Introduction

The major water soluble vitamins such as Vitamin B1 (Thiamine), Vitamin B2 (Riboflavin), Vitamin B3 (Nicotinic Acid and Nicotinamide), Vitamin B5 (Pantothenic Acid), Vitamin B6 (Pyridoxamine, Pyridoxal, and Pyridoxine), Vitamin B7 (Biotin), Vitamin B9 (Folic Acid) and Vitamin B12 (Cyanocobalamin) are essential nutrients required for normal body functioning that can either cannot be synthesized by the body at all or in insignificant amounts. These compounds are synthesized by the body at all or in insignificant amounts. These compounds are acquired from the diet and can be toxic in large doses and can cause significant medical issues when deficient.

A simple, sensitive, specific and accurate quantitative analytical method was developed for the chromatographic baseline separation and measurement of the water soluble vitamins in human serum. A Poroshell 120 EC-CN column on an Agilent 1200 HPLC and 6460 Mass Spectrometer system was used for this method.

Experimental

Reagents, Standards, Calibrators and Controls

The following standards were obtained from Isolociences Standards
- Biotin
- Pyridoxal
- Pyridoxine
- Pyridoxamine
- Riboflavin
- Thiamine
- Riboflavin-13C4, 16N2
- Pyridoxamine
- Pyridoxine-2H3
- Biotin-2H8
- Pantothenic Acid-13C3,16N1
- Pyridoxal-2H3
- Nicotinic Acid
- Biotin
- Folic Acid
- Cyanocobalamin

The following standards were obtained from Cerilliant
- Nicotinic Acid
- Nicotinamide
- Pantothenic Acid

The following standards were obtained from Sigma-Aldrich
- Thiamine
- Pyridoxine
- Riboflavin
- Folic Acid
- Cyanocobalamin

Sample Preparation

- 200 μl of serum sample, calibrators, controls was taken and 10 μl ISTD at 1000 ng/ml were added to each
- 400 μl of HPLC grade Water was added to each tube and vortexed briefly prior to centrifugation for 10 minutes at 13,000 rpm
- The supernatant was transferred to MS vials for analysis
- All in-house calibrators were prepared in DC Mass Spec Gold Serum (Golden West Biological, Inc)

Method

HPLC Conditions

Agilent 1200 Infinity HPLC series binary pump, well plate, thermo-statted column compartment
- Column: Agilent Technologies Poroshell 120, EC-CN, 2.1 x 100 mm
- Column Temperature: 25 °C
- Injection Volume: 5 μl
- Autosampler Temperature: 4 °C
- Needle Wash: Flush port (60%MeOH:50%Water) 5 seconds
- Mobile Phase A: 0.1% Formic Acid+5mM Ammonium Formate Water
- Mobile Phase B: 0.1% Formic Acid in Methanol
- Flow Rate: 0.3 ml/min
- Gradient: 0 min- 100%A:95%B
- 0 min- 5%A:95%B
- Run/Stop time: 5 minutes/3 minutes

MS Conditions

Agilent 6460 Triple Quadruple Mass Spectrometer- Dynamic MRM
- Ion mode: Agilent Jet Stream Positive Mode
- Gas Flow: 8 L/min
- Nebulizer: 38 psi
- Sheath Gas Temperature: 400°C
- Sheath Gas Flow: 112 L/min
- Capillary Voltage: 21000V
- Nozzle Voltage: 0V
- Q1/Q2 Resolution: 60 / 0.7 unit
- Delta EMV/CAV: +400V/2

Results and Discussion

Linearity

The assay was linear over the calibration curve shown in the table below with a mean of coefficient of determinations (R2) > 0.998

<table>
<thead>
<tr>
<th>Compound</th>
<th>Curve Range (ng/ml)</th>
<th>LOD/LOQ (ng/ml)</th>
<th>S/N</th>
<th>%CV C1 2.5 ng/ml</th>
<th>%CV C2 25 ng/ml</th>
<th>%CV C3 250 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>0.1 - 1000</td>
<td>0.1</td>
<td>812.3</td>
<td>6.42</td>
<td>3.59</td>
<td>2.92</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>0.1 - 1000</td>
<td>0.1</td>
<td>270.3</td>
<td>7.23</td>
<td>3.65</td>
<td>2.45</td>
</tr>
<tr>
<td>Pyridoxal</td>
<td>0.25 - 500</td>
<td>0.25</td>
<td>220.9</td>
<td>11.2</td>
<td>6.58</td>
<td>4.56</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.1 - 1000</td>
<td>0.1</td>
<td>775.1</td>
<td>7.62</td>
<td>4.57</td>
<td>1.66</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.1 - 1000</td>
<td>0.1</td>
<td>67.9</td>
<td>9.89</td>
<td>4.63</td>
<td>N/A</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>0.1 - 1000</td>
<td>0.1</td>
<td>67.7</td>
<td>7.2</td>
<td>3.6</td>
<td>2.89</td>
</tr>
</tbody>
</table>

Conclusions

- Baseline separation of the water soluble vitamins was achieved within a 5 minute run on a Poroshell 120 EC-CN column. Other columns were evaluated but did not offer the same degree of fast separation
- Excellent linearity (>998) of calibration curves with great accuracy, precision and reproducibility was also achieved down to low clinical levels for the majority of the analytes except for Folic Acid, Riboflavin and Nicotinic Acid
- Further investigation into the best sample preparation will be carried out in order to achieve lower LOD and to achieve consistent results for all the clinically relevant water soluble vitamins

References

- Quantitative Analysis of Water-Soluble B-Vitamins in Cereal Using Rapid Resolution LC/MS/MS
- Multi-analyte Quantification of Vitamin B6 and B12 species in the Nanomolar Range in Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry
  Clinical Chemistry, 2005, 52, 1206-1216