Amino Acid Analysis: Ion Exchange vs Liquid Chromatography Tandem Mass Spectrometry

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Why do we analyse Amino Acids?

- Diagnosis of inherited defects of amino acid catabolism, biosynthesis or transport
- Analysis of amino acids needs to be able to detect increased and decreased concentrations of amino acids in biological fluid
- Consider amino acid analysis in a range of clinical situations
  - Hyperammonaemia
  - Lethargy, coma, seizures, vomiting
  - Metabolic acidosis/lactic acidosis
  - Metabolic decompensation
  - Unexplained developmental delay
  - Follow up of NBS
  - Dietary monitoring of patients with known IMD
What do clinicians want from AA analysis?

“Always interested in timely result, especially same day in acutely unwell patient. Needs to be accurate. Cost is secondary”

“TAT and a rapid screen for treatable amino acidopathies”

**Analytical requirements?**
- No formal acceptance criteria
- Precision
- Accuracy
- Traceability
- Robust system
- Capable of rapid TAT
- Clear, unambiguous interpretation of results for clinicians
## Routinely used methods for Quantitative Plasma Amino Acid Analysis

### Data from ERNDIM Quantitative Amino Acid Scheme

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>LC-MS/MS</td>
<td>0</td>
<td>2.8%</td>
<td>9.9%</td>
<td>12.0%</td>
<td>23.4%</td>
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<tr>
<td>RP-HPLC</td>
<td>12.7%</td>
<td>13.5%</td>
<td>14.0%</td>
<td>13.7%</td>
<td>14.0%</td>
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<tr>
<td>IEC</td>
<td>85.8%</td>
<td>82.3%</td>
<td>70.8%</td>
<td>66.8%</td>
<td>54.0%</td>
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<td>LC-MS</td>
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<td></td>
<td></td>
<td></td>
<td>5.7%</td>
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<tr>
<td>Other</td>
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<td>1.1%</td>
<td>5.3%</td>
<td>6.6%</td>
<td>3.0%</td>
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<tr>
<td><strong>Total participants</strong></td>
<td><strong>134</strong></td>
<td><strong>178</strong></td>
<td><strong>243</strong></td>
<td><strong>241</strong></td>
<td><strong>265</strong></td>
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Following User Survey in 2017, method group data being reviewed
Includes a statistical comparison of method groups
Ion Exchange Chromatography (IEC)

- Traditionally IEC referred to as the gold standard methodology
- Routinely used in the clinical laboratory
- Cation exchange chromatography with post column ninhydrin detection
- Stepwise elution of a series of lithium citrate buffers
- Start with acidic buffer – amino acids retained by the resin
- Then increase the pH and ionic strength with each step
- More acidic species elute first
- Post column derivitisation – eluent from the column is mixed with ninhydrin in the reaction coil at 135°C
- Dual detection at 440nm (yellow) and 570 nm (purple)
Ion Exchange Chromatography

Amino Acid Chromatogram
Advantages of Ion Exchange Chromatography

• Minimal sample prep required - no derivitisation step
• Stable and precise
• Large dynamic range, approx 3 -4 orders magnitude
• Suitable for sulphur containing amino acids
• Separates citrulline from ammonia
• Identifies all analytes of interest
• Identifies atypical amino acids
• Commercial kit (reagent rental)
• In routine use for a number of years
Disadvantages of Ion Exchange Chromatography

• Long analysis time ~ 130 min for standard profile
• Dedicated instruments, often running at capacity
• Structural analogue internal standard
• Single point calibration, sporadic frequency
• Method lacks specificity
  • identification based on Rt alone
  • co-eluting substances
  • interferences from drugs/ninhydrin positive compounds
  • poor resolution of some analytes e.g. sulphocysteine
• Operator experience
• Identifies atypical AA
• Manufacturer promotes ‘accelerated’ 90 minute method but ? lacks robustness
Co-eluting Compounds: Phenylalanine & 5-ALA

<table>
<thead>
<tr>
<th>Scale Standard Deviations</th>
<th>Scale µmol/L</th>
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<tbody>
<tr>
<td>&gt;3SD</td>
<td>&gt;43.0</td>
</tr>
<tr>
<td>2-3SD</td>
<td>37.0 - 43.0</td>
</tr>
<tr>
<td>1.5 - 2.0SD</td>
<td>34.1 - 37.0</td>
</tr>
<tr>
<td>1.0 - 1.5SD</td>
<td>31.1 - 34.1</td>
</tr>
<tr>
<td>0.5 - 1.0SD</td>
<td>28.1 - 31.1</td>
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<tr>
<td>0.0 - 0.5SD</td>
<td>25.1 - 28.1</td>
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<tr>
<td>-0.5 - 0.0SD</td>
<td>22.2 - 25.1</td>
</tr>
<tr>
<td>-1.0 - -0.5SD</td>
<td>19.2 - 22.2</td>
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<tr>
<td>-1.5 - -1.0SD</td>
<td>16.2 - 19.2</td>
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<tr>
<td>-2 - -1.5SD</td>
<td>13.3 - 16.2</td>
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<td>-3 - -2SD</td>
<td>7.31 - 13.3</td>
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<tr>
<td>&lt;-3SD</td>
<td>&lt;7.31</td>
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</table>

**All Labs results**

<p>| | |</p>
<table>
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<tr>
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<tbody>
<tr>
<td><strong>n</strong></td>
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<tr>
<td><strong>Mean</strong></td>
<td>25.1</td>
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<tr>
<td><strong>Median</strong></td>
<td>25.0</td>
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<tr>
<td><strong>SD</strong></td>
<td>5.95</td>
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</table>
Co-eluting Compounds: Methionine & Homocitrulline
Sulphocysteine?

570:440 ratio Scys = 4.3, patient ratio  = 10.0
Spike sample with Scys and reanalyse
Dedicated short IEC program does exist ~50 mins
Just another interfering compound...
• Derivitisation of sample to produce a stable and well retained product which ionises well e.g. Applied Biosystems iTRAQ kit
• Derivatives separated by reverse phase HPLC C18 column (2.1x100x1.6um)
• Gradient elution
• Mobile phase is ACN/formic acid/water with ion pair reagent
• Detection with triple quad MS
• Ability to quantitate 45+ amino acids in single injection
Example of full profile amino acid chromatogram by LCMSMS

Representative chromatogram showing analysis of 45 amino acids using aTRAQ™ reagents and LC/MS/MS analysis. Total run time is 18 minutes.
Advantages of LCMSMS

• Rapid analysis time e.g. 18 mins
• Stable isotope internal standards
• Superior specificity - combination of chromatographic separation and MRM detection enables identification of most isobaric compounds
• Stable and precise
• Suitable for sulphur containing amino acids
• Commercial kits are available that include columns, reagents, standards and controls e.g. Applied Biosystems iTRAQ,
• Ability to analyse multiple amino acids in a single injection
Disadvantages of LC-MS/MS

- Smaller linear range
- Not as precise as IEC – between batch CV ~ 10% vs 5% IEC
- Derivitisation required
- Ion pair reagent results in a dedicated LC-MS/MS system
- Limited number of MRMs in a given experiment – only see selected amino acids
- Isobaric compounds e.g. isoleucine/leucine/allo/hydroxyproline
- Operator expertise
- Cost (instrument and stable isotopes)
BUT
The disadvantages of LC-MS/MS are being overcome

- Technology has improved in last 5 years
- Instruments have faster scan speeds/shorter dwell times/improved pos neg switching
- A given experiment can contain more MRMs and still produce quantitative data
- Analysis of full amino acid profile is possible **without** sample derivitisation or use of an ion pair reagent
- **Open access instrument** - other analytes can be measured simultaneously
Plasma Amino Acids by LC-MS/MS at Viapath

• Method validated on Waters Xevo TQS with Acquity UPLC
• Comprehensive amino acid profile
• 15 minute run time
• 6 time functions, 5 positive ionisation, one negative ionisation
• 37 analytes, 26 stable isotope internal standards
• Total of 63 MRMs
• Using SpotOn reagents (calibrators, QC and stable isotopes)
• Simple sample prep; protein crash, no derivitisation required
• Chirobiotic column with 50% Acetonitrile, 0.025% formic acid mobile phase

• Method introduced into routine use June 2018
• Sacrificed sensitivity and specificity of some analytes to optimise performance of others
• Specificity achieved by use of MRMs for most analytes
• Some analytes also required chromatographic resolution
• Some analytes required other techniques to be employed
  » alternative daughter ions
  » sub-optimal collision energy
  » correction factor
• Challenge was optimising method to get adequate sensitivity and specificity for wide range of analytes, with concentration range of 3 orders magnitude whilst maintaining a rapid analysis time
**METHIONINE**

**Methionine: Biochrom vs Xevo**

- **Equation:** $y = 1.107x - 3.2815$
- **$R^2$:** 0.9983

**Methionine: Well Child vs Viapath**

- **Equation:** $y = 0.9956x + 0.74$
- **$R^2$:** 0.9979
Potential Pitfalls & Limitations

- Lack of stable isotope internal standards for some compounds
  - External cals (ASA & anhydride, carnosine, anserine)
  - Non ideal IS (homocystine, sarcosine, cystathionine, 1 & 3-methylhistidine)
- Linearity is analyte dependent, some analytes non linear at 500uM
- Sensitivity of smaller molecules e.g. glycine and alanine
- Isobaric compounds e.g allo & iso
- Require 2nd line testing of any samples with increased ‘total isoleucine’
- Not a dedicated instrument
Conclusion

- Advantages of LC-MS/MS outweigh the disadvantages
- Rapid analysis time
- Ability to analyse clinically urgent samples
- Ability to generate SD plot for every sample automatically
- Backlogs easily rectifiable!
- Next step is addition of other analytes