An HPLC-MS/MS Method for the Quantification of Serum Methylmalonic Acid (MMA).

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Background

- Vitamin B12 deficiency has an estimated prevalence of 10.8% in the USA, with 2 – 3% of >60 year olds thought to be deficient1,2.
- Deficiency of B12 can result in haematological dysfunction and potentially irreversible neurological impairment.
- Serum B12 is the most common clinically used marker for deficiency but has suboptimal sensitivity2,3.
- B12 depletion results in accumulation of methylmalonic acid (MMA) and homocysteine.

Aim: Validate a method to quantify serum MMA by LC-MS/MS.

Methods

- Samples spiked with internal standard were passed through a Waters OSTR™ protein precipitation and phospholipid removal plate.
- Chromatographic separation was optimised using the ACQUITY UPLC T3 100 x 2.1 mm column.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow rate (mL/min)</th>
<th>%A (0.3% Formic (aq))</th>
<th>%B (0.1% Formic (MeOH))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0.2</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>3.0</td>
<td>0.2</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>3.7</td>
<td>0.2</td>
<td>98</td>
<td>2</td>
</tr>
</tbody>
</table>

- Mass detection was performed with the Xevo™ TQ-S operating in negative electrospray ionisation (ESI-) mode.
- Phospholipids were detected in positive ESI mode with daughter scans (parent m/z 350 to 800 daughter m/z 184).
- Matrix effects were determined by calculating the Matrix Factor percentage in six individual serum samples.

Table 1: Inert method validation.

Matrix Factor = Response in the presence of matrix / Response in the absence of matrix.

Results

Chromatography

- Figure 2: Chromatographic separation of isobaric MMA and succinic acid - <6 minutes injection to injection.
- Figure 3: Phospholipid daughter scan of serum sample (red trace) with (A) protein precipitation or (B) with protein precipitation with OSTRO™ demonstrating the removal of phospholipid interferences from the serum sample.

Sample Preparation

- Interference testing demonstrated lithium heparin plasma samples may also be used with this method.
- Mean Matrix Factor = 84% (range 70-91%) demonstrating 16% ion suppression.

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Calibration

- Linearly assess the MMA spiked serum covering a range of 0.09 – 1.5 µmol/L, n = 4 per concentration.

Calibration curve

- Figure 5: Calibration curve. 1/1 X weight to account for heteroscedasticity seen in linearity experiments. Range = 0.09 – 1.4 µmol/L, n = 4.

Imprecision and recovery of lower concentration calibrators of MMA spiked PBS, n = 6.

<table>
<thead>
<tr>
<th>Type</th>
<th>Specimen</th>
<th>Concentration (µmol/L)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low cal 50%</td>
<td>Spiked PBS</td>
<td>≤4%</td>
<td>2.3</td>
</tr>
<tr>
<td>Low cal 50%</td>
<td>Inject after 24 hrs</td>
<td>≤4%</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Ninhydrin method

- Figure 6: Method comparison with a reverse-phase MS/MS method (n = 53). Denoting regression analysis revealed -20% constant bias, p=0.005.

Table 2: Method validation criteria and results.

Conclusion

- Partial validation of a method to quantify serum MMA by LC-MS/MS.
  - Successfully covers recognised decision limits (<0.27 µmol/L for repletion, >0.37 µmol/L for deficiency)2.
- Chromatographic separation of MMA and succinic acid.
  - Uses reverse-phase methodology for easy implementation in clinical laboratories.

Future work

- Complete validation experiments.
  - Expand linearity assessment >1.4 µmol/L and perform accuracy experiments with certified reference material (CRM) to determine cause of method comparison bias.
- Develop method to measure homocysteine in tandem.
  - Use of multiple markers are known to improve clinical performance2.

Acknowledgements

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