<Brief Note>

# A simplified HPLC method for thiamine and its phosphate esters in whole blood

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**Summary** We developed a simple whole-blood assay for thiamine and its three phosphate esters: thiamine monophosphate (TMP); thiamine diphosphate (TDP); and thiamine triphosphate (TTP). We used HPLC, fluorometric detection of the effluent, and pre-column oxidation of thiamine to thiochrome. The phosphate esters were oxidized in the same way to their respective thiochrome esters. Thiamine was used as an external standard in the assay for all the thiamine derivatives, thereby simplifying the method. Prior to the HPLC separations and with known concentrations of thiamine and its phosphate esters, we determined the ratios of the fluorescence intensity per mol of each ester to that of thiamine. The ratios were 0.75 for TMP/thiamine; 0.90 for TDP/thiamine; and 0.97 for TTP/thiamine. The ratios depend on both the concentrations and fluorescence intensity per mol of thiamine and its esters. The above ratios were determined on a daily basis to verify the reproducibility and the precision of the method. For example, the concentration of TMP can be calculated from: concn. TMP= (concentration of thiamine)×(TMP fluorescence/thiamine fluorescence)/0.75. The ratio, 0.75, corrects for the lower fluorescence intensity per mol of TMP vs. that of thiamine, and the concentration of thiamine must of course be known. Values obtained by this method correlated significantly ($p < 0.001$) with the procedure in which four standards, i.e., thiamine, TMP, TDP, and TTP, are used in the calibration.

**Key words:** Vitamin $B_1$, Thiamine, Thiamine phosphate ester, Thiochrome, High-performance liquid chromatography

## 1. Introduction

Thiamine, like several other B-vitamins, is essential for normal development, growth, reproduction, lactation, physical performance, and well being. It is involved in releasing energy from the macronutrients that provide energy, especially from carbohydrates[1)]. Nadel and Burger reported that patients fed intravenously for long periods were at risk for thiamine deficiency[2)]. When large amounts of glucose were administered intravenously for a prolonged period of time and without added thiamine, the concentration of

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Table of abbreviations: HPLC, high-performance liquid chromatography; TMP, thiamine monophosphate; TDP, thiamine diphosphate; TTP, thiamine triphosphate; TPN, total parenteral nutrition; CV, coefficient of variation

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pyruvate in the blood increased. Pyruvate dehydrogenase (EC 1.2.4.1) requires thiamine diphosphate as a cofactor to convert pyruvate to acetyl coenzyme A. With an accumulation of pyruvate in blood, the blood lactate concentration increases, resulting in lactic acidosis that can be fatal. Based on these observations, the addition of thiamine to total parenteral nutrition (TPN) fluids is recommended by the Ministry of Health, Labour and Welfare of Japan[3-6]. Thiamine is not in a coenzyme form; however, the liver converts thiamine to bioavailable thiamine triphosphate (TTP) and thiamine diphosphate (TDP). The latter is hydrolyzed to thiamine monophosphate (TMP) that is also not bioavailable. Therefore, it is important to separately measure thiamine and its phosphate esters in blood, since blood concentrations are useful markers of thiamine status.

Nowadays, thiamine and its phosphate esters in blood can be assayed by the thiochrome method using separation by HPLC and fluorometric detection[7, 8]. The currently used HPLC methods for thiamine and its phosphate esters are time consuming owing to the required use of four calibrators: thiamine, TMP, TDP, and TTP. In the study described here, we assumed that the ratios of fluorescence intensity of the thiochrome phosphate esters to that of thiochrome depended only on the ester being measured, its fluorescence intensity per mol relative to thiamine, and the concentration of thiamine. We determined three molar ratios of fluorescence intensity for each analyte: TMP/thiamine, TDP/thiamine, and TTP/thiamine. Our goal was to test the above hypothesis to see if a single standard, i.e., thiamine, could be used to simplify the method.

## 2. Materials and methods

1. Standard solutions

A 200 nmol/l standard solution of thiamine hydrochloride was prepared fresh daily from a 1.48 mmol/l solution from Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). TMP was purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA); TDP and TTP were from Wako. Standard stock solutions (2.0 mmol/l) of thiamine phosphate esters were prepared in 100 mmol/l hydrochloric acid and stored at −20℃. Working solutions were prepared fresh each day from stock solutions to the required concentrations in distilled water. The currently used standard concentration was ca. 200 nmol/l for each thiamine phosphate ester. The accuracy of concentrations of these solutions were checked by measuring the absorbance at 248 nm according to the method described by Penttinen[9, 10]. All other reagents described below were from Wako and were of the highest quality available.

2. Protein precipitating reagent

A 0.2 mol/l potassium acetate buffer in distilled water was prepared from sodium acetate (Wako) and titrated with glacial acetic acid to a pH of 4.5. An aqueous 10% solution of trichloroacetic acid (TCA) was prepared by dissolving 25 g TCA (Wako) in enough distilled water to make 100 ml.

3. Other reagents

Bromine water (2-3%, w/v) was prepared fresh each day from bromine (Wako); cyanogen bromide was freshly prepared by titrating the bromine water with 10% potassium cyanide. A 90 mmol/l, pH 8.6 phosphate buffer was prepared from $KH_2PO_4$ and $K_2HPO_4$.

4. HPLC analysis

We combined 0.2 ml of each of the four standard solutions or a whole blood specimen (collected in heparin or EDTA) with 0.2 ml of 0.2 mol/l, pH 4.5 potassium acetate and 0.2 ml of 10% TCA. After standing for 10 minutes at room temperature, the mixture was centrifuged at 12,000 rpm for 5 minutes; a clear supernatant was obtained in all cases. We then mixed 0.2 ml of the supernatant with 0.03 ml of 4 mol/l sodium acetate and then added, in order, 0.02 ml of cyanogen bromide and 0.02 ml of 2 mol/l NaOH. For each assay, 0.05-ml aliquots were injected into a polyamino-filled HPLC column (Asahipack, NH2P-50 4E, 4.6 mm ID×250 mm L; Shodex, Tokyo, Japan). The best separation of thiamine and its phosphate esters was achieved by using isocratic elution with the 90 mmol/l, pH 8.6 phosphate buffer-acetonitrile mixture (40:60, v/v) at a flow rate of 1.2 ml/minutes at

40℃. The spectrofluorometer was set with the excitation wavelength at 375 nm and the emission wavelength at 430 nm.

### 3. Results and discussion

Fig. 1A shows a typical chromatogram of whole blood from a patient being given a TPN solution containing thiamine. Two major peaks eluted at 2.2 and 5.6 minuts and three minor peaks appeared at 3.9, 5.6 and 16.1 minutes. Retention times of these fractions corresponded to those for mixtures of standard solutions of thiamine, 2.2 minutes; TMP, 5.6 minutes; TDP, 7.5 minutes; and TTP, 13.5 minutes. (See Fig. 1B). A peak at 3.9 minutes was not identified. Fluorescence intensity, i.e., the areas under each peak were expressed as $\mu$V*s, and the fluorescence intensity per mol was greatest for thiamine hydrochloride followed in decreasing order by TTP, TDP, and TMP (See Table 1). The fluorescence intensity was linear from a concentration of about 2 to 500 nmol/l for thiamine and its phosphate esters. We recovered 94% to 116% of thiamine and its esters by HPLC (Table 2). At equal concentrations of thiamine and its esters, the ratios of the peak areas of thiamine to one of the three esters were: TMP/thiamine, 0.75; TDP/thiamine, 0.90; and TTP/thiamine, 0.97. Our results were in good agreement with those reported by Ishi et al.[11] who found the ratios, in the same order as above, to be 0.63-0.65, 0.80-0.83, and 0.85, when cyanogen bromide was used as an oxidizing agent. In addition, the ratios for TMP/thiamine and TDP/thiamine were reported to be 0.8 and 0.9, respectively[12], while Lewin and Wei reported that equimolar amounts of thiamine and its phosphate esters produced equal thiochrome fluorescence values[13]. The difference observed between our results and the values by Lewin and Wei would be due to the formation of different amounts of

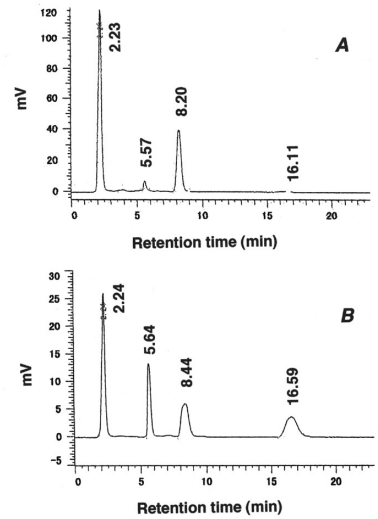


Fig. 1 Typical chromatograms of whole blood (A: upper) and mixture of standard solutions of thiamine and its phosphate esters (B: bottom). Mixture of standard solutions of thiamine and its phosphate esters contained thiamine hydrochloride (200 nmol/l); TMP (164 nmol/l); TDP (152 nmol/l); and TTP (200 nmol/l).

Table 1 Fluorescence intensities ($\mu$V*s) of thiamine and its phosphate esters at various concentrations

| | Thiamine | TMP | TDP | TTP |
|---|---|---|---|---|
| 10 nmol/l | 11,000 | 7,784 (0.71) | 9,569 (0.87) | 9,536 (0.86) |
| 100 nmol/l | 107,500 | 82,600 (0.77) | 92,100 (0.86) | 93,200 (0.87) |
| 200 nmol/l | 214,400 | 163,200 (0.76) | 195,000 (0.91) | 208,500 (0.97) |
| 500 nmol/l | 509,500 | 391,600 (0.77) | 490,200 (0.96) | 574,500 (1.13) |
| Mean±SD | | 0.75±0.03 | 0.90±0.05 | 0.96±0.13 |

Ratios of fluorescence intensities of thiamine phosphate esters to that of thiamine in parentheses.

Table 2 Recovery of thiamine and its phosphate esters

| Thiamine | | | TMP | | | TDP | | | TTP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial | Added | Found (% REcovery) | Initial | Added | Found (% REcovery) | Initial | Added | Found (% REcovery) | Initial | Added | Found (% REcovery) |
| 7 | 5 | 6 (100) | 2 | 5 | 4 (114) | 59 | 5 | 37 (116) | 0 | 5 | 0 ( 0) |
| 7 | 50 | 28 ( 98) | 2 | 50 | 25 (104) | 59 | 50 | 51 ( 94) | 0 | 50 | 24 ( 96) |
| 7 | 250 | 132 (103) | 2 | 250 | 124 ( 98) | 59 | 250 | 147 ( 95) | 0 | 250 | 126 (101) |

All values are indicated in nmol/l.
The "% Recovery" is the [Found/(Initial+Added)/2] ×100.

nonfluorescent oxidation products, since they used potassium ferricyanide as an oxidizing agent.

The fluorescence intensity of each fraction varied from day to day: the CVs of the fluorescence intensity were 6.5% for TMP, 8.3% for TDP, and 19.5% for TTP; however, the CVs of the ratios were 4.9% for TMP, 5.7% for TDP, and 9.3% for TTP. We tested whether the ratios varied depending on the eluent flow rate of the HPLC. Increasing the flow rate shortened the retention times of each fraction, but the ratios were little changed at flow rates between 1.2 and 2.0 ml/minute.

In an older HPLC method, individual calibrators for thiamine and its phosphate esters are required, i.e., there are four 200 nmol/l standard solutions. They have to be prepared fresh daily because of lability even at 4℃. Furthermore, standard solutions should be prepared separately because TMP, TDP, and TTP degraded to thiamine, and the formation of thiamine was accelerated when the four standards were combined. In the HPLC method described here, where only thiamine is used as a standard, and with the ratios as described above, concentrations of the thiamine esters are easily determined as follows: TMP concentration＝concn. of thiamine×1/0.75×(peak area for TMP)/(peak area for thiamine). The other phosphate esters are calculated the same way but with the appropriate ratios for each ester. When we assayed thiamine and its phosphate esters in whole blood from patients using thiamine calibration only vs. separate standards for thiamine, TMP, TDP, and TTP, we obtained the following slopes and intercepts with Y being thiamine and X being one of the phosphate esters in each case: Y＝1.01X－0.10, Sy.x＝2.0, r＝0.999, n＝58 for TMP; Y＝1.03X－2.0, Sy.x＝10, r＝0.998, n＝28 for TDP; and Y＝1.25X－1.1, Sy.x＝0.30, r＝0.987, n＝9 for TTP (Figures not shown). Our calibration technique facilitates routine assays for thiamine and its phosphate esters in blood and significantly simplifies the HPLC method.

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