Rapid HPLC Measurement of Thiamine and its Phosphate Esters in Whole Blood

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BACKGROUND: Thiamine (vitamin B1) deficiency is associated with severe diseases such as beriberi and Wernicke encephalopathy. Although most Americans have sufficient dietary intake, thiamine deficiency is observed in the alcohol-dependent and elderly populations. Measurement of thiamine concentration in whole blood provides an assessment of vitamin B1 status in at-risk individuals.

METHOD: We used TCA to precipitate proteins in whole blood. Thiamine and its phosphate esters were derivatized using potassium ferricyanide to thiochromes, which were separated by gradient elution on a reversed-phase HPLC column and detected by fluorescence. The method was validated for linearity, limit of quantification, imprecision, accuracy, and interference. Results obtained with this method were compared with those produced by the method currently used in our clinical laboratory. Reference values of thiamine and its phosphate esters were determined in samples obtained from self-reported healthy adults who were not taking vitamin supplements. To shorten analysis time, our method used whole blood rather than washed erythrocytes, did not require lengthy enzymatic dephosphorylation, and had a simple mobile phase.

RESULTS: The method was linear to 4000 nmol/L. The lower limit of quantification was 3 nmol/L. The within-run CV was <3.5% and total CV was <9.4%. This method correlated with our current method (r = 0.97). Approximately 90% of the total thiamine content in whole blood was present as thiamine diphosphate (TDP). The means (ranges) for an apparently healthy population were 114 (70–179) nmol/L for TDP and 125 (75–194) nmol/L for total thiamine. Results for separation and measurement of free thiamine and thiamine phosphate esters in whole blood were obtained within 5.5 min.

CONCLUSION: We developed an HPLC method that allows separation and measurement of free thiamine and thiamine phosphate esters in whole blood and provides more rapid results than other methods.

The vitamin B1 family consists of thiamine, a pyrimidyl-substituted thiazole, and its phosphate esters: thiamine monophosphate (TMP), thiamine diphosphate (TDP), and thiamine triphosphate. The principal biologically active form of thiamine is the diphosphate, TDP, a required coenzyme for the essential decarboxylation reactions by which carbohydrates, fats, and alcohol are metabolized to produce energy. Thiamine triphosphate is necessary for the regulation of ion channels in the nervous system. Thiamine deficiency causes deleterious changes in the cardiovascular and nervous systems that are effectively reversed by administration of the vitamin (1, 2).

The majority of the total thiamine content of whole blood is found in erythrocytes as TDP. Because TDP concentrations are depleted from erythrocytes at a rate similar to depletion from other organs, measurement of TDP in erythrocytes provides a good indication of the adequacy of body stores (1). Talwar et al. showed that the TDP concentration in erythrocytes correlates with that in whole blood, a characteristic that allows the use of whole blood, rather than washed erythrocytes, for thiamine assessment (3).

HPLC methods for determination of thiamine and its esters in blood have been reviewed (4). Because of the low biological concentrations of these analytes, most methods employ precolumn or postcolumn oxidation of thiamines followed by fluorescent detection of thiochrome compounds. Total thiamine concentration can be measured by dephosphorylation of thiamine esters. Overnight enzymatic hydrolysis using acid phosphatase converts thiamine esters to free thiamine, and total thiamine concentration is measured (5). Although still in use, this method is time-consuming and
fails to provide direct information on TDP, the principal biologically active form of the vitamin. Methods for the simultaneous determination of thiamine and its esters have been developed using isocratic, ion-pairing, and gradient elution. Herve et al. used isocratic elution to separate the 4 thiamine compounds within 15 min, but baseline resolution was not achieved (6). In the isocratic method of Baines, later improved by Lynch et al., thiamine was extracted directly into methanol, a component of the mobile phase (7, 8). This step obviated the necessity for removal or neutralization of trichloroacetic acid (TCA) following precipitation, and resolved one peak thought to correspond solely to TDP. This otherwise rapid and convenient method did not perform well in our hands owing to incomplete protein precipitation from whole blood by the weak organic solvent methanol and coelution of the peak front with TDP in biological samples. Losa et al. and Bettendorf et al. used ion-pairing to better separate the thiamine mixture (9, 10). The use of ion-pair reagents introduces complexity in the method. Tallaksen et al. used a step-gradient method and an amine column to measure individual thiamine compounds. However, the separation required 20 min (11). We developed a step-gradient HPLC method with which we sought to overcome the weaknesses of previously reported thiamine assays and provide more rapid results for baseline separation of thiamine compounds. To shorten analysis time, our method used whole blood rather than washed erythrocytes, did not require lengthy enzymatic dephosphorylation, and had a simple mobile phase.

Materials and Methods

CHEMICALS AND REAGENTS
Thiamine, TMP, TDP, potassium ferricyanide \([K_2Fe(CN)_6]\), and dibasic sodium phosphate were purchased from Sigma. Hydrochloric acid, methanol, methyl-tert-butyl ether, phosphoric acid, and TCA were obtained from JT Baker. All reagents were HPLC grade. Solutions were filtered through a 0.22-μm filter before use. Water was generated using a nanopure water system. Erythrocytes from expired blood bank units were obtained from ARUP Blood Services.

CHROMATOGRAPHIC CONDITIONS
The HPLC system was an Agilent Rapid Resolution High-Throughput 1200 with the autosampler set at 10 °C and configured for a 96-well microtiter plate. Separation was accomplished at 25 °C using an Agilent Zorbax Eclipse plus C\(_{18}\) Rapid Resolution HT analytical column (3.0 × 50 mm, 1.8 μm), protected with a Phenomenex Security C\(_{18}\) guard cartridge (4 × 2.0 mm). Mobile phase A was dibasic sodium phosphate (25 mmol/L, pH 7.0):methanol (90:10, vol/vol); mobile phase B was dibasic sodium phosphate (25 mmol/L, pH 7.0):methanol (30:70, vol/vol). Gradient steps were programmed as follows: 0%–12.5% B in 1 min, ramped to 50% B in 0.5 min, held at 50% B for 1.5 min, returned to initial conditions during 1 min, and equilibrated for 1.5 min. Analytes were eluted at a flow rate of 0.6 mL/min. Injection volume was 20 μL. The compounds were detected at an excitation wavelength of 375 nm and emission wavelength of 435 nm. Peak area was measured.

CALIBRATORS
Stock solutions of thiamine, TMP, and TDP (approximate concentrations 1 mmol/L) were prepared in 0.1 mol/L hydrochloric acid. Stock solutions were divided into aliquots and stored at −70 °C. Exact concentrations of thiamine and TDP solutions were determined by spectrophotometric measurements at 246 nm, using a molar absorptivity of 14 200 L/mol per cm for thiamine and of 13 000 L/mol per cm for TDP (12). The concentration of TMP was determined by weighing the dry chemical. These solutions were stable for 5 months.

Calibrators were prepared fresh for each run by diluting the stock solutions with water to concentrations of 0, 100, 200, and 400 nmol/L for TDP and 0, 10, 20, and 40 nmol/L for TMP and thiamine, to mimic the biological concentrations of these thiamine compounds in human blood. Calibrators were treated in the same way as blood hemolysates and assayed at the beginning and end of each run.

SAMPLE PREPARATION
Venous blood was collected into EDTA- or heparin-containing tubes. Specimens were frozen immediately. Thawed hemolysates (500 μL), water (blank), calibrators, and controls were treated with 500 μL of 10% TCA to precipitate proteins. Samples were vortex-mixed vigorously for 15 s and left standing on ice for 15 min. The precipitated samples were centrifuged for 6 min at 10 °C. Each supernatant was transferred into a glass tube and washed twice with water-saturated methyl-tert-butyl ether (750 μL) to remove TCA. Aliquots of the solution (80 μL) were transferred from each tube to a corresponding well of a 96-well microtiter plate. Methanol (20 μL) was added to intensify the fluorescent signal and enhance stability of the product. Before injection onto the column, samples were derivatized by addition of 50 μL of freshly prepared potassium ferricyanide in 15% sodium hydroxide (Fig. 1). Final concentrations were 2 × 10\(^{-4}\) mol/L for potassium ferricyanide and 5% for sodium hydroxide (13). After mixing, the plate was covered with a sealing tape and placed on the autosampler.
ASSAY VALIDATION

Chromotographic conditions. Isocratic, ion-pairing, and gradient methods were evaluated to resolve the structurally similar thiamine and thiamine phosphate esters in a short run-time.

Linearity and detection limit. To study linearity of the assay, duplicates at each of 5 concentrations of all calibrators over the range of 0–4000 nmol/L were analyzed. The limit of quantification was determined by assaying samples with low thiamine concentrations. Serial dilutions of expired blood bank erythrocytes were analyzed in duplicate 3 times. The target CV for these measurements was <10%.

Imprecision. For the imprecision study, 1 lyophilized whole blood control and 2 pools of washed erythrocytes with added TDP (at 3 different concentrations) were assayed. Six replicates of each control were used to estimate within-run SDs. Total SDs were estimated by assaying each control in duplicate every day over a period of several months.

Recovery. Recovery studies were performed by analyzing a pool of washed erythrocytes of known concentration before and after addition of 50 nmol/L and 100 nmol/L thiamine, TMP, and TDP. Recovery was calculated as: [(final concentration – initial concentration)/added concentration].

Materials and method comparison. Patient sample analysis results obtained with the new method were compared to results obtained with the method currently used in our laboratory. In this assay, acid phosphatase is used to remove phosphate groups from the phosphorylated forms of thiamine, and total thiamine is determined. Precolumn derivatization of thiamine yields a fluorescent thiochrome that is isolated by isocratic elution on a reversed-phase HPLC column and detected by fluorescence. The new method detects and measures thiamine and its phosphate esters; the sum of the individual concentrations was used for comparison with total thiamine.

Interference. Interference was investigated by analyzing samples containing known concentrations of vitamins B2 and B6 and whole blood samples to which we added caffeine (5 mg/L) or common over-the-counter and prescription drugs. Drugs were added to obtain concentrations at the upper end of the therapeutic range: acetaminophen (10 mg/L), aspirin (3 mg/L), diphenhydramine (0.1 mg/L), ephedrine (100 mg/L), ibuprofen (50 mg/L), naproxen (20 mg/L), and pseudoephedrine (0.1 mg/L).

Stability of the products. To assess the stability of the thiochrome derivatives, controls and patients samples (n = 25) were assayed immediately after potassium ferricyanide oxidation, then stored at 10 °C in the dark and reassayed after 24 and 48 h.

Reference values. Nonfasting whole blood specimens were obtained from self-reported healthy laboratory staff members [19 females and 24 males, age 21–63 years (mean 34 years)] who were not taking vitamin supplements. For these 43 samples, concentrations of thiamine, TMP, and TDP were measured and total thiamine concentrations and the percentage distributions of the vitamers were calculated. All studies with samples from human subjects were approved by the Institutional Review Board of the University of Utah and participants provided informed consent.

Results

CHROMATOGRAPHY

TMP and TDP, which eluted at high aqueous concentration, were separated from the peak front and resolved from free thiamine, which eluted at high organic-phase concentrations, using a ramp from 10% to 40% methanol during a 90-s period. Chromatographic profiles corresponding to a calibration solution and a whole blood extract are shown in Fig. 2. Thiamine, TMP, and TDP peaks were well resolved. Thiamine compounds were identified by comparing peak retention times with those of the calibrators and quantified using a calibration curve generated from 4 calibrators. Approximate analyte elution times were: thiamine, 3.4 min; TMP, 2.8 min; and TDP, 2.2 min. The chromatographic profile of blank injections prepared from an aliquot of water subjected to oxidation by K3Fe(CN)6 showed no interference. No significant drift was observed in calibrator solutions analyzed at the beginning and end of a run.
ASSAY VALIDATION

The peak area measurements of thiamine, TMP, and TDP were linear to 4000 nmol/L. Imprecision for serial dilutions of expired blood bank erythrocytes was less than the desired target of 10% for concentrations as low as 3 nmol/L. This concentration was accepted as the lower limit of quantification for the assay. Imprecision data for TDP at 3 concentrations are listed in Table 1. Within-run CVs were <3.5%; total CVs were <9.4%. Within-run and between-run CVs for thiamine, TMP, and the sum of the analytes (thiamine + TMP + TDP) were <4% and <8%, respectively.

Table 1. Imprecision of TDP measurements in washed erythrocyte pools and lyophilized whole blood control material.

<table>
<thead>
<tr>
<th></th>
<th>Within day (n = 6)</th>
<th>Total (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean, nmol/L</td>
<td>SD</td>
</tr>
<tr>
<td>Pool 1</td>
<td>85.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Pool 2</td>
<td>195.7</td>
<td>4.6</td>
</tr>
<tr>
<td>Control</td>
<td>169.3</td>
<td>4.1</td>
</tr>
</tbody>
</table>

When 50 nmol/L and 100 nmol/L thiamine, TMP, and TDP were added to pools of washed erythrocytes, recoveries were not significantly different from 100%. Actual mean (SD) recoveries (n = 5) were 100% (3%), 101% (5%), and 103% (2%), respectively, for 50 nmol/L and 99% (2%), 97% (2%), and 95% (2%), respectively, for 100 nmol/L.

As shown in Fig. 3, method comparison yielded the Deming regression equation: $y = 0.95x - 3.63, r = 0.97$. The new method agreed well with our current clinical method.

Vitamins B$_2$ and B$_6$, caffeine, and common over-the-counter drugs did not alter thiamine concentration. Concentrations of thiochrome products stored in the dark at 10 °C decreased by no more than 5% in 48 h. TDP measured in specimens subjected to 3 freeze-thaw cycles decreased by no more than 5%. Both of these changes are within the day-to-day variation of the assay. TDP in calibration solutions showed no change in concentration when exposed to room light for 7 h. The 3-ring structure of the thiochrome results in excellent stability of the derivatives; thiochromes were stable for at least 48 h. The analytical column remained stable for approximately 500 injections.

REFERENCE VALUES

As expected, approximately 90% of the total thiamine content in whole blood analyzed using this method was present as TDP. Concentrations and percentage distri-
Table 2. Measurement of thiamine (T), TMP, and TDP in whole blood obtained from 43 self-reported healthy adults who were not taking vitamin supplements.

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>TMP</th>
<th>TDP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.4</td>
<td>3.5</td>
<td>114.0</td>
<td>124.8</td>
</tr>
<tr>
<td>(Range)</td>
<td>(3.3–12.4)</td>
<td>(1.6–6.5)</td>
<td>(70.3–178.6)</td>
<td>(75.2–193.8)</td>
</tr>
<tr>
<td>Mean percentage of total</td>
<td>5.9</td>
<td>2.8</td>
<td>91.3</td>
<td>100</td>
</tr>
<tr>
<td>(Range)</td>
<td>(3.4–10.8)</td>
<td>(1.2–4.5)</td>
<td>(85.6–95.2)</td>
<td></td>
</tr>
</tbody>
</table>

butions for each thiamine vitamer are listed in Table 2. No significant differences were observed in results for samples from male and females ($P = 0.23$). Whole blood concentrations determined for this group of apparently healthy reference individuals were 70–180 nmol/L for TDP and 75–195 nmol/L for total thiamine.

Discussion

Although we receive approximately equal numbers of whole blood and plasma specimens for thiamine assessment, whole blood is the superior sample type for analysis of thiamine concentration. Losa et al. showed that plasma contains mainly thiamine and TMP, whereas TDP predominates in erythrocytes (9). Our data also demonstrate that TDP accounts for 90% of the thiamine content in whole blood. As has been reported previously (3, 9, 11), thiamine triphosphate was not detected in whole blood specimens. For monitoring TDP, the specific index of thiamine nutrition, whole blood is the ideal specimen type. No significant difference was noted in results from blood samples anticoagulated with EDTA or heparin. This finding is consistent with results reported by Ihara et al. (14).

Talwar et al. (3) reported significant discrepancies between TDP concentrations in whole blood stored at $–20 ^\circ C$ and $–70 ^\circ C$. We found that incompletely frozen specimens yielded low thiamine concentrations. Some samples kept at $–20 ^\circ C$ for 24 h did not produce good hemolsates. These samples yielded higher concentrations following overnight storage at $–70 ^\circ C$. Specimens must be frozen completely to ensure lysis of the erythrocytes.

Because approximately 90% of all thiamine in whole blood is TDP, measurement of the diphosphate ester should be representative of thiamine status. In fact, we believe that monitoring of this physiologically active form of thiamine is preferable to the analysis of total thiamine. For clinical assessment, our laboratory reports TDP concentration in whole blood.

The reference interval for TDP concentration has been reported as 90–140 nmol/L for whole blood (15). Our population reference range for TDP is comparable with this published interval. Vitamin concentrations depend solely on dietary intake. Diets are influenced by many factors and vary geographically. Therefore a local reference interval is essential for recommendations for optimal intake of vitamins to maintain health (3).

In conclusion, we developed an HPLC method that allows measurement of free thiamine and its phosphate esters in whole blood. Well-resolved peaks elute in 5.5 min using a simple gradient, and consumption of mobile phase and other reagents is much less for this method than for other methods.

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References


