GCMS Technical Overview & use for VLCFA

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What are we going to cover?

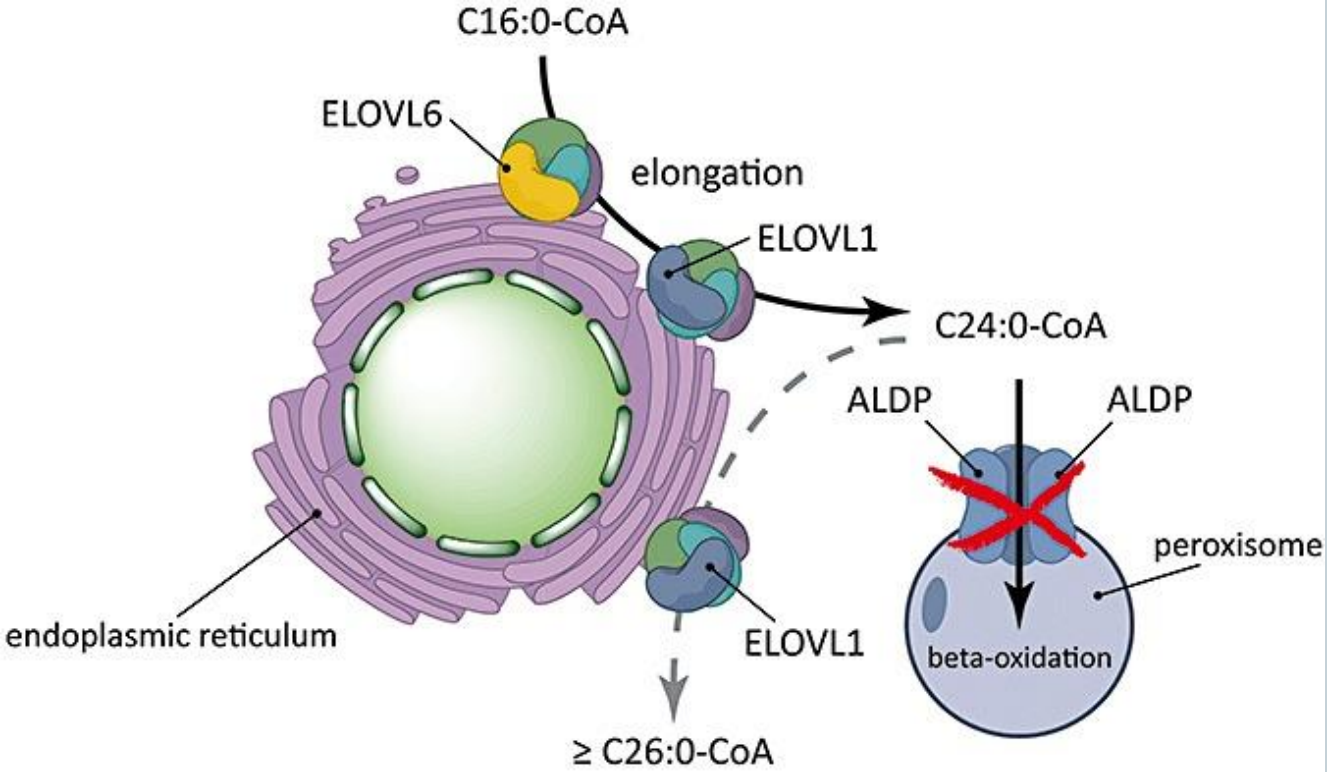
- What are VLCFAs?
- Disorders associated with VLCFAs
- Brief overview of our method
- GCMS basics
- Isotope Dilution Mass Spectrometry
- Validation and Troubleshooting

VLCFA's – What are they?

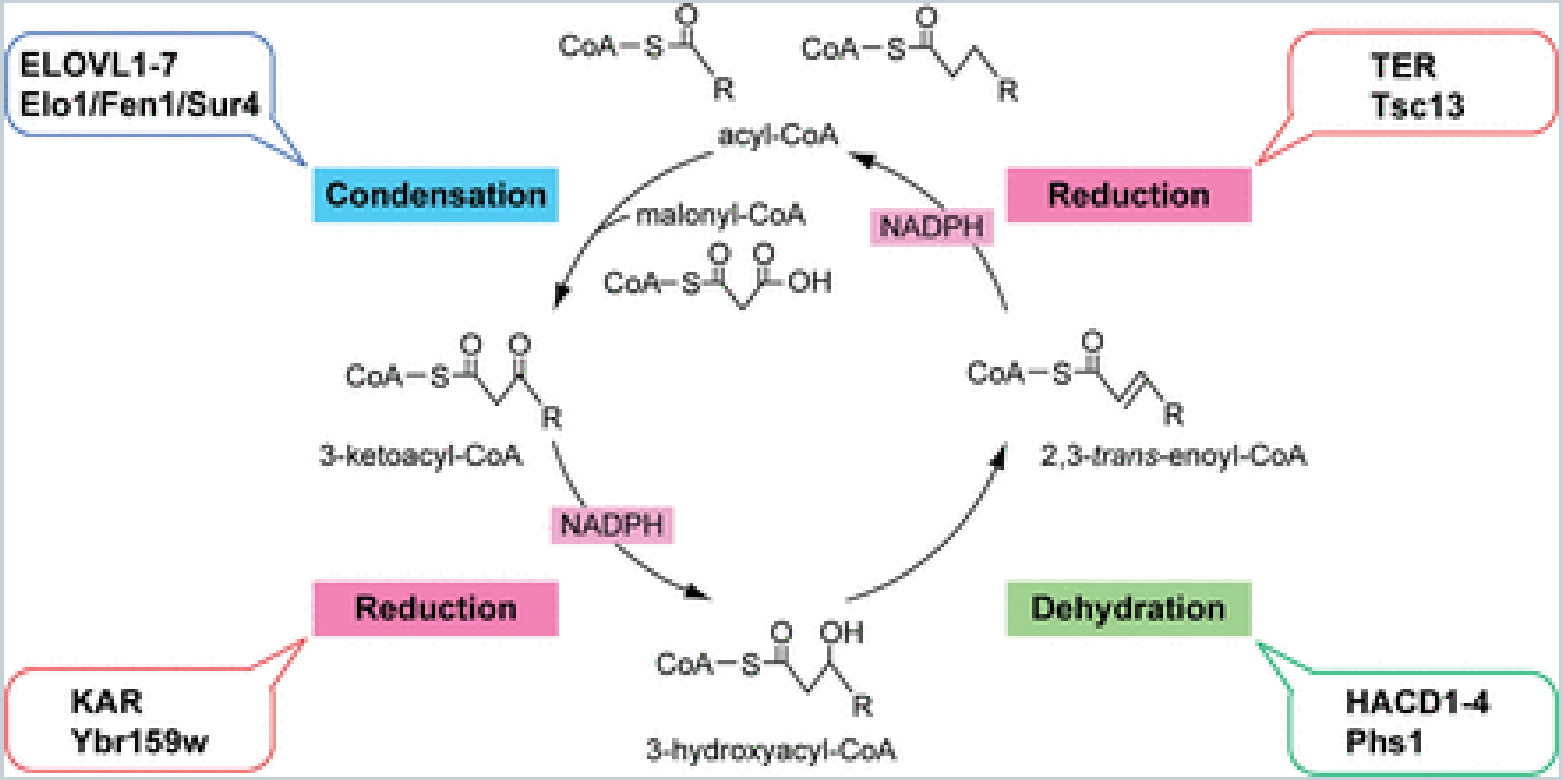
- VLCFA's are essential components of cell membranes.
- They are present in many foods but are also produced in the body by chain elongation of shorter fatty acids to longer fatty acids.
- In healthy patients, VLCFAs are catabolised by conversion to CoA esters and then degraded via β-oxidation exclusively in the peroxisomes.
- The analysis of VLCFA in plasma is therefore an important investigation in suspected peroxisomal disorders.

VLCFA's – What are they?

- Abnormal levels of VLCFA's in blood and tissues are caused by inherited peroxisomal disorders.



VLCFA's – What are they?



Fatty Acid Elongation Cycle

Disorders associated with VLCFAs

- Disorders of peroxisome biogenesis: *Zellweger syndrome, neonatal adrenoleukodystrophy (ALD)* and *infantile Refsum disease.*
- Disorders of peroxisomal beta-oxidation: *Pseudo-Zellweger syndrome and pseudoneonatal ALD.*
- *Rhizomelic chondrodysplasia punctata*
- *X-linked ALD*
- *Refsum disease*
- *α-Methyl-acyl-CoA racemase*

Disorders associated with VLCFAs

- Clinical features of these disorders include:

- Neurological abnormalities – encephalopathy, hypotonia, seizures, deafness and abnormal MRI scans.
- Skeletal abnormalities – short proximal limbs, calcific stippling.
- Dysmorphic features – craniofacial abnormalities (severe forms)
- Hepatointestinal dysfunction – neonatal hepatitis, hepatomegaly, cholestasis, cirrhosis etc. (severe forms).

Expected results from VLCFAs

Disorder	C22	C24	C26	Phytanate	Pristinate
Peroxisomal biogenesis disorders	↓ - Normal		↑↑	↑	↑
Peroxisomal beta-oxidation disorders	↑	↑	↑		
X-linked ALD	↑	↑	↑	N	N
Rhizomelic chondrodysplasia punctata	N	N	N	↑	↓- Normal
Refsum disease (adult)	N	N	N	↑	↓
α-Methyl-acyl-CoA racemase	N	N	N	↑	↑

GCMS – The Basics

- GCMS is the analysis method of choice for simple molecules such as steroids, fatty acids and hormones.
- GC = Gas chromatography – the sample is vaporised and separated using a capillary column packed with a stationary phase. As the components separate, they elute from the column at different times, known as their retention time.
- MS = Mass spectrometry – The eluted samples are ionised by the mass spectrometer and accelerated through the quadrupole mass analyser. The ions are then separated based on their different *m/z* ratio.

Isotope Dilution Mass Spectrometry

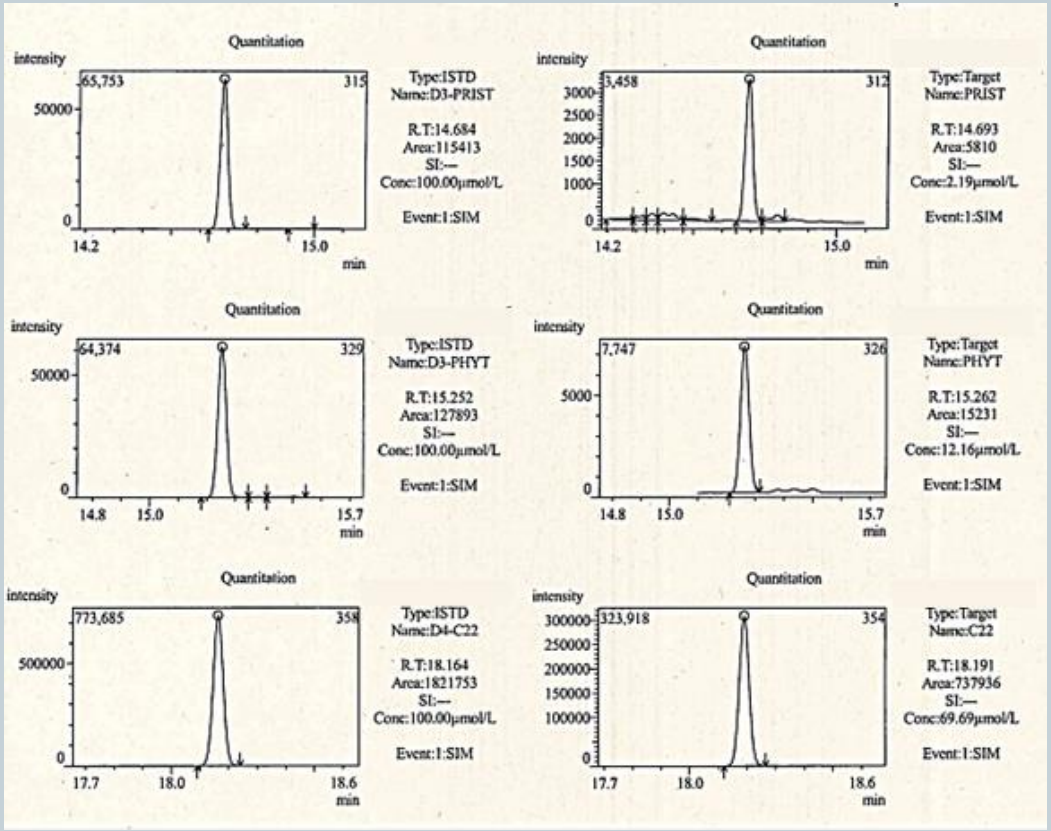
- We use isotope dilution mass spectrometry to determine VLCFAs in plasma.
- This involves adding known amounts of an isotopically-enriched substance to the patient sample.
- This addition effectively ‘dilutes’ the isotopic enrichments of the standard.
- This new isotope composition in the mixture is then measured on the GCMS where it provides the concentration of the analyte in the sample after simple calculations.

VLCFA analysis on the GCMS

- Our method involves the addition of stable isotope internal standards to patient samples.
- Samples are then methylated and the fatty acids are extracted from plasma using methanol/benzene.
- Acetyl chloride is used to transfer methyl groups to the fatty acids followed by the addition of an aqueous solution.
- The extracts are then removed and analysed on the GCMS.

VLCFA analysis on the GCMS

- The isotope internal standards allows for quantitation on GCMS.
- The peak area ratios for a characteristic fragment ion for each native compound and it's stable isotope are used to read off standard curves giving concentrations for plasma samples.



Method Validation

- In 2017, we started method validation to allow the VLCFAs to be run on a new analyser (Shimadzu 2020).
- To meet ISO 15189 standards the following method validation and verification criteria had to be met:
  - LoB, LoD, and LoQ
  - Linearity
  - Inter and Intra assay precision
  - Bias
  - Carry over
  - Patient comparison

Method Validation – Limit of detection and Linearity

The VLCFA assay consists of five measured parameters (C22, C24, C26, phytanate and pristanate) and two calculated ratios (C24/22 and C26/22).

- Linearity:

C22	200µmol/L
C24	200µmol/L
C26	200µmol/L
Phytanate	200µmol/L
Pristanate	160µmol/L

- Limit of detection and quantitation:

  Limit of blank (LoB) = blank mean + 1.645*(blank SD)
  Limit of detection (LoD) = limit of blank + 1.645*(SD low concentration sample)
  Limit of quantitation = lowest concentration at which minimum criteria for imprecision is met (in this case defined to be %CV <40%)

Method Validation – Intra and Inter Precision

- Intra and Inter Assay Precision – Intra precision describes the variation of results with a data set obtained from day (i.e. the 10 results). Inter precision describes the variation of all the results obtained over the 10 days.

- All %CV for the intra and inter precision were ≤10% for all parameters which indicated acceptable precision for a manual assay.

Method Validation – Bias

- Bias was assessed by running 10 EQA samples from the ERNDIM special assays serum scheme and comparing the results with the method mean.
- All analytes showed good correlation and a level of bias that is acceptable.

Method Validation – Carry Over

- To assess carry over on the GCMS, a blank initial sample was run followed by then alternating a blank sample with 5 high calibrants.
- There was no effect on the C22, C24, Phytanate and Pristanate.
- C26 showed a small increased (0.15 µmol/L), however the effect was so small that it is non-physiological.

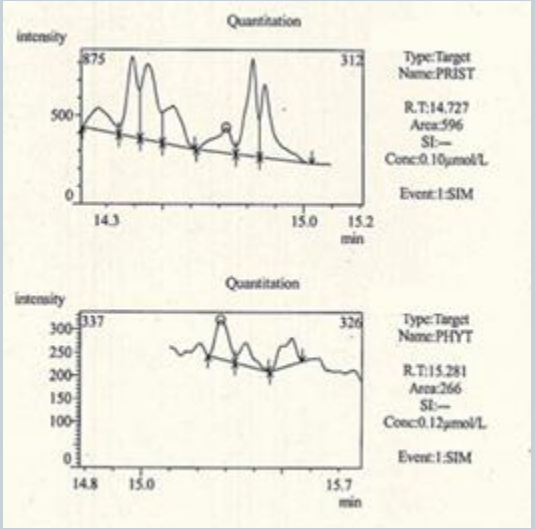
Method Validation – Patient Comparison

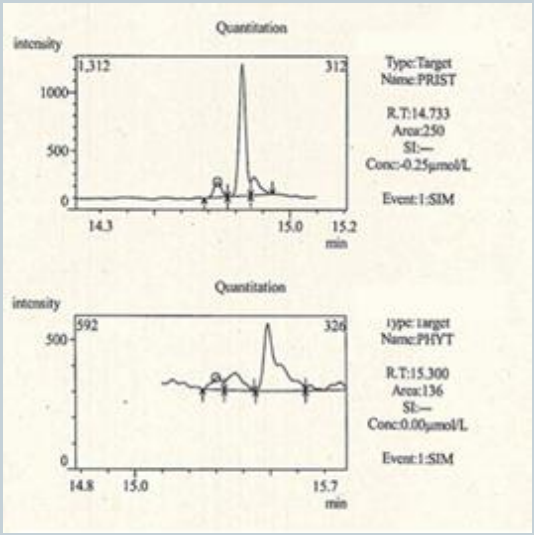
- 34 Patient sample were run on both the old method and the new method in order to assess comparability of results.
- Results were very comparable (see below)

Analyte	R^2	Bias constant	Slope	Proportional Bias	%bias
C22	0.998	-0.10	1.023	0.023	2%
C24	1.00	0.34	1.020	0.020	2%
C26	0.999	0.03	0.876	-0.12	-2%
Phytanate	0.998	-0.47	1.006	0.006	1%
Pristanate	0.956	0.01	1.069	0.069	6.9%

Method Troubleshooting

- Sporadic results – e.g. high C26 that when repeated is normal. These cases are usually caused by unclean glass vials.
- Messy baselines on ‘blank’ samples – caused by ‘gone off’ reagent.





Thank you for listening ☺

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