AACC Better health through laboratory medicine.

Mass Spectrometry and Separation Sciences for Laboratory Medicine

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

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- 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

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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

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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.

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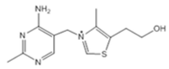
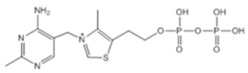
Thiamine and Thiamine Derivatives

- **Thiamine is mainly the transport form of vitamin B1**

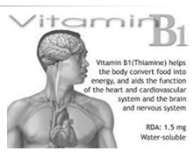


- **Thiamine derivatives**
  - Thiamine monophosphate (ThMP)
  - Thiamine diphosphate (ThDP)/thiamine pyrophosphate (TPP)



  - Thiamine triphosphate (ThTP)
  - Adenosine thiamine triphosphate (AThTP)
  - Adenosine thiamine diphosphate (AThDP)

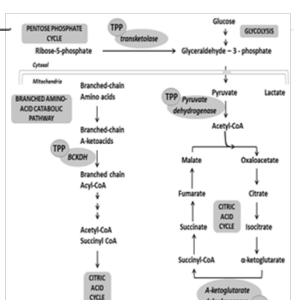
Functions of Vitamin B1



- 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#ixzz3Y9fbB2CO

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.

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Diseases Caused by Thiamine Deficiency

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

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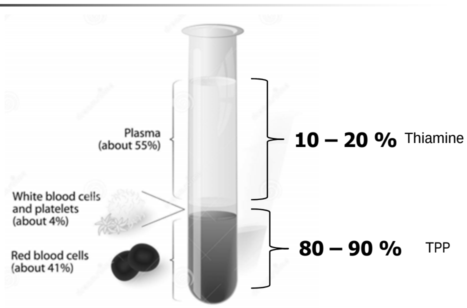
Vitamin B1 Distribution in Whole Blood



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Thiamine Measurement

- HPLC – Fluorescence
- LC-MS/MS

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HPLC – Fluorescence Detection

- Lyse RBC and precipitate proteins
- Th, TMP, TPP + $K_3[Fe(CN)_6]$ $\xrightarrow{\text{Alkaline}}$ Fluorescence
- HPLC
- Fluorimetric Detection

Mancinelli et al. 2003

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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

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LC-MS/MS

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

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Instruments

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

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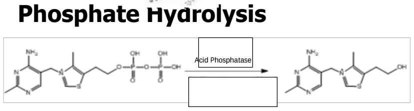
Sample Preparation

- **Cell Lysis and Protein Precipitation**

- **Phosphate Hydrolysis**



- **Stop Hydrolysis and Extract Sample**

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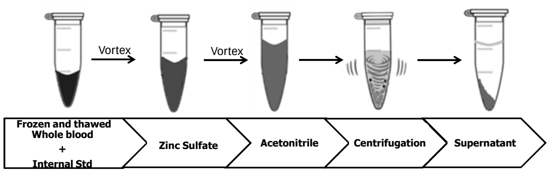
Cell Lysis and Protein Precipitation



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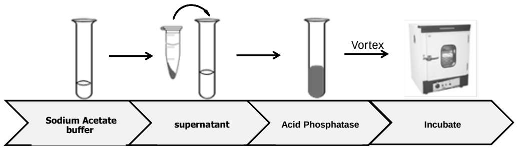
Phosphate Hydrolysis



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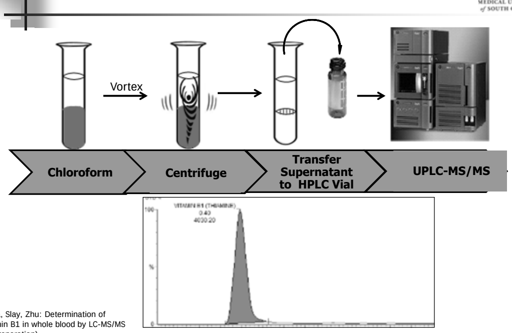
Sample Extraction



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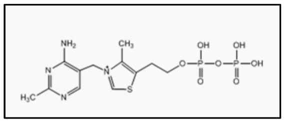
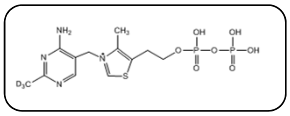
Standard and Internal Standard



TPP



TPP-D3

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Imprecision

- Within-Run

N=20	Mean	SD	% CV
Low	59.73	2.24	3.7
Med	95.52	3.23	3.4
High	214.97	5.57	2.6

- Between-Run

N=40	Mean	SD	% CV
Low	54.98	4.01	7.3
Med	81.2	8.4	10.4
High	210.64	16.47	7.8

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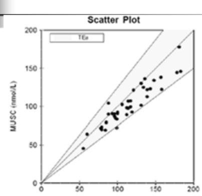
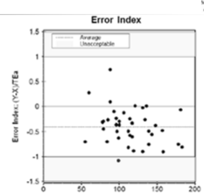
Accuracy by Comparison





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 = Slope * X + 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)

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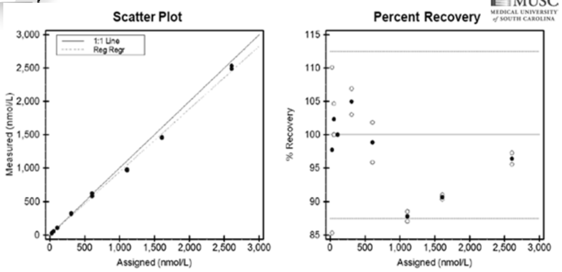

Range: 14.3 nmol/L – 2600 nmol/L

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Functional sensitivity

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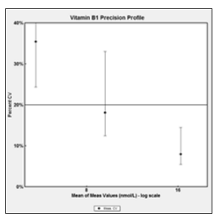
Sample and target Conc. (N=10)	Mean	SD	CV
1:4 (3.58 nmol/L)	5.44	1.93	35.4
1:2 (7.15 nmol/L)	9.21	1.67	18.1
1 (14.3 nmol/L)	16.34	1.30	7.9



Range: 14.3 nmol/L – 2600 nmol/L

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Carryover

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

	Mean	SD
High-Low	31.02	1.13
Low-Low	31.46	3.35
Carryover (HL – LL)	-0.44	

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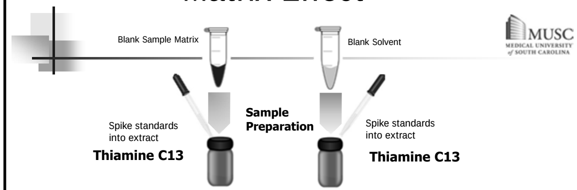
Matrix Effect



$$\textit{Ion suppression or enhancement}\ (\%) = \left(\frac{\textit{Responce of Post Spiked Extraction Matrix}}{\textit{Responce of Post Spiked Extraction solvent}} - 1\right) \times 100$$

	LOW (40 nmol/L)		HIGH (400 nmol/L)	
	Blank	Matrix	Blank	Matrix
Mean (n=10)	15147	14318	158296	137306
SD	1304	1061	3294	11381
CV	9%	7%	2%	8%
ion suppression (%)	**-5.48**		**-13.26**	

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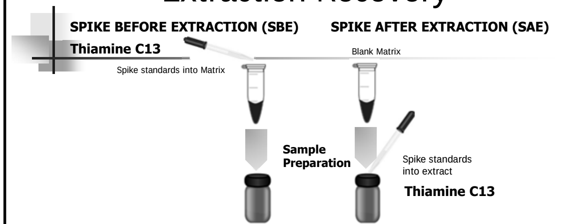
Extraction Recovery



$$\% \text{ Recovery} = \frac{\textit{Responce of Analyte in Exracted Spiked Matrix (SBE)}}{\textit{Responce of Analyte in Extracted Blank Matrix (SAE)}} * 100$$

	LOW (40 nmol/L)		MEDIUM (150 nmol/L)		HIGH (400 nmol/L)	
	SBE	SAE	SBE	SAE	SBE	SAE
Average (n=6)	2724.67	2933.33	10051.33	10574.83	25317.00	27079.50
Recovery	**93%**		**95%**		**93%**	

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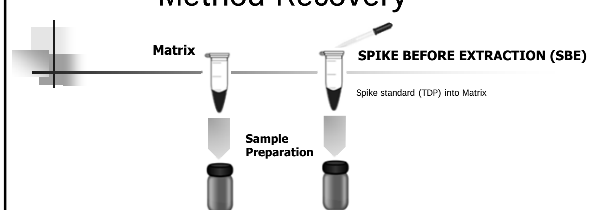
Method Recovery



$$\%\ \textit{Recovery} = \frac{\textit{Final Concentration} - \textit{Initial Concentration}}{\textit{Spiked Concentration}} * 100$$

	INITIAL	LOW (70 nmol/L)	MED (150 nmol/L)	HIGH (350 nmoL/L)
Average (n=6)	115	181	254	439
Final – Initial		65	139	323
Recovery		**93%**	**93%**	**92%**

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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^{o}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

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Acknowledgement

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Thank you

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