Measurement of Water-soluble Vitamins by UPLC-MS/MS

Yusheng Zhu, PhD, DABCC, FACB
Associate Professor
Director of Clinical Chemistry & Toxicology

Financial Disclosure Information

Grant/Research Support
- Fujirebio Diagnostics, Inc.
- Helena Laboratories
- NIH
- AHA
- AACC CPOCT

Learning Objectives
- Describe the clinical significance of determination of water-soluble vitamins
- Develop LC-MS/MS methods for testing water-soluble vitamins
- Validate LC-MS/MS assays for the measurement of water-soluble vitamins
Vitamins

- **Fat-soluble vitamins**
  - A
  - D
  - E
  - K
- **Water-soluble vitamins**
  - B: thiamin (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5) pyridoxine (B6), biotin (B7), folate (folic acid, B9), Cobalamin (B12)
  - C

Functions of Water-Soluble Vitamins

- **B1 (Thiamine):** energy metabolism; important to nerve function
- **B2 (Riboflavin):** energy metabolism; important for normal vision and skin health
- **B3 (Niacin):** energy metabolism; important for nervous system, digestive system, and skin health
- **B5 (Pantothenic acid):** energy metabolism; nerve function
- **B6 (Pyridoxine):** protein metabolism; helps make Hb
- **B7 (H, Biotin):** energy metabolism
- **B9 (Folate, Folic acid):** making DNA and new cells, especially red blood cells
- **B12 (Cobalamin):** making new cells; important to nerve function
- **C (Ascorbic acid):** Antioxidant; protein metabolism; important for immune system health; aids in iron absorption

Features of Water-soluble Vitamins

- Water-soluble vitamins dissolve in water.
- The body cannot store them.
- Leftover amounts of the vitamin leave the body through the urine.
- Need a continuous supply of such vitamins in diet.
Thiamine and Thiamine Derivatives

- Thiamine is mainly the transport form of vitamin B1
- Thiamine derivatives
  - Thiamine monophosphate (ThMP)
  - Thiamine diphosphate (ThDP)/thiamine pyrophosphate (TPP)
  - Thiamine triphosphate (ThTP)
  - Adenosine thiamine triphosphate (AThTP)
  - Adenosine thiamine diphosphate (AThDP)

Functions of Vitamin B1

- Carbohydrate metabolism
- Lipid metabolism
- Amino acid metabolism
- Production of the neurotransmitters
  - Glutamic acid
  - Gamma-Aminobutyric acid (GABA)
Thiamine Deficiency

- An essential vitamin required for carbohydrate metabolism, brain function, and peripheral nerve myelination.
- Approximately 80% of all chronic alcoholics are thiamine deficient due to poor nutrition.
- Deficiency also can occur in individuals who are:
  - elderly
  - have chronic gastrointestinal problems
  - have marked anorexia
  - on cancer treatment
  - receiving diuretic therapy.

Diseases Caused by Thiamine Deficiency

- Beriberi
- Alcoholic brain disease-Wernicke-Korsakoff syndrome
- Optic neuropathy
- Alzheimer's disease
- Heart failure

Vitamin B1 Distribution in Whole Blood

- Plasma (about 50%)
- White blood cells and platelets (about 4%)
- Red blood cells (about 47%)

- 10 – 20% Thiamine
- 80 – 90% TPP
Thiamine Measurement

- HPLC – Fluorescence
- LC-MS/MS

HPLC – Fluorescence Detection

- Lyse RBC and precipitate proteins
- Alkaline
  
  Th, TMP, TPP + K₃[Fe(CN)₆] → Fluorescence

- HPLC
- Fluorimetric Detection

Issues with HPLC Method

- Labor and time consuming
- Derivatization
- Fluorescence detector
- Alkaline condition (NaOH) damages the column
- The fluorescence intensity is pH dependent and reaches a plateau at certain pH levels
- Lack of ideal internal standards
LC-MS/MS

- Simultaneously detect multiple water-soluble vitamins
- Use stable isotope labeled internal standard
- Improved resolution, speed, sensitivity, and specificity

Instruments

- LC: ACQUITY UPLC system (Waters)
- MS/MS: TQ (Tandem Quadrupole Detector (Waters))

Sample Preparation

- Cell Lysis and Protein Precipitation
- Phosphate hydrolysis
- Stop hydrolysis and extract sample
Cell Lysis and Protein Precipitation

- Frozen and thawed whole blood + internal std
- Zinc sulfate
- Antioxidant
- Centrifugation
- Supernatant

Phosphate Hydrolysis

- Sodium acetate buffer
- Supernatant
- Acid phosphatase
- Incubate

Sample Extraction

- Chloroform
- Centrifuge
- Transfer supernatant to HPLC vial
- UPLC-MS/MS
### Standard and Internal Standard

- **TPP**
- **TPP-D3**

### Imprecision

<table>
<thead>
<tr>
<th></th>
<th>N=20</th>
<th>Mean</th>
<th>SD</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>59.73</td>
<td>2.24</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td>Med</td>
<td>95.52</td>
<td>3.23</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>214.97</td>
<td>5.57</td>
<td>2.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>N=40</th>
<th>Mean</th>
<th>SD</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>54.98</td>
<td>4.01</td>
<td>7.3</td>
<td></td>
</tr>
<tr>
<td>Med</td>
<td>81.2</td>
<td>8.4</td>
<td>10.4</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>210.64</td>
<td>16.47</td>
<td>7.8</td>
<td></td>
</tr>
</tbody>
</table>

### Accuracy by Comparison

- Within-Run
- Between-Run
Analytical Measureable Range (AMR)

Range: 14.3 nmol/L – 2600 nmol/L

Sample and target

<table>
<thead>
<tr>
<th>Conc.</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4 (3.58 nmol/L)</td>
<td>5.44</td>
<td>1.93</td>
<td>35.4</td>
</tr>
<tr>
<td>1:2 (7.15 nmol/L)</td>
<td>9.21</td>
<td>1.67</td>
<td>18.1</td>
</tr>
<tr>
<td>1 (14.3 nmol/L)</td>
<td>16.34</td>
<td>1.30</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Range: 14.3 nmol/L – 2600 nmol/L

Functional sensitivity

Carryover

<table>
<thead>
<tr>
<th>Sample Result LOW-LOW</th>
<th>HIGH-LOW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low 1</td>
<td>31.9</td>
</tr>
<tr>
<td>Low 2</td>
<td>36.1</td>
</tr>
<tr>
<td>Low 3</td>
<td>29.5</td>
</tr>
<tr>
<td>High 1</td>
<td>506.5</td>
</tr>
<tr>
<td>High 2</td>
<td>472.8</td>
</tr>
<tr>
<td>Low 4</td>
<td>32.5</td>
</tr>
<tr>
<td>High 3</td>
<td>500</td>
</tr>
<tr>
<td>High 4</td>
<td>467.5</td>
</tr>
<tr>
<td>Low 5</td>
<td>31.9</td>
</tr>
<tr>
<td>Low 6</td>
<td>27.2</td>
</tr>
<tr>
<td>Low 7</td>
<td>32.1</td>
</tr>
<tr>
<td>Low 8</td>
<td>32.4</td>
</tr>
<tr>
<td>High 5</td>
<td>454.6</td>
</tr>
<tr>
<td>High 6</td>
<td>456.8</td>
</tr>
<tr>
<td>Low 9</td>
<td>30.2</td>
</tr>
<tr>
<td>High 7</td>
<td>472.6</td>
</tr>
<tr>
<td>High 8</td>
<td>503.8</td>
</tr>
<tr>
<td>Low 10</td>
<td>29.9</td>
</tr>
<tr>
<td>High 9</td>
<td>495</td>
</tr>
<tr>
<td>High 10</td>
<td>477.7</td>
</tr>
<tr>
<td>Low 11</td>
<td>30.6</td>
</tr>
</tbody>
</table>

Satya N Narla, Brian Slay, Yusheng Zhu: Determination of vitamin B1 in whole blood by LC-MS/MS (in preparation).
Matrix Effect

<table>
<thead>
<tr>
<th>Sample Preparation</th>
<th>Spike standards into extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Sample Matrix</td>
<td>Blank Solvent</td>
</tr>
<tr>
<td>Thiamine C13</td>
<td>Thiamine C13</td>
</tr>
</tbody>
</table>

Ion suppression or enhancement (\%): \[ \left( \frac{\text{Sample Matrix Concentration}}{\text{Blank Matrix Concentration}} \right) \times 100 \] ± 15%

<table>
<thead>
<tr>
<th>Low (40 nmol/L)</th>
<th>High (400 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Spike standards</td>
</tr>
<tr>
<td>Concentration</td>
<td>Concentration</td>
</tr>
<tr>
<td>Blank Matrix</td>
<td>Blank Matrix</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean (n=10)</th>
<th>SD</th>
<th>CV ion suppression (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank Matrix</td>
<td>Matrix</td>
<td></td>
</tr>
<tr>
<td>11547</td>
<td>14518</td>
<td>158296</td>
</tr>
<tr>
<td></td>
<td></td>
<td>137 (8% )</td>
</tr>
<tr>
<td>9%</td>
<td>7%</td>
<td>2%</td>
</tr>
</tbody>
</table>

Positive Value: Ion Enhancement
Negative Value: Ion Suppression

Extraction Recovery

<table>
<thead>
<tr>
<th>Spike Before Extraction (SBE)</th>
<th>Spike After Extraction (SAE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine C13</td>
<td>Thiamine C13</td>
</tr>
</tbody>
</table>

Response of Analyte in Extracted Matrix (SBE) + 100
Response of Analyte in Extracted Blank Matrix (SAE)

<table>
<thead>
<tr>
<th>Low (40 nmol/L)</th>
<th>Medium (150 nmol/L)</th>
<th>High (400 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike standards</td>
<td>Concentration</td>
<td>Concentration</td>
</tr>
<tr>
<td>Blank Matrix</td>
<td>SBE</td>
<td>SAE</td>
</tr>
<tr>
<td>2724.67</td>
<td>2933.33</td>
<td>10051.33</td>
</tr>
<tr>
<td>Recovery</td>
<td>93%</td>
<td>95%</td>
</tr>
</tbody>
</table>

Method Recovery

<table>
<thead>
<tr>
<th>Spike Before Extraction (SBE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine C13</td>
</tr>
</tbody>
</table>

Final Concentration – Initial Concentration

<table>
<thead>
<tr>
<th>Initial</th>
<th>Low (70 nmol/L)</th>
<th>Medium (150 nmol/L)</th>
<th>High (350 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spike</td>
<td>Concentration</td>
<td>Concentration</td>
<td>Concentration</td>
</tr>
<tr>
<td>Standard (TDP) into Matrix</td>
<td>Blank Matrix</td>
<td>SBE</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>181</td>
<td>254</td>
<td>419</td>
</tr>
<tr>
<td>Recovery</td>
<td>93%</td>
<td>93%</td>
<td>92%</td>
</tr>
</tbody>
</table>

Satya N Narla, Brian Slay, Yusheng Zhu: Determination of vitamin B1 in whole blood by LC-MS/MS (in preparation)
Stability

- **Sample Type:** Whole blood EDTA or Lithium Heparin
- **Room temperature (RT)**
  - froze 5 samples at 0, 2, 5, 12, and 24 h
  - Recovery: 2h: 94%, 5h: 101%, 12h: 98%, 24h: 120%
- **After thawing at 4°C**
  - Stable for 4 days after thawing
  - Recovery: day 2: 102%, day 3: 95%, day 4: 102%
- **Freeze thaw cycles**
  - Stable for 5 freeze thaw cycles
  - Recovery: cycle 2: 95%, cycle 3: 92%, cycle 4: 97%, cycle 5: 96%
- **After sample extraction**
  - Stable for 24h in amber vials (102% recovery) and plain vials (99% recovery)
- **Sensitivity to light (RT)**
  - No significant difference observed for samples processed in normal tubes vs amber tubes

Summary

- A sensitive and specific UPLC-MS/MS assay for whole blood total vitamin B1 quantification has been developed and validated
- The assay has acceptable imprecision and wide measurement range
- The assay is accurate and reliable
- Short total runtime

Acknowledgement

MUSC
- Satya N Narla, PhD, NRCC (Clinical Chemistry Postdoctoral Fellow)
- Brian Slay, MT (ASCP), MHSA
- Joyce Foster, MHS, MT (ASCP), SPB

Marshfield Labs
- Joyce Flanagan, PhD, DABCC, FACB
- Dale Whipple
Thank you