Introduction

The major water soluble vitamins include Vitamin B1 (Thiamine), Thiamine Pyrophosphate), Vitamin B2 (Riboflavin), Vitamin B3 (Nicotinic Acid and Nicotinamide), Vitamin B5 (Panthenic Acid, Pantethine and Pantenol), Vitamin B6 (Pyridoxal, Pyridoxine and Pyridoxal-5-Phosphate), Vitamin B7 (Biotin), Vitamin B9 (Folic Acid, Folinic Acid and 5-Methyltetrahydrofolate) and Vitamin B12 (Cyanocobalamin, Adenosylcobalamin, Methylcobalamin and Hydroxocobalamin).

Therefore, we developed LC/MS/MS analytical methods and evaluated various columns and solvent combinations in order to demonstrate the chromatographic separation, detection and quantification of the water soluble vitamins in blood. The sample preparation was a simple protein precipitation and the methodologies were developed on an Agilent 1200 HPLC and 6460 Mass Spectrometer with a 8 minute analytical gradient method in positive ionization mode.

Experimental

Sample Preparation

- 200 µL of serum sample, calibrators, controls was taken and 20 µL ISTD at 1000 ng/mL were added to each
- 100 µL of 5% Trichloroacetic Acid was added to each tube and vortexed for 5 minutes prior to centrifugation for 10 minutes at 13000 g
- 25 µL of 1N Sodium Hydroxide was added to quench the acid and prevent damage to the column
- The supernatant was transferred to MS vials for analysis

HPLC Conditions

Agilent 1260 Infinity HPLC series binary pump, well plate, thermostatted column compartment

Column:
Agilent Technologies Poroshell 120, SB-AQ, (or Extend) 3 x 100 m, 2.7 µm
Agilent Technologies Poroshell 120 Fast Guard 2.1 x 5 mm

Column Temperature:
25°C

Injection Volume:
5 µL

Autosampler Temperature:
4°C

Needle Wash:
Flush port (50%MeOH:50%Water) 5 seconds

Mobile Phase A:
0.1% Formic Acid+5mM Ammonium Formate Water

Mobile Phase B:
100%A:5%B

Flow Rate:
0.5 ml/min

Gradient:
0 min - 100%A:5%B
6 min - 5%A:95%B
13 min - 5%A:95%B
18 min - 5%A:95%B

Run/Stop time:
8 minutes/3 minutes

Method

LC/MS/MS Quantitative Analysis of Water Soluble Vitamins in Blood

Reagents

Biotin: Isosciences Biotin-13C6,15N1: Isosciences
Riboflavin: Isosciences Riboflavin-13C4,15N2: Isosciences
Pyridoxal: Isosciences Pyridoxal-2H3: Isosciences
Thiamine: Isosciences Thiamine-13C4: Isosciences
Pyridoxamine: Isosciences Pyridoxamine-2H3: Isosciences
Nicotinamide-13C3,15N: Isosciences
Nicotinamide-13C3,15N: Isosciences
Biotin: Isosciences Biotin-2H8: Isosciences
Sodium Hydroxide: Sigma-Aldrich
Pantothenic Acid: Isosciences Pantothenic Acid-13C3: Isosciences

Standards/Calibrators

Thiamine: Thiamine-13C4,15N2: Isosciences
Riboflavin: Riboflavin-13C4,15N2: Isosciences
Pyridoxal: Pyridoxal-2H3: Isosciences
Thiamine: Thiamine-13C4,15N2: Isosciences
Nicotinamide-13C3,15N: Isosciences
Nicotinamide-13C3,15N: Isosciences
Biotin: Biotin-2H8: Isosciences
Folic Acid: Sigma-Aldrich Folic Acid-13C6: Isosciences
Folic Acid: Sigma-Aldrich Folic Acid-13C6: Isosciences

Folate: Sigma-Aldrich
Pantothenic Acid: Sigma-Aldrich
Hydroxocobalamin: Sigma-Aldrich
Adenosylcobalamin: Sigma-Aldrich
Methylcobalamin: Sigma-Aldrich
Cyanocobalamin: Sigma-Aldrich
Thiamine Pyrophosphate: Sigma-Aldrich
Pyridoxal-5-Phosphate: Sigma-Aldrich

Results and Discussion

Table 1: MRM Acquisition Table - * Quantifier Ion

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
<th>Compound</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>0.1</td>
<td>0.25</td>
<td>Pyridoxine</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Thiamine Pyrophosphate</td>
<td>10</td>
<td>25</td>
<td>Pyridoxal-5-Phosphate</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>5</td>
<td>10</td>
<td>Biotin</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.1</td>
<td>0.25</td>
<td>Folic Acid</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.1</td>
<td>0.25</td>
<td>Folic Acid</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>Pantethine</td>
<td>2.5</td>
<td>5</td>
<td>5-Methyltetrahydrofolate</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Pantothanol</td>
<td>0.25</td>
<td>0.5</td>
<td>Cyanocobalamin</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>Pantotheine</td>
<td>1</td>
<td>2.5</td>
<td>Adenosylcobalamin</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>0.25</td>
<td>0.5</td>
<td>Methylcobalamin</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>0.1</td>
<td>0.5</td>
<td>Hydroxocobalamin</td>
<td>0.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Table 2: Sensitivity

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Thiamine Pyrophosphate</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Riboflavin</td>
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<td>10</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Pantethine</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>Pantothanol</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Pantotheine</td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Conclusions

- Baseline separation of the water soluble vitamins was achieved within a 8 minute run on a Poroshell 120 SB-AQ column.
- Excellent linearity (>99%) of calibration curves with great accuracy, precision and reproducibility was achieved for all vitamins
- Further evaluate different sample preparation techniques and water soluble vitamin free serum matrices available to determine which gives the best results while maintaining low cost and ease of use.
- Further evaluate the different Water Soluble Vitamins per a group method rather than a global method for better analytical needs, chromatography and results.

For research use only. Rat’s treated with Mifepristone (RU-486) and/or Naltrexone were compared with sham operated controls. Data was normalized to a sham control and graphed as a percent effect relative to sham control.