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

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- 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)

Vitamin B1 (thiamine) | University of Maryland Medical Center
http://umm.edu/health/medical/altmed/supplement/vitamin-b1-thiamine#ixzz3Y9fb2C0
Vitamin B1 Functions

BCKD: branched chain α-ketoacid dehydrogenase complex;
CoA: coenzyme A
TPP: thiamine pyrophosphate
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 55%)**: 10 – 20 % Thiamine
- **White blood cells and platelets (about 4%)**: 80 – 90 % TPP
- **Red blood cells (about 41%)**: 80 – 90 % TPP
Thiamine Measurement

- HPLC – Fluorescence
- LC-MS/MS
HPLC – Fluorescence Detection

- Lyse RBC and precipitate proteins

  Alkaline

- Th, TMP, TPP + $\text{K}_3[\text{Fe(CN)}_6]$ --- $\rightarrow$ Fluorescence

- HPLC

- Fluorimetric Detection

Mancinelli et al. 2003
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

Puts et al. 2015
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

Acetonitrile

Centrifugation

Supernatant

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

Sodium Acetate buffer → supernatant → Acid Phosphatase → Incubate

Vortex
Sample Extraction

Chloroform → Vortex → Centrifuge → Transfer Supernatant to HPLC Vial → UPLC-MS/MS

Narla, Slay, Zhu: Determination of vitamin B1 in whole blood by LC-MS/MS (in preparation)
Standard and Internal Standard

TPP

TPP-D3

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

**Within-Run**

<table>
<thead>
<tr>
<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>
</tr>
<tr>
<td>Med</td>
<td>95.52</td>
<td>3.23</td>
<td>3.4</td>
</tr>
<tr>
<td>High</td>
<td>214.97</td>
<td>5.57</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Between-Run**

<table>
<thead>
<tr>
<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>
</tr>
<tr>
<td>Med</td>
<td>81.2</td>
<td>8.4</td>
<td>10.4</td>
</tr>
<tr>
<td>High</td>
<td>210.64</td>
<td>16.47</td>
<td>7.8</td>
</tr>
</tbody>
</table>

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

Key Statistics:
- Average Error Index: -0.40
- Error Index Range: -1.08 to 0.74
- Coverage Ratio: --

Evaluation Criteria:
- Allowable Total Error: 25%
- Reportable Range: --

Deming Regression Statistics:
- $Y = \text{Slope} \times X + \text{Intercept}$
- Correlation Coeff (R): 0.9325
- Slope: 0.852 (0.749 to 0.956)
- Intercept: 4.632 (-7.623 to 16.888)
- Std. Err of Estimate: 9.890
- N: 40 of 41

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Analytical Measureable Range (AMR)

Range: 14.3 nmol/L – 2600 nmol/L

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

<table>
<thead>
<tr>
<th>Sample and target Conc. (N=10)</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
### Carryover

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

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-Low</td>
<td>31.02</td>
<td>1.13</td>
</tr>
<tr>
<td>Low-Low</td>
<td>31.46</td>
<td>3.35</td>
</tr>
<tr>
<td>Carryover (HL – LL)</td>
<td>-0.44</td>
<td></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

**Sample Preparation**

Spike standards into extract

---

**Thiamine C13**

- Blank Sample Matrix
- Blank Solvent
- Sample
- Spike standards into extract

**Ion suppression or enhancement (%)**

\[
\text{Ion suppression or enhancement (\%) = } \left( \frac{\text{Response of Post Spiked Extraction Matrix}}{\text{Response of Post Spiked Extraction solvent}} - 1 \right) \times 100
\]

<table>
<thead>
<tr>
<th></th>
<th>LOW (40 nmol/L)</th>
<th>HIGH (400 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>Matrix</td>
</tr>
<tr>
<td>Mean (n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>15147</td>
<td>14318</td>
</tr>
<tr>
<td>SD</td>
<td>1304</td>
<td>1061</td>
</tr>
<tr>
<td>CV</td>
<td>9%</td>
<td>7%</td>
</tr>
<tr>
<td>Ion suppression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td>-5.48</td>
<td></td>
</tr>
</tbody>
</table>
# Extraction Recovery

## SPIKE BEFORE EXTRACTION (SBE)

### Thiamine C13

- Spike standards into Matrix

## SPIKE AFTER EXTRACTION (SAE)

### Blank Matrix

- Spike standards into extract

### Sample Preparation

- Thiamine C13

\[
\text{% Recovery} = \frac{\text{Response of Analyte in Extracted Spiked Matrix (SBE)}}{\text{Response of Analyte in Extracted Blank Matrix (SAE)}} \times 100
\]

<table>
<thead>
<tr>
<th></th>
<th>LOW (40 nmol/L)</th>
<th>MEDIUM (150 nmol/L)</th>
<th>HIGH (400 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SBE</td>
<td>SAE</td>
<td>SBE</td>
</tr>
<tr>
<td><strong>Average</strong> (n=6)</td>
<td>2724.67</td>
<td>2933.33</td>
<td>10051.33</td>
</tr>
<tr>
<td><strong>Recovery</strong></td>
<td><strong>93%</strong></td>
<td><strong>95%</strong></td>
<td><strong>93%</strong></td>
</tr>
</tbody>
</table>
Method Recovery

**Matrix**

**SPIKE BEFORE EXTRACTION (SBE)**

Spike standard (TDP) into Matrix

\[
\% \text{ Recovery} = \frac{\text{Final Concentration} - \text{Initial Concentration}}{\text{Spiked Concentration}} \times 100
\]

<table>
<thead>
<tr>
<th></th>
<th>INITIAL</th>
<th>LOW (70 nmol/L)</th>
<th>MED (150 nmol/L)</th>
<th>HIGH (350 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average (n=6)</td>
<td>115</td>
<td>181</td>
<td>254</td>
<td>439</td>
</tr>
<tr>
<td>Final – Initial</td>
<td>65</td>
<td>139</td>
<td>323</td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td><strong>93%</strong></td>
<td><strong>93%</strong></td>
<td><strong>92%</strong></td>
<td></td>
</tr>
</tbody>
</table>

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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%, 12 h: 98%, 24 h: 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 24 h 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

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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

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