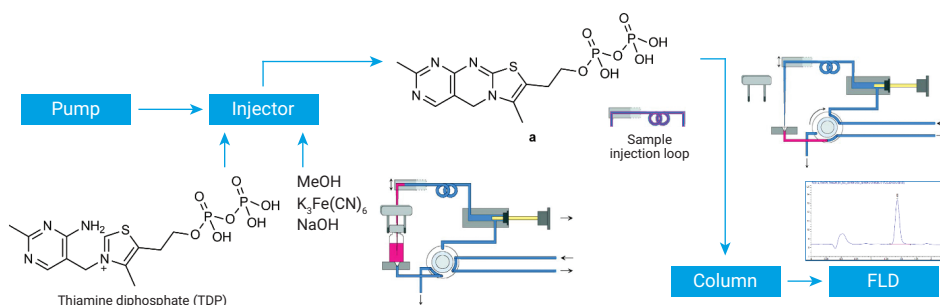**Agilent**

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# High-Throughput Determination of Thiamine in Whole Blood and Erythrocytes

Agilent 1290 Infinity LC with fluorescence detection and online precolumn derivatization

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## Abstract

Thiamine (vitamin B1) deficiency is prevalent in Cambodian women due to poor dietary diversity that stems from their heavy dependence on white rice. Thiamine deficiency is associated with fatal diseases such as beriberi. To understand the extent of this public health issue, it is necessary to obtain population-representative data. A high-throughput, ultrahigh-performance liquid chromatography (UHPLC) method has been developed and validated using online precolumn derivatization, which allows the sample to be freshly derivatized and immediately injected into the column without sample degradation. This approach greatly increased the capacity of the test to address the need for testing a large quantity of samples in a baseline clinical study. In this study, whole blood and erythrocyte samples are purified and derivatized online via the injector program, separated by gradient elution on a reversed-phase analytical column, and measured by the fluorescence detector. An Agilent 1290 Infinity LC is used in this workflow. The method limit of detection and limit of quantification are 10.0 and 30.2 nmol/L, respectively. The recovery ranges from 95 to 106%, with precision of <4% relative standard deviation. The method was used to successfully analyze a total of 1,487 nationally representative blood samples from Cambodian women of childbearing age and their children.

## Introduction

The vitamin B1 family is made up of thiamine and its phosphate esters: thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate (TTP). The biologically active form of thiamine is TDP, which is a required cofactor for essential enzymatic reactions such as decarboxylation.[1] Therefore, thiamine plays a fundamental role in energy metabolism. Vitamin B1 deficiency may lead to adverse health outcomes such as beriberi, a disease that can be fatal if not diagnosed.[2] Furthermore, inadequate thiamine levels in early stages of life have detrimental impacts on neurological and cognitive development, especially in infants.[3]

In Cambodia, low thiamine intake stems from poor dietary diversity and high dependency on white rice, which contains little thiamine.[4] Previous studies found suboptimal thiamine status in Cambodian women of childbearing age, pregnant and lactating women, as well as their infants.[5] However, these studies only recruited a small population. Therefore, a more extensive study was conducted with a population of over 1,500, including lactating women and their infants, to better understand the prevalence of thiamine deficiency in Cambodian women and children.

Traditionally, thiamine status is assessed using the erythrocyte transketolase activity coefficient as an indicator.[6] However, there are several drawbacks of this method including lack of specificity, low sensitivity, and poor precision. As most thiamine in whole blood is found as TDP in erythrocytes, and it is correlated to the erythrocyte transketolase activity coefficient, the measurement of TDP concentrations in erythrocytes provides a good indication of thiamine status.[7] Current methods use HPLC with manual derivatization and fluorescence detection to determine TDP levels in blood samples.[8] The reaction mechanism is depicted in Figure 1. However, the derivatized products are not stable, especially when a large number of samples are subjected to analysis; the samples are prone to degradation while waiting for injection in the sampler.

To circumvent the issue of degradation, we developed the online derivatization method to leverage the injector program function, whereby the samples are freshly derivatized and injected onto the column with minimum residence time in the autosampler. The online derivatization method enhanced the repeatability and precision of the test method.

In this study, we developed a new UHPLC-FLD method with online derivatization for thiamine analysis on a 1290 Infinity LC. We used this new method to measure the thiamine levels in the erythrocyte samples from 726 women of childbearing age and 761 children. In this application note, the development and validation of the method is presented.

## Experimental

### Equipment
Agilent 1290 Infinity LC

- Agilent 1290 Infinity Binary pump (G4220A)
- Agilent 1290 Infinity Autosampler (G4226A)
- Agilent 1290 Infinity Thermostat (G1330B)
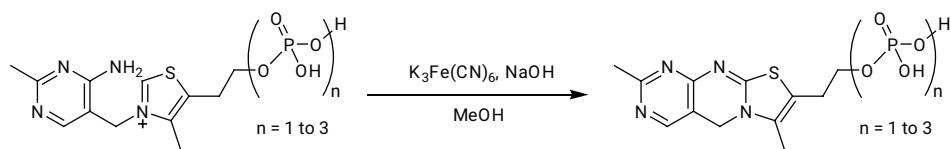- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1260 Infinity Fluorescence Detector (G1321B)

### Software
OpenLab CDS ChemStation Edition C.01.05

### Column
- Agilent ZORBAX Eclipse Plus C18 Rapid Resolution HT analytical column (3.0 × 50 mm, 1.8 µm)
- C18 guard cartridge (4 × 2.0 mm)

### Chemicals
Trichloroacetic acid (TCA), potassium ferricyanide (III), dibasic sodium phosphate, and sodium hydroxide were analytical grade, methanol was LC/MS grade, and *tert*-butyl methyl ether (MTBE) was HPLC grade. All these chemicals and reagents were purchased from Sigma-Aldrich. TDP: certified reference material purchased from Sigma-Aldrich, catalog number PHR1369, lot number LRAA1473, purity 94.5%. Thiamine monophosphate (TMP): purchased from Santa Cruz Biotechnology, catalog number SC-215965, lot number G1013, purity 99.7%. Free thiamine: purchased from Sigma-Aldrich, catalog number T4625, lot number SLBF5810V, purity 99%. Water was generated using a Millipore pure water system. Vitamin B1 whole blood control samples level 1 (TDP certified value of 61.3 nmol/L with a range of 42.9–79.7 nmol/L) and level 2 (TDP certified value of 225 nmol/L with a range of 157–292 nmol/L) were purchased from ChromSystems (catalog number 0164, lot number 1814). The two levels of blood control sample were prepared from the lyophilized solid and stored as per the supplier's instruction.



**Figure 1.** Derivatization mechanism of thiamine and its phosphate esters.

## Sample preparation

Erythrocyte samples were thawed on ice from the −78 °C freezer in a dark room. Thawed erythrocyte samples, whole blood control samples, blank water, and calibration standards (0.25 mL) were treated with 0.75 mL of 10% TCA to precipitate proteins. Samples were vortex-mixed vigorously for 15 seconds and left to set in a cold environment for 15 minutes. The samples were centrifuged at 13,000 g for 6 minutes at 4 °C. Each supernatant was transferred into a microcentrifuge tube and washed twice with 0.75 mL of water-saturated MTBE to remove TCA. The remaining bottom layer (cleaned-up sample) was transferred into an HPLC autosampler 96-well microplate before UHPLC analysis.

## Calibration standard preparation

The stock solution of TDP was prepared in 0.1 M hydrochloric acid. Calibration standards were prepared freshly by diluting the stock solution with water to concentrations of 30, 60, 120, 180, and 240 nmol/L. Ultrapure water was used as a blank.

# Results and discussion

Method validation was performed on whole blood control samples in the assessment of specificity, repeatability, LOD/LOQ, linearity, precision, and accuracy.

## Specificity

A mixture solution of three different forms of thiamine (TDP, TMP, and T) was analyzed using this test method without an HPLC guard column attached. TDP was well resolved from TMP and T as shown in Figure 2.

## Instrument method

| Parameter | Value |
|---|---|
| Solvent | A) dibasic sodium phosphate buffer (pH 7.0)/methanol (90:10, vol/vol)<br>B) dibasic sodium phosphate buffer (pH 7.0)/methanol (30:70, vol/vol) |
| Gradient | 0.00 min: 0% B<br>1.00 min: 12.5% B<br>1.80 min: 12.5% B<br>2.20 min: 50% B<br>2.70 min: 50% B<br>3.00 min: 0% B<br><br>Stop time: 3 min |
| Flow rate | 0.600 mL/min |
| Temperature | 25 °C |
| Detection | Excitation wavelength of 375 nm and emission wavelength of 435 nm<br>Data rate: 20 Hz |
| Injection | Injection volume: 15 µL<br>Sampling speed: draw speed: 100 µL/min; eject speed: 100 µL/min<br>Draw position offset: 0.0 mm<br>Sample temperature: 4 °C<br>Needle wash: 15 s in water/acetonitrile (50/50) |
| Injector Program | See Table 1 |

**Table 1.** Injector program of TDP derivatization.

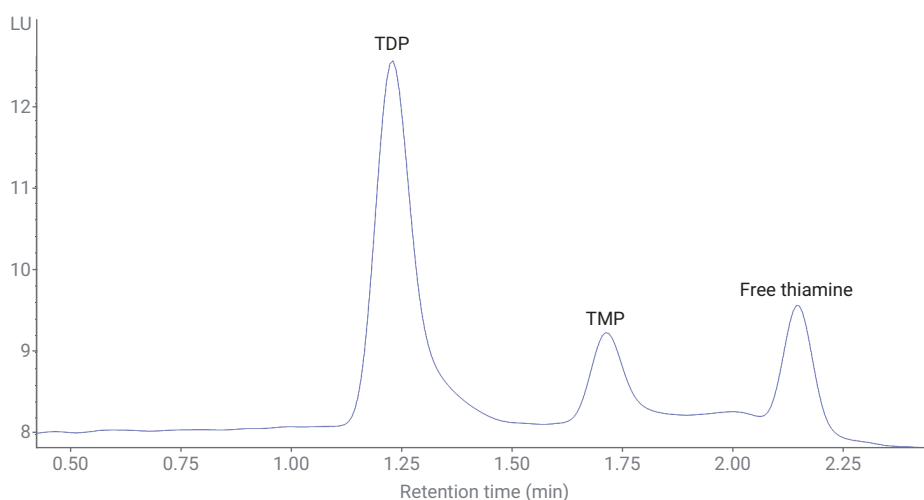| Step | Parameter |
|---|---|
| 1 | Draw 1 µL from location P1-A-02 (methanol) with a speed of 100 µL/min |
| 2 | Draw 4 µL from the sample with a speed of 50 µL/min |
| 3 | Mix 3.5 µL from the air with default speed three times |
| 4 | Draw 2.5 µL from location P1-A-01 (0.6 mmol/L potassium ferricyanide in 15% NaOH) with a speed of 100 µL/min |
| 5 | Mix 4 µL from the air with default speed 10 times |
| 6 | Wait 1 min |
| 7 | Inject |



**Figure 2.** Chromatogram of the separation of thiamine from its phosphate esters.

## Repeatability, LOD, and LOQ

Injection repeatability was evaluated by seven injections of the TDP calibration standard at a concentration of 120 nmol/L (Figure 3). The injections showed excellent repeatability, with area RSD at 2.8% and retention time RSD at 0.2%.

A blank blood matrix in which thiamine is absent was unavailable. The commercially available blood standard samples were used to determine the LOD and LOQ. The LOD and LOQ were estimated to be 3x and 10x, respectively, the standard deviation of seven repeated injections of blood control sample level 1 (61.3 nmol/L). The LOD and LOQ were determined to be 10.0 nmol/L and 30.2 nmol/L, respectively, in this manner.

## Linearity

Linearity was evaluated as the coefficient of determination ($R^2$). The calibration curves with concentrations over the range of 32.9 to 263.6 nmol/L on six days showed excellent linearity with $R^2 \geq 0.9978$ and residuals within 5.1% (Figure 4).

## Accuracy and precision

Vitamin B1 whole blood control samples with certified values were used for the accuracy and precision test. The control samples were tested together with the study samples as a quality control measure. The recovery of 95 to 106%, and the low RSD of 3.5% on average over different testing days, indicated the high accuracy and precision of this method, as shown in Table 2.

The precision tests were performed on six days with two whole blood control samples each day, of which the RSD of less than 4% were achieved (Table 2).
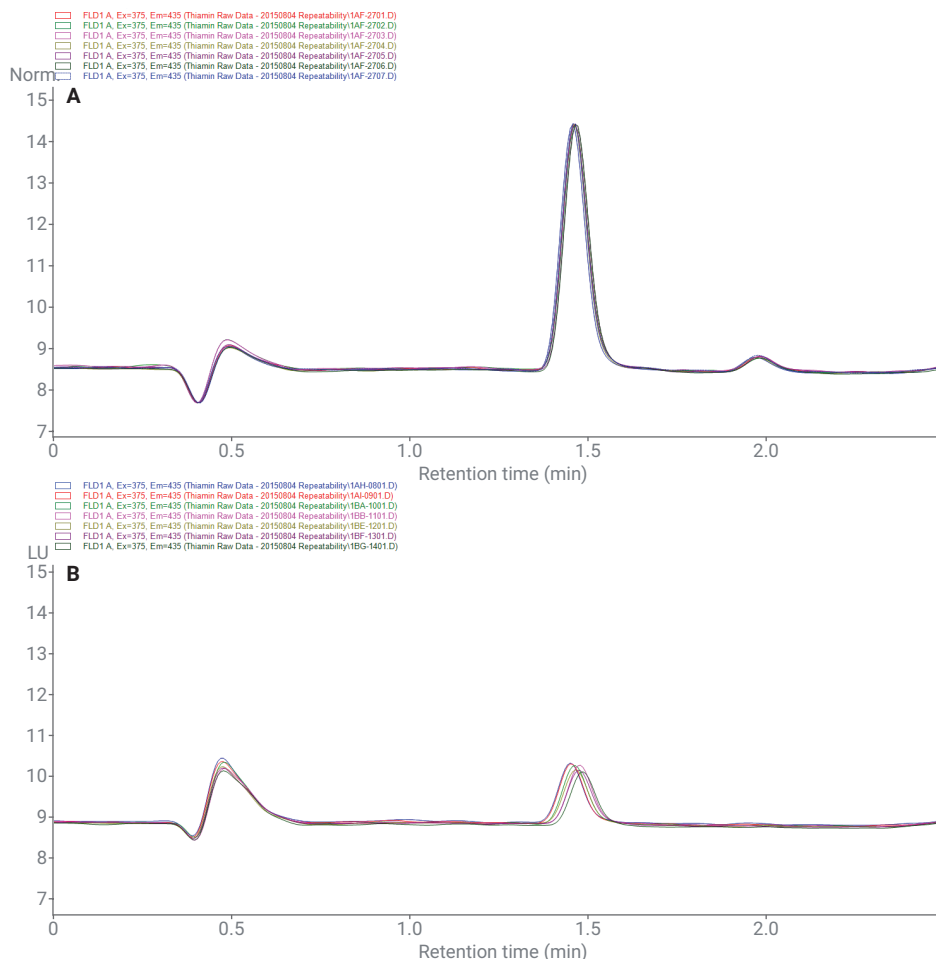


**Figure 3.** Repeatability and LOD/LOQ of the method. A) Seven replicate injections of TDP calibration standard 120 nmol/L. B) Seven replicate injections of blood control sample level 1 (61.3 nmol/L).
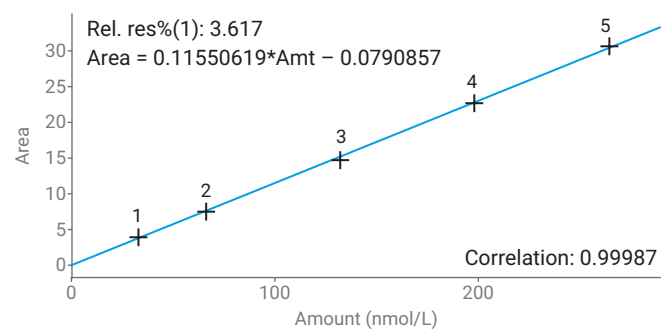


Rel. res%(1): 3.617
Area = 0.11550619*Amt − 0.0790857

Correlation: 0.99987

**Figure 4.** Calibration curve of TDP standards from 32.9 to 263.6 nmol/L with a correlation of 0.99987.

## Sample preparation and online derivatization

Erythrocyte samples have high viscosity. We used positive pipettes (Eppendorf Xstream) with slow drawing and dispensing speed to ensure the accuracy of the sampling volume and dilute the sample with water before the treatment with TCA.

The MTBE extraction step to remove TCA is a tedious procedure while handling a few hundred samples a day, and increases the likelihood of operational errors. Thanks to the use of the autosampler injector program, fewer steps and reagents were required for the sample preparation. Using the online derivatization method, samples were freshly derivatized before injection. The cleaned-up samples were stable for at least 72 hours in the UHPLC autosampler at 4 °C, compared to offline derivatization methods,[8] where samples were found to degrade within 16 hours (Figure 5). In addition, automation ensured high throughput, repeatability, and precision.

## Method validation

The method has high specificity because it involves a highly selective ring closure reaction during the derivatization, and the product is measured at a specific excitation/emission wavelength of the fluorescence detection. The selectivity on TDP among different forms in the class of thiamine is shown by the good separation of TDP from the others on the analytical column. The accuracy of the method was verified by recovery of TDP whole blood control samples. The recovery of whole blood control samples was satisfactory at the two measured concentration levels, which covered the typical range of the TDP level in study test samples as shown in Figure 6.

**Table 2.** Accuracy and precision of blood control samples.

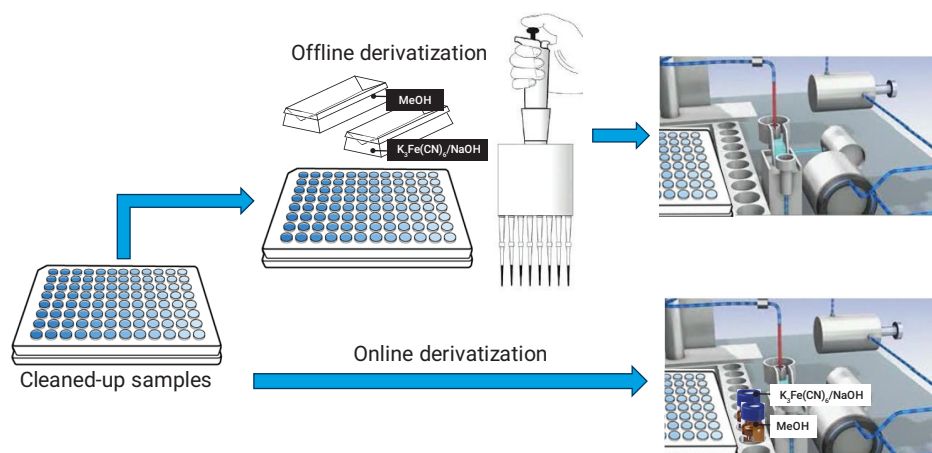|  | Control Level 1 (61.3 nmol/L) | | Control Level 2 (225 nmol/L) | |
|---|---|---|---|---|
|  | Measured Concentration (nmol/L) | Recovery | Measured Concentration (nmol/L) | Recovery |
| Day 1 | 64.8 | 106% | 236 | 105% |
| Day 2 | 60.9 | 99.3% | 229 | 102% |
| Day 3 | 62.0 | 101% | 233 | 104% |
| Day 4 | 60.9 | 99.3% | 228 | 101% |
| Day 5 | 59.5 | 97.1% | 222 | 98.7% |
| Day 6 | 58.2 | 94.9% | 216 | 96.0% |
| Average | 61.0 | 99.6% | 227 | 101% |
| RSD | 3.8% | – | 3.2% | – |



**Figure 5.** Comparison of offline and online derivatization workflow.
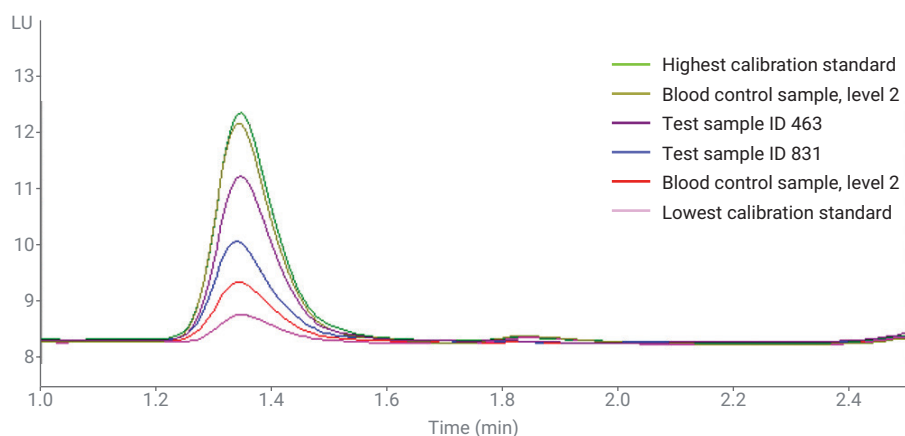


**Figure 6.** Representative overlaid chromatograms of TDP calibration standards, blood control samples, and test samples.

This online derivatization method has been used to successfully analyze 1,487 erythrocyte samples and contributed to the assessment of thiamine status among Cambodian mothers and their children by Whitfield et al.[9]

## Conclusion

A new method involving precolumn online derivatization was developed for the determination of thiamine in whole blood and erythrocyte samples using UHPLC-FLD. This method allows high-throughput analysis with excellent repeatability and precision due to the automation of the UHPLC system. The method was validated for injection repeatability, specificity, LOD, LOQ, accuracy, and precision. A total of 1,487 blood samples were analyzed using this method. The analysis has allowed the completion of a nationwide study on the thiamine status evaluation in Cambodian women and children by Whitfield et al.[9] This high-throughput method may be used to assess the thiamine levels in future similar studies.

## References

1. Lonsdale, D. A Review of the Biochemistry, Metabolism and Clinical Benefits of Thiamin(e) and its Derivatives. *eCAM* **2006**, *3*, 49–59. DOI: 10.1093/ecam/nek009.

2. Soukaloun, D. *et al.* Erythrocyte Transketolase Activity, Markers of Cardiac Dysfunction and the Diagnosis of Infantile Beriberi. *PLoS Neglect. Trop. D.* **2011**, *5*. DOI: 10.1371/journal.pntd.0000971.

3. Ross, A. C. *et al.* Modern Nutrition in Health and Disease: Eleventh edition, Wolters Kluwer Health Adis (ESP), **2012**.

4. Anderson, W. Beriberi, White Rice, and Vitamin B: A Disease, a Cause, and a Cure. By Kenneth J Carpenter. *Q. Rev. Biol.* **2002**, 77, 367–368. DOI: 10.1086/345272.

5. Coats, D. *et al.* Thiamine Deficiency in Cambodian Infants with and without Beriberi. *J. Pediatr.* **2012**, *161*, 843–847. DOI: 10.1016/j.jpeds.2012.05.006.

6. Gibson, R. S. Principles of Nutritional Assessment, 2nd. Oxford University Press: New York, Auckland, N.Z., **2005**.

7. Talwar, D. *et al.* Vitamin B1 Status Assessed by Direct Measurement of Thiamin Pyrophosphate in Erythrocytes or Whole Blood by HPLC: Comparison with Erythrocyte Transketolase Activation Assay. *Clin. Chem.* **2000**, *46*, 704–710.

8. Lu, J.; Frank, E. L. Rapid HPLC Measurement of Thiamine and its Phosphate Esters in Whole Blood. *Clin. Chem.* **2008**, *54*, 901–906. DOI: 10.1373/clinchem.2007.099077.

9. Whitfield, K. C. *et al.* High Prevalence of Thiamine (Vitamin B1) Deficiency in Early Childhood Among a Nationally Representative Sample of Cambodian Women of Childbearing Age and Their Children. *PLoS Neglect. Trop. D.* **2017**, *11*. DOI: 10.1371/journal.pntd.0005814.

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