ERNDIM
QUALITY ASSURANCE IN LABORATORY TESTING FOR IEM

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ORGANIC ACIDS
QUALITATIVE ANALYSIS IN URINE BY GCMS

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ORGANIC ACIDS
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General

a) Purpose of examination

Acids, like all chemical compounds, are classified as either organic or inorganic. Organic acids occur in, or can be produced from, animal and vegetable matter. In addition to hydrogen, organic acids always contain carbon and at least one other element.
Organic acids occur as physiological intermediates in a variety of metabolic pathways. Organic acidurias are a group of disorders in which one or more of these pathways are blocked, resulting in a deficiency of normal products and an abnormal accumulation of intermediate metabolites (organic acids) in the body. These excess metabolites are excreted in the urine.
Organic acidurias typically present with either an acute life-threatening illness in early infancy or unexplained developmental delay with intercurrent episodes of metabolic decompensations in later childhood.

b) Principle and method of the procedure used for the examination

Organic acids are extracted from acidified, salt saturated urine. The extracts are evaporated to dryness under nitrogen and trimethylsilyl (TMS) derivatives formed using N_2-O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine.
TMS derivatives are identified using gas chromatography mass spectrometry (GCMS) electron impact.

Derivatisation is necessary to produce compounds that are thermally stable, chemically inert and volatile below 300°C, so that separation can be achieved by GC. Compounds are partitioned between a moving inert carrier gas (He) and a stationary phase (non-volatile liquid) coated directly onto the inner surface of the capillary column, and elute at characteristic times (retention time). The eluent from the GC enters the electron ionisation chamber of the mass spectrometer where molecules are ionised and fragmented by collision with an electron beam, giving a unique fragmentation pattern (mass spectrum).
A compound is identified on the basis of its retention time and mass spectrum.

c) Analytical performance characteristics

This is a qualitative assay. Internal standards are used to determine the sensitivity; abundances of internal standards have to meet minimum criteria before an analysis is accepted.

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Hazards and Precautions

d) Environmental and safety controls

COSHH

Hazardous Substance

For all of the following wear purple nitrile gloves, handle only in a fume cupboard away from sources of ignition.

Concentrated hydrochloric acid :	corrosive
Ethyl acetate :	highly flammable.
Diethyl ether :	extremely flammable, may form explosive peroxides
Pyridine :	highly flammable, harmful
BSTFA :	Flammable, corrosive, toxic by inhalation and skin contact.

Discarding TMS Extracts

The extracts are discarded into a yellow sharps bin in a fume cupboard after the sample has been reported. Please check in telepath if in doubt!

Samples must not be left by the side of the GCMS or discarded into waste for incineration bins in the open lab – the vials are not sealed after sampling and contain noxious harmful chemicals (BSTFA and Pyridine)

When necessary pool the extracts for the test mix, aliquot the test mix into glass vials and store in the cold room.

Fire Hazard.

Hazardous Substance

- Ethyl Acetate Highly flammable

Store Winchester in flammable bin, store decanted bottle in fume cupboard

- Diethyl Ether Extremely flammable

Store Winchester in flammable bin, store decanted bottle in fume cupboard, change decanted bottle every 6 months to reduce risk explosion due to build up of oxides

- Pyridine highly flammable

Store bottle in fume cupboard, do not fill decanted bottle with more than 10ml liquid

- BSTFA flammable

Store bottle in fume cupboard, after use blow in nitrogen to keep inert.

For full health and safety information relating to this examination procedure you must also read the latest MSDS for all associated reagents.

Pre-Examination processes

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e) Patient preparation

None is required
In the event of a patient presenting with particular symptoms e.g. fits or hypoglycaemia it is important to obtain samples as near to the episode as possible.

f) Interferences and cross reaction

Samples collected using **Borate** as preservative are **not suitable**.

g) Type of samples

Random urine samples. Store at -20°C until analysed.
Extracts for GCMS (prepared by requesting lab).
Repeat samples may be required to reveal some abnormalities.
Post mortem CSF samples or Urine/bladder wash samples may be used if no other sample available.

Urgent samples
Urgent samples can either be run in isolation ASAP or analysed with the batch and run at the beginning of the batch.
Same day analysis is only guaranteed if the sample arrives in the lab by 1.30pm.
Urgent analysis can sometimes be requested out of hours, analysis is arranged by the consultant clinical scientist covering the on call period.

Repeat analysis on diagnostic sample
If a sample from a batch is abnormal and indicative of a primary organic acidaemia then the sample is analysed in isolation ASAP to confirm the findings before the result is telephoned to the clinician.
Use the same chromatography number with an R on the end e.g. 6052R

Reruns and repeat extractions on the same sample
Use the same chromatography number as the original extraction with an R at the end e.g. 6052R

h.) Type of container and additives

20ml universal no preservative

Examination processes

i) Required equipment, reagent and consumables

PPE
Lab coat or plastic apron
Purple nitrile gloves

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Equipment

Benchtop GCMS :	Shimadzu QP2010 Series GCMS system Serial no C704643 70030
GC column :	'Agilent J and W GC COlumn'. Length 25m, diameter 0.2 mm. Part number 19091S-602
Centrifuge	Jouan G4i
Heating/Drying Block	Techne DB3
Sample concentrator	Techne
Rotary Mixer	
Fume Cupboard	

Consumables

Glass Pasteur pipettes	Western laboratory Services-short form PP-150-S100
Plastic vials	Burke Analytical 30111P-1232
Snap on Tops	Agilent 5182-0550
7.5ml pyrex tubes	Sigma Z28,111-5
screw tops	Sigma Z14,508-4
Pipettes	Gilson P100 ,……

Reagents

5M Hydrochloric acid	Fisher J/4270/17 Acid Bin 1 Bottle in use in OA skip
Sodium chloride	Analar 27810.262 chemical cupboard A Tub in use on bench- topped up from store
Anhydrous sodium sulphate (granular)	Analar 1.06637 chemical cupboard A Tub in use in fume cupboard- topped up from store
Ethyl Acetate	Rathburns RH1013 Solvent Bin 2 Bottle in use in fume cupboard
Diethyl ether	Rathburns RG2013 Solvent Bin 2 Bottle in use in fume cupboard
BSTFA	Superlco fume cupboard
Pyridine	Rathburns RH1046 fume cupboard Decant into small bottle for in use
Heptadecanoic acid	Sigma H-3500 green no 59
Methanol.	Rathburns RH1019 Solvent Bin 2

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Heptanoyl glycine MW = 187	available from Nigel Manning (green no 66)
5M HCl If need to make it up	Dilute 570ml deionised water with 430ml 37% HCl SG1.18 (fisher H/1200/PB17 Acid Bin 1) Aliquot in use in OA skip
Internal Standards	
Working standard A	75 mg heptadecanoic acid in 200 ml methanol. Aliquot into 5ml glass tubes store in small freezer Aliquot in use at 4°C in metabolic fridge-stable one week – new aliquot at beginning of each week and write date removed from freezer on lid of aliquot.
Stock Standard B Heptanoyl glycine 2.990 mmol/L.	56 mg heptanoyl glycine in 100 ml water Store at -20°C in aliquots in small freezer
Working standard B Heptanoyl glycine 0.299 mmol/L	Dilute 10 ml stock standard to 100 ml with water. Aliquot in 5 ml amounts. Store at -20°C in metabolic storage freezer Aliquot in use at 4°C in metabolic fridge-stable one week – thaw aliquot at beginning of each week and write date thawed on lid of aliquot.

Please make a note in the relevant logs of any standards, QC or reagents made up and any lot changes

Solvent/Acid logs	attached to relevant solvent bins
QC	log in QC and calibration SOP folder
Standard logs	attached to this SOP
Chemicals –	write the date opened on the container Update details on the entry in the reagent database on G drive when a new reagent is received.

j) Calibration procedures and metrological traceability

Internal calibrations should be performed including changing the column and running the hydrocarbons for automatic annotation.

k) Procedural Steps

Sample Preparation

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1. Make a worksheet, the telepath code is OA, ensure the patient details on the request forms and details on the worksheet match. The maximum number on a batch is 10, if more than 10 please check with the metabolic duty senior before proceeding, they will assess the requests and chose 10 based on the age of the patient and the clinical details.
2. Defrost the samples in the water bath and mix thoroughly by inversion, ask AN Other to check samples against the worksheet.
3. Look up the creatinine result in telepath and write the result on the worksheet.
4. Look up the patient details for previous organic acid results, if there are any write the previous chromatography number on the worksheet (in the blank column),otherwise write N/A.
5. Transfer the pH and ketone (ACET2) stix test results to the worksheet. If these results are not available please see SOP 122004 for stix test methods.
6. Write the current chromatography number on the worksheet, refer to the previous GCMS worklist and the extracts on the GCMS carousel for the next available number.
7. Enter the results from the worksheet in to telepath. Reprint the worksheet.
8. Work out the amount of urine to be extracted and the amount of water to be added and write on the far right hand side of worksheet.

Urines are extracted to a creatinine of 2 mmol/L.

Calculation:-

Urine volume (ml) = 2 ÷creatinine (mmol/l)

Creatinine less than 0.8 mmol/l

If the urine volume required exceeds 2.5ml (i.e creat < 0.8) use two tubes or more for the extraction and label them with the number on the worksheet and "**a,b"**, etc Only add internal standards to the tube numbered "**a**". Combine all the extracts for that sample into one tube at the drying down with nitrogen stage.

The drying down of the extract can be started before all the extraction steps are completed, after each extraction step add the extract in the tube with the sodium sulphate added, then decant the extract into the tube that is being dried down under nitrogen.

Creatinine greater than 8.0 mmol/l

Always extract 250µl urine

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Insufficient urine to extract calculated amount
NB please check with senior BMS before using all the sample available as the urine may be needed for other tests.!!!!

Neatly cross out with one line the calculated amount and write the actual amount used. The method for the GCMS will alter according to the amount of urine extracted e.g. if only half the amount of calculated urine volume or less is used then the high sensitivity method is used on the GCMS, otherwise use the OA method

GCMS extract – extracted by other labs
Write patient details on the worksheet and allocate a chromatography number
Add 20 µl pyridine to the extract and transfer into labelled GCMS plastic vial.

Extraction

Label tubes in triplicate (in black) numbered according to the worksheet.
(3 rows of tubes)

1 Using the EDP 2000, pipette the calculated volume of urine into the appropriately labelled clean glass tube, and make up to 2 ml with distilled water. (discard any cracked or broken tubes).

2 Add 6 drops 5M HCL (check pH is less than 2 with pH paper).

3 Add 100 µl internal standard A and 100 µl internal standard B.
NB if more than one tube used only add IS to tube "**a"**

4 Add solid sodium chloride to saturate. Mix on vortex mixer.
Use an initial amount of approx. 1cm depth in the tube, add more if required during the extraction to ensure saturation.

Transfer the analysis to the fume cupboard. Wear purple nitrile gloves

5 Add 2 ml ethylacetate, cap the tubes with a well fitting top containing a plastic seal and mix for 2 minutes in a rack on rotary mixer in fume cupboard.

6 Centrifuge for 3 minutes at 1000 rpm using Juan G4i (ORG program)

7 Using a glass pasteur take off top organic layer into the second labelled tube.

8 Repeat steps 5-7

9 Repeat steps 5-7 but use 2 ml diethyl ether

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10 Dry combined extracts in the second tube by adding approx. 2g anhydrous sodium sulphate.

11 Decant dried extract into the third labelled tube and evaporate to dryness under nitrogen at 37°C in dry block heater, approx 20 minutes

12 To derivatise add 75µl of room temp. BSTFA and 20µl of pyridine to each dried extract, cap and vortex mix.
(Refer to COSHH assessments for BSTFA and pyridine).

13 Place tubes in heating block at 80ºC for 30 minutes (check temperature)

14 Transfer the cooled extracts into GCMS plastic vials labelled with the chromatography number allocated to that sample (see worksheet details). Cap tubes and place on the GCMS carousel – last 2 numbers of the chromatography number refer to the position on the carousel

Leave the glass extraction tubes in the fume cupboard overnight. Next morning the tubes can be placed in a bucket of water and sent for washing if there is no smell of solvent.

Writing the GCMS sample list

Sample information is entered into the batch table.
Method name is 'OA' and this injects a volume of 2 µl.
Place the request forms and worksheet on the desk next to the VDU screen for the GCMS.
The printed out worklist for the GCMS should be checked by another member of staff e.g. Clinical Scientist / qualified BMS. and the GCMS set to run. This person must wait until the first sample has been injected, the initialled worksheet and attached request forms can then be placed on the shelf next to the VDU.

GCMS Aquisition parameters

For details see GCMS Methods in GCMS SOP file in B1 Copies of the methods are backed up on the hard drive.

l) principle of the procedure for calculating results including, where relevant the measurement of uncertainty of measured values

N/A This is a qualitative method

m) Instructions for determining quantitative results where a result is not within the measurement interval

N/A This is a qualitative method.

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n) Quality Control procedures

Internal Quality Control
Blank - monthly (extract 2mL water)
MCAD QC - monthly, extract 250µL urine. (Stored in metabolic assays freezer).

Refer to the document on internal QC and calibration material for further details. SOP 129005 and 129005_1

The abundance for the internal standards and the abnormal acylglycines for the IQC are recorded. See SOP 124204 for acceptance criteria for the MCAD and blank QC.

EQA
ERNDIM Qualitative Organic Acid Scheme – Heidelberg
This is a qualitative scheme where the abnormal metabolites have to be identified and a diagnosis made and clinical advice given. There are three sets of three samples per year. Reports for this scheme are located on the shelves in the metabolic “office” area.

Post -examination process

The Chromatogram and the “macros” are printed out when the batch has finished running (usually first thing the next morning so that any abnormalities or problems arising can be dealt with ASAP). The macros are a series of selective ion searches set up to be carried out automatically by the software.

The metabolites looked for are :	Short form
Heptanoylglycine.	Hept Gly
Hexanoylglycine	Hex Gly
Suberylglycine	Sub Gly
Isovalerylglycine	IVG
Phenylpropionylglycine	PPG
Tiglylglycine	Tig Gly
3-methylcronylglycine	3 Me Crot Gly
Isobutyrylglycine	IBG
4-OH Butyrate	4HB
Orotic acid	ORT
Succinylacetone	Succac
Succinylacetoacetate	Succacetoac
3-OH Glutarate	3OH Glut
Mevalonolactone	Mev
3,6 epoxytetradecanedioate	3,6,epoxy

The chromatograms and their accompanying “macro” print out are then stapled together and placed with the request form. Write the age and clinical details on the

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left hand side of the chromatogram just below the details for the laboratory number etc. Make a note of the relevant IS abundance and any acyl glycines detected on the "macros".
Give the chromatograms and request forms to a clinical scientist/metabolic senior BMS to check that there are no significant abnormalities or any that need repeating. The chromatograms can then be placed in the basket for annotation check in the GCMS room.

o) Biological reference intervals or clinical decision values
refer to SOP 124204

p) Reportable interval of examination results
N/A

q) Alert/critical values, where appropriate
refer to SOP 124204

r) Laboratory clinical interpretation

Reporting
Profiles are reported by a Clinical Scientist.

s) Potential sources of variation
Changes in the column over time
Aging of the liner
Time of specimen collection.
Deterioration of the sample.

t) References

Diagnosis of Organic Acidaemias by Gas Chromatography Mass Spectrometry
Goodman and Markey, Alan R Liss, Inc., New York, 1981.

Organic Acids in Man: Analytical Chemistry, Biochemistry and Diagnosis of the Organic Acidurias
Chalmers and Lawson, Chapman and Hall, 1982.