Liquid-Chromatographic Determination of the Total Thiamin Content of Blood

Mieko Kimura,¹ Tomio Fujita,² and Yoshinori Itokawa¹,³

A liquid-chromatographic method for determining the total thiamin content of blood is presented. Blood is deproteinized and incubated with Aspergillus oryzae carboxyl proteinase (EC 3.4.23.8; Takadiastase) to convert thiamin phosphate esters to free thiamin. An aliquot of the sample is applied to the column (Shodex OH-Pak M-414) of a high-performance liquid chromatograph. A 100 mg/L solution of potassium ferricyanide in 150 g/L sodium hydroxide is added to the column effluent with a proportioning pump, to convert thiamin into a fluorophore. The intensity of the fluorophore is measured with a spectrophotometer and recorded graphically. The total thiamin in each blood sample appears as a single peak, and no co-eluting substance was detected. This method is simple, highly reproducible, and rapid, and its sensitivity is sufficient for determination of the thiamin content of 0.1-mL blood samples.

Additional Keywords: detecting thiamin deficiency • enzymic methods • reference interval

Various biochemical tests have been developed for the detection of thiamin deficiency, including measurements of thiamin in blood or urine or of erythrocyte transketolase (EC 2.2.1.1) activity (1).

A close correlation exists between the development of thiamin deficiency and the decreasing excretion of thiamin, as measured in 24-h urine samples (1, 2). However, estimation of thiamin status by use of untimed urine samples may be inaccurate (3), and it is not usually feasible to collect 24-h urine samples.

Measurement of the activity of erythrocyte transketolase, a thiamin-dependent enzyme, is considered a convenient and sensitive method to detect thiamin deficiency (4, 5). The method involves incubation of hemolyzed erythrocytes with ribose-5-phosphate as a substrate in both the presence and the absence of excess thiamin pyrophosphate (ThPP). After the incubation, the production of sedoheptulose-7-phosphate is measured (6). The percentage stimulation of transketolase activity produced by adding thiamin pyrophosphate (the "ThPP effect") represents the amount of the apoenzyme not saturated with coenzyme (4). A decrease in activity from that originally present and an increase of the ThPP effect indicate thiamin deficiency. However, in these assay methods, effects of other non-thiamin-dependent enzymes cannot be excluded. Moreover, erythrocyte transketolase frequently fails to respond to the in vitro addition of ThPP in patients with nervous diseases or hepatic diseases, even in the presence of severe thiamin deficiency (7, 8).

Therefore, determination of total thiamin in blood is assumed to be the most nearly accurate way to evaluate the nutritional status of thiamin in humans. Berger et al. (9) discovered that a fluorescent substance (thiochrome) was formed from thiamin by reaction with potassium ferricyanide in an alkaline solution, and later Fujiwara and Matsumi (10) found that thiochrome was produced when thiamin was mixed with cyanogen bromide. Both of those reactions were widely used for the quantitative determination of the total thiamin content of blood. However, in these methods, at least 3 mL of blood is necessary for accurate estimation, and technical difficulties are involved.

In these situations, we have contrived a simple and sensitive method for determining the total thiamin content of blood by "high-performance" liquid chromatography.

Materials and Methods

Instrumentation. The following instruments were used: Pump for high-performance liquid chromatography, model LC-3A; sample injector, model SII-1A; column, Shodex OH pak M-414 (4 mm i.d. × 250 mm); column oven, CTO-2A (35 °C); mixing coil, Teflon tube (0.3 mm i.d. × 2 m); proportioning pump for thiochrome reaction, PRR-2A (Tytgon tube); detector, fluorospectrophotometer RF500-LCA (excitation wavelength, 375 nm; emission maximum, 450 nm; square-shaped flow cell of 12 μL capacity); recorder and computer, R-112 and Chromatopac C-RIA. All items of the equipment are products of Shimadzu Co., Kyoto, Japan.

Reagents. Thiamin HCl was purchased from Wako Chemical Co., Osaka, Japan. De-ionized distilled water was used to prepare all reagents. For the mobile phase, 0.2 mol/L NaH₂PO₄ was used. A 100 mg/L solution of K₂Fe(CN)₆ in 150 g/L NaOH was used to convert thiamin into the fluorophore (thiochrome reaction). The Aspergillus oryzae carboxyl proteinase (EC 3.4.23.8; Takadiastase) was a product of Sanyko Co., Tokyo, Japan. This material also contains alpha-amylase, phosphatase, and protease. We find that it works rather better than the purified enzyme. All other chemicals were of the purest grade commercially available.

Procedures. To 0.1 mL of blood in a 1.1 × 5 cm polypropylene centrifuge tube, 0.4 mL of 100 g/L trichloroacetic acid was added and vortex-mixed vigorously. The sample was then centrifuged at 35 000 × g for 30 min, the pellet discarded, and 400 μL of the supernatant solution adjusted to pH 4.6 with 60 μL of 4 mol/L sodium acetate. Forty microliters of Takadiastase was added, to give a final concentration of 0.5 g/L, and

Fig. 1. Schematic diagram of the analytical system

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Table 1. Thiamin Concentration in Blood of Human, Rat, and Rabbit

<table>
<thead>
<tr>
<th></th>
<th>No. samples</th>
<th>Thiamin concentration, (\mu g/L) (mean ± SE)</th>
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<tbody>
<tr>
<td>Human</td>
<td>20</td>
<td>46.2 ± 2.3</td>
</tr>
<tr>
<td>Rat</td>
<td>7</td>
<td>243.3 ± 12.1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>5</td>
<td>257.8 ± 11.1</td>
</tr>
</tbody>
</table>

0.5 mL of the mixture was incubated at 37 °C for 8–10 h, to convert thiamin phosphate esters into free thiamin.

Figure 1 shows a schematic diagram of this system. The mobile phase (0.2 mol/L NaH₂PO₄) was pumped at a flow rate of 0.3 mL/min into the column of a high-performance liquid chromatograph (HPLC). A 100-µL aliquot of the supernatant solution was loaded on the sample loop and then injected onto the column after the zero time had been marked. Potassium ferricyanide and sodium hydroxide solution were added to the column effluent at the rate of 0.3 mL/min with a proportioning pump and mixed with it to convert thiamin into thiochrome. The fluorescence of the thiochrome was measured with a spectrofluorophotometer and recorded graphically. For quantification, the peak height was used as compared with a standard calibration curve for thiamin. A blank study was run without potassium ferricyanide.

Results and Discussion

Fluorescence intensities and amounts of standard thiamin were linearly related within a range of concentrations from 100 ng/L to 500 µg/L. This suffices for the determination of the thiamin content of human blood.

Figure 2 illustrates elution profiles for a thiamin standard, a blood sample, a blood sample supplemented with thiamin, and a blank (blood without thiochrome reaction). Thiamin was detected as a single peak, with a retention time of 16 min. The blood sample contained a fluorescent peak other than thiochrome at the retention time of 11 min (Figure 2b) but no co-eluting substance was found in the position of the peak of thiamin, as indicated in Figure 2d. Addition of thiamin to the sample increased the peak height of thiamin as shown in Figure 2c. Analytical recoveries of 1 ng of thiamin with this system were calculated to be 97.0 ± 2.1% (mean ± SE; n = 7).

The concentration of total thiamin in the same blood samples was determined simultaneously by this method and by the conventional thiochrome method (10). The regression line is shown in Figure 3. The correlation coefficient is 0.94.

Table 1 shows the thiamin concentration in samples of blood taken from normal humans (age range: 25–48 years), rats, and rabbits as determined by this method.

Recently the technique of liquid chromatography has progressed considerably and has been applied to the differential determination of thiamin and of its phosphate esters (11-15). However, these methods are not suitable for the determination of thiamin in blood, because thiamin phosphate esters are easily interconvertible by various enzyme systems in blood and errors are increased when thiamin is estimated from the sum of each peak height of thiamin and thiamin phosphate esters.

In contrast, by the use of the present method, the total thiamin content can be determined as a single peak and the sensitivity is such that thiamin can be determined in 0.1-mL blood samples. Moreover, this method is simple, highly reproducible, and rapid. We assume that this method can be used as a tool for evaluating the nutritional status for thiamin in clinical studies. However, further studies on isolation and independent chemical identification of the chromatographic peak are necessary to establish the accuracy and specificity for thiamin in this method.

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