide. 2014; Available at: https://www.phenomenex.com/ViewDocument?id=gc+troubleshooting+guide Accessed 12th September, 2018.

(8) Saudubray J, Baumgartner M, Walter J editors. Inborn Metabolic Diseases, Diagnosis and Treatment. 6th ed.: Springer; 2016.

(9) Kumps A, Duez P, Mardens Y. Metabolic, nutritional, iatrogenic and artifactual sources of urinary organic acids: a comprehensive table. . Clin Chem 2002;48(5):708-717.

(10) Rinaldo P. Organic Acids. In: Blau N, Duran M, Gibson K, editors. Laboratory Guide to the Methods in Biochemical Genetics. : Springer; 2008. p. 137-169.

(11) Meeting the Traceability requirements of ISO17025: An Analyst's guide. 2nd Edition, November 2003, LGC Ltd

(12) Roux-Petronelli C, Weykamp C. ERNDIM - Quantitative Schemes Quantitative Organic Acids Annual Report. 2018; Annual Report ERNDIM-EQAS 2017.