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DETERMINATION OF THIAMINE AND ITS PHOSPHATE ESTERS IN HUMAN AND RAT BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

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SUMMARY

A method for the determination of thiamine and thiamine phosphate esters in human and rat blood by liquid chromatography with post-column derivatization, and distribution of thiamine and its phosphate esters in human and various animals is demonstrated. Blood is deproteinized and centrifuged. Aliquots of the samples are applied to a μBondapak C_{18} column attached to a high-performance liquid chromatograph. Addition of potassium ferricyanide sodium hydroxyide solution to the column effluent with a proportioning pump converts thiamine phosphate into fluorophores, the intensities of which are measured with a spectrofluorophotometer. In human blood, thiamine pyrophosphate was present in the greatest concentration, followed by thiamine triphosphate; thiamine monophosphate and thiamine were present in small amounts. In rat blood, thiamine pyrophosphate was found in the largest amount, followed by thiamine monophosphate. Thiamine triphosphate and thiamine were present in small amounts.

INTRODUCTION

Various biochemical tests have been developed for the detection of thiamine deficiency, including measurements of thiamine in blood or urine or of erythrocyte transketolase activity[1]. A close correlation exists between the development of thiamine deficiency and the decreasing excretion of thiamine as measured in 24-h urine samples[1,2]. However, the determination of thiamine status by the 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 thiamine-dependent enzyme, is considered to be a convenient and sensitive method for detecting thiamine deficiency[4,5]. However, in these assay methods, effects of other non-thiamine dependent enzymes cannot be excluded. Moreover, erythrocyte transketolase frequently fails to respond to the *in vitro* addition of TPP in patients with nervous or hepatic diseases, even in the presence of severe thiamine deficiency[6,7]. The determination of total thiamine in blood is assumed to be the most accurate way of evaluating the nutritional status of thiamine in humans.

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Berger *et al.*[8] discovered that a fluorescent substance (thiochrome) was formed from thiamine by reaction with potassium hexacyanoferrate(III) in alkaline solution, and later Fujiwara and Matsui[9] found that thiochrome was produced when thiamine was mixed with cyanogen bromide. Both of these reactions have been widely used for the determination of the total thiamine content of blood. However, in these methods, at least 3 ml of blood are necessary for accurate determination, and technical difficulties are involved.

In view of this, we have developed a simple and sensitive method for determining the total thiamine content of blood[10] and the erythrocyte transketolase activity using high-performance liquid chromatography (HPLC)[11] with post-column derivatization.

In animal tissues, there exist four different forms of thiamine: free thiamine, thiamine monophosphate (TMP), thiamine pyrophosphate (TPP) and thiamine triphosphate (TTP).

Recently, several microquantitative methods for the determination of thiamine and its phosphate esters by HPLC have been developed[12–19]. We have previously reported a reversed-phase HPLC method with post-column derivatization for separating and determining thiamine phosphate esters[20]. Here, we describe an improved method for the determination of as little as 30 fmol of thiamine and its phosphate esters in human and animal blood.

EXPERIMENTAL

Apparatus

The following instruments were used: pump for HPLC, Model LC-3A; sample injector, Model SIL-1A; column, μBondapak C_{18} (25 cm × 4 mm I.D.); proportioning pump with Tygon tubing for thiochrome reactions, PRR-2A; detector, RF500-LCA spectrofluorophotometer (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. The column was obtained from Waters Assoc. (Milford, MA, U.S.A.) and all other equipment from Shimadzu (Kyoto, Japan).

Reagents

Thiamine hydrochloride was obtained from Wako (Osaka, Japan) and TMP and TPP from Sigma (St. Louis, MO, U.S.A.). TTP was donated by the Central Research Division of Takeda Chemical (Osaka, Japan). All other chemicals were of the best grade commercially available. De-ionized, distilled water was used to prepare all reagents.

For the mobile phase, we used a 0.2 *M* solution of NaH_2PO_4 in 0.3% aqueous acetonitrile. For post-column conversion of phosphate esters into fluorophores, we used a 0.1% solution of $K_3Fe(CN)_6$ in 15% NaOH[10].

Preparation of samples

Collect human, rat, mouse, guinea pig, rabbit, dog, pigeon and chick blood with a heparinized syringe. Centrifuge the blood quickly after collecting at 1100 *g* for 15 min to separate erythrocytes from plasma. To 0.2 ml of 10% trichloroacetic acid in a 1.5-ml polyethylene centrifuge tube, add 0.2 ml of blood, erythrocytes or

plasma and vortex mix vigorously, then centrifuge at 35 000 *g* for 5 min. Use the supernatant solution as the sample.

Procedures

Inject 100 μl of the sample into the chromatograph. The flow-rate of the mobile phase is 1.0 ml/min. Add hexacyanoferrate(III)–sodium hydroxide solution to the column effluent at 0.5 ml/min with a proportioning pump to convert thiamine phosphate esters into thiochrome phosphates. Measure and record with the spectrofluorophotometer. For quantification compare the peak heights of the samples with a standard calibration graph for thiamine and its phosphate esters.

We also chromatographed samples without adding the potassium hexacyanoferrate(III) to check the blank values.

Animal experiments

Use male Wistar rat weighing approximately 150 g. Administer thiamine (10 mg/kg body weight) intraperitoneally or orally. Collect blood at 0, 5, 15, 30, 60, 120 and 180 min after administration and measure thiamine and its phosphate esters in these samples. Also, collect blood and incubate it at 37°C on a water-bath without or with thiamine *in vitro*. At 0, 5, 15, 30, 60, 120 and 180 min after incubation measure the thiamine and its phosphate esters in the blood.

RESULTS AND DISCUSSION

Elution profiles for a standard solution of thiamine, TMP, TPP and TTP, a human blood sample and a blank (without thiochrome reaction) are shown in Fig. 1 TTP, TPP, TMP and thiamine were each detected as single peaks, with retention times of 3.1, 3.8, 5.0 and 8.0 min, respectively. In the blood sample, TTP, TPP and TMP were detected as single peaks, but thiamine was usually not detected. Moreover, the blood contained three fluorescent peaks other than thiochrome, with retention times of 2.0, 6.25 and 12.0 min, but no substance was found to be eluted at the position where the peaks for TTP, TPP, TMP and thiamine would be eluted. Thiamine and its phosphate esters were not detected in the solution after passing it through a zeolite column[9], which adsorbs thiamine and its phosphate esters.

Fig. 2 shows the calibration graph obtained for standard solutions of thiamine, TMP, TPP and TTP. The detection limit is 30 fmol of thiamine and its phosphate esters. Fig. 1 illustrates elution profiles for a human blood sample to which TTP, TPP, TMP and thiamine had been added. Analytical recoveries of 1 pmol each of added thiamine and its phosphate esters in samples of blood taken from normal humans were 95.0–100.2%. In normal blood, the concentration of TPP was highest, followed by TTP; TMP and thiamin were hardly detectable. In erythrocytes, only TTP and TPP were found; TMP and thiamine were not detected. Plasma, on the other hand, contained small amounts of TMP and thiamine, but no TTP and TPP. In blood kept at room temperature for 1 h after collection, the TTP content decreased in proportion to the time interval, but no significant changes in TPP, TMP and thiamine contents were observed.

Fig. 3 illustrates elution profiles for whole blood, erythrocytes and plasma samples of a rat. In the whole blood, TTP, TPP, TMP and thiamine were detected

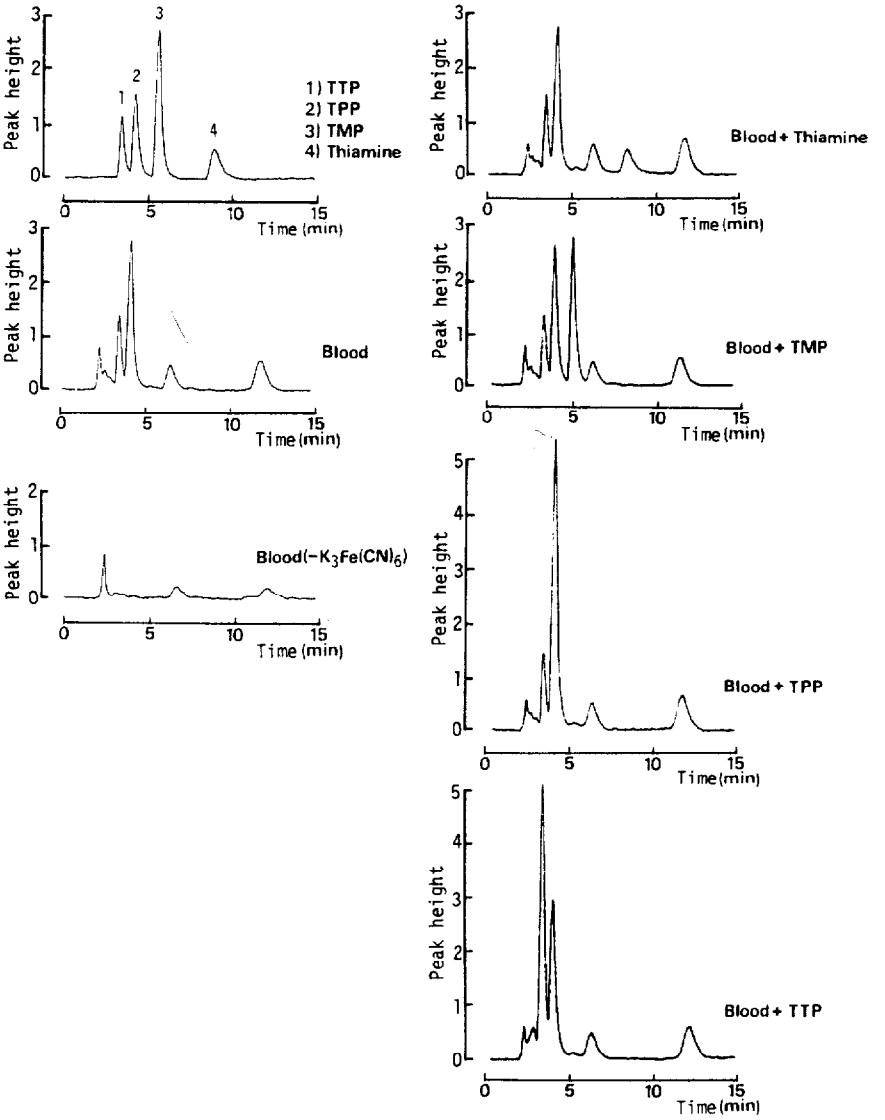

Fig. 1. Elution profiles for standard solutions of thiamine, TMP, TPP and TTP; a human blood sample, a human blood sample without thbiochrome reaction and human blood samples supplemented with thiamine, TMP, TPP and TTP.

as single peaks. TTP and TPP were found in the erythrocytes, and larger amounts of TMP and thiamine were detected in the rat plasma than in the human sample. The finding that a considerable amount of TMP was found in rat plasma is consistent with the results of Rindi *et al.*[21]. Concentrations of thiamine and its phosphate esters in whole blood of various species are shown in Table I. It is worth noting that a large amount of TMP was found in the blood of various animals. These results suggest

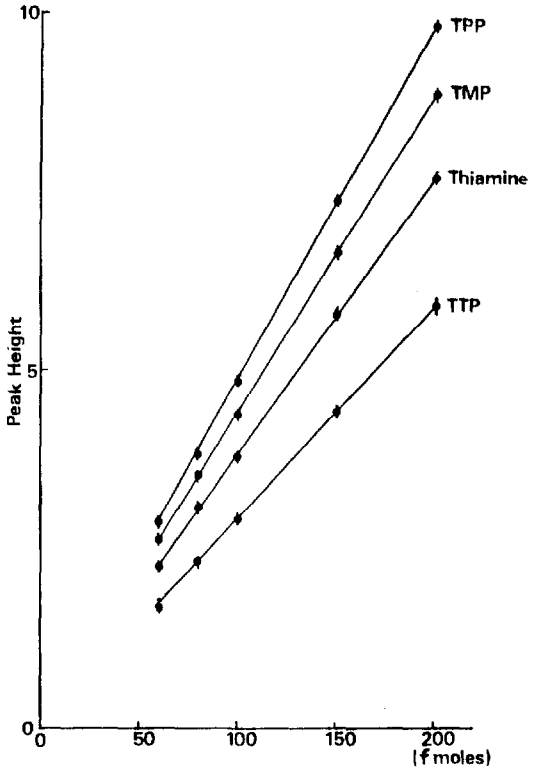

Fig. 2. Calibration graphs obtained for standard solutions of thiamine, TMP, TPP and TTP.

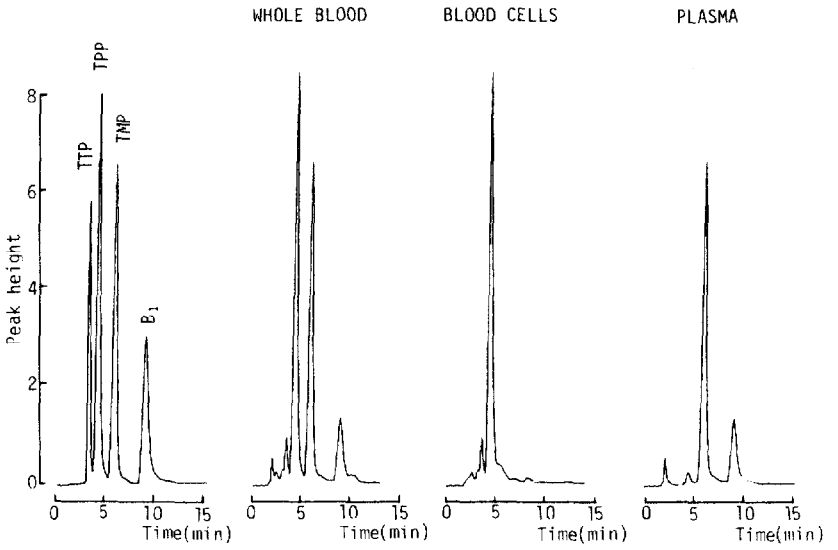

Fig. 3. Elution profiles for a standard solution, whole blood, blood cells and plasma of a rat.

that the distribution of thiamine and its phosphate esters in blood will differ according to the animal species or thiamine nutritional status.

A 10-mg amount of thiamine hydrochloride was administrated i.p. or orally to rats *in vivo*, and blood was collected at various intervals after administration. The changes in concentration of thiamine and its phosphate esters in whole blood are shown in Fig. 4A and B. With i.p. administration, the thamine concentration increased quickly up to a very high level ($4 \cdot 10^{-6}$ *M*) within 5 min, then decreased.

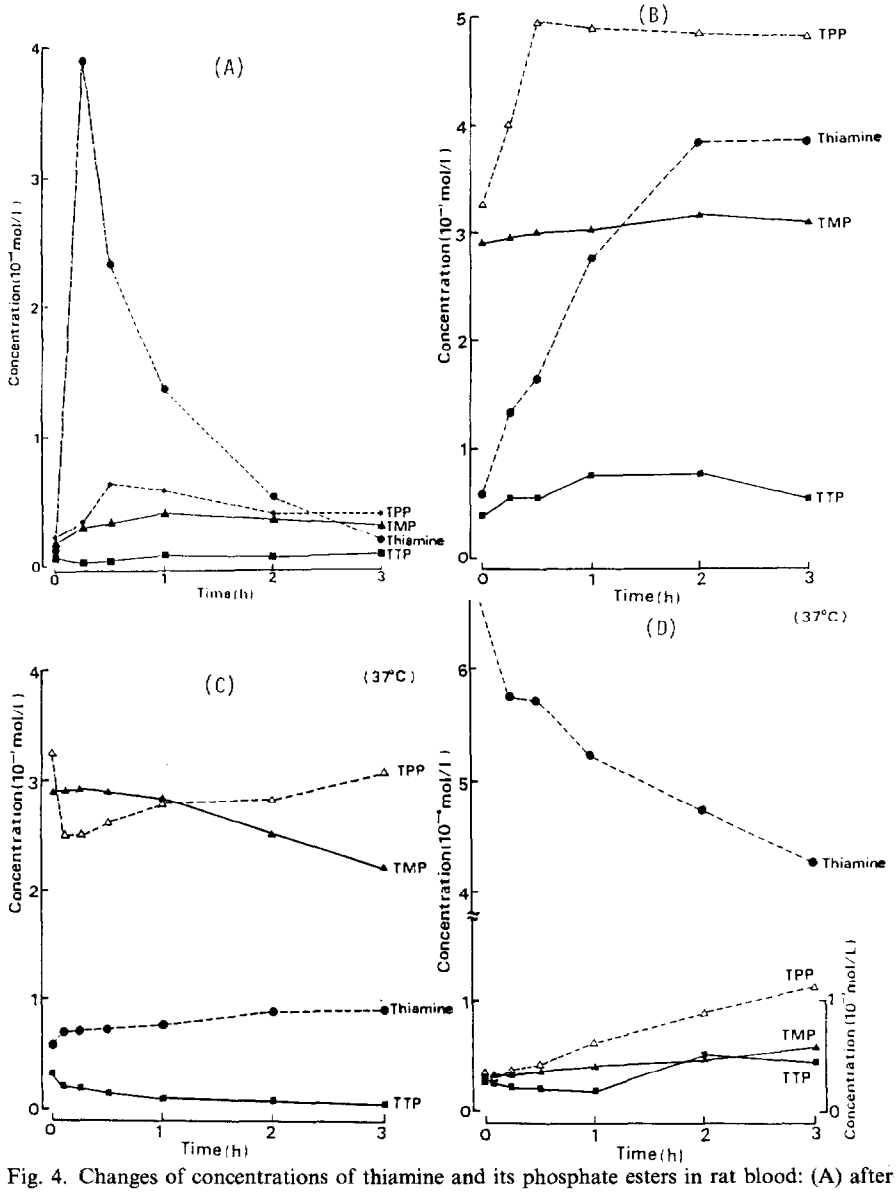

Fig. 4. Changes of concentrations of thiamine and its phosphate esters in rat blood: (A) after thiamine administration i.p. *in vivo*; (B) after thiamine administration orally *in vivo*; (C) blood kept at 37°C *in vitro*; (D) blood kept at 37°C after thiamine administration *in vitro*.

TABLE I

THIAMINE AND ITS PHOSPHATE ESTERS CONCENTRATION IN BLOOD OF VARIOUS ANIMALS (n = 3)

Blood	*Concentration (10^{-7} mol/l)*			
	Thiamine	*TMP*	*TPP*	*TTP*
Mouse	4.86 ± 0.33	4.92 ± 0.41	7.80 ± 0.56	0.50 ± 0.02
Rat	1.03 ± 0.10	4.42 ± 0.31	3.45 ± 0.25	0.42 ± 0.02
Guinea pig	0.66 ± 0.03	1.28 ± 0.11	7.16 ± 0.21	0.55 ± 0.03
Rabbit	0.07 ± 0.00	1.79 ± 0.09	4.06 ± 0.34	0.62 ± 0.04
Dog	0.20 ± 0.01	0.39 ± 0.01	1.34 ± 0.08	0.24 ± 0.02
Pigeon	0.22 ± 0.01	1.28 ± 0.10	6.79 ± 0.36	0.23 ± 0.01
Chick	0.22 ± 0.01	0.32 ± 0.02	1.23 ± 0.06	0.11 ± 0.01

The TPP and TMP concentrations increased gradually. On the other hand, with of oral administration, the thiamine concentration increased gradually and reached a peak after 2 h. TPP increased more quickly than thiamine, and TTP and TMP did not change much. Fig. 4C and D also shows the changes in the concentrations of thiamine and its phosphate esters in rat blood *in vitro*. Rat blood was collected and placed in a test-tube in a 37°C water-bath. The TPP level decreased over 5 min, then increased again gradually; the TMP and thiamine levels increased. When thiamine was added to rat blood, its concentration decreased quickly and the TTP, TPP and TMP levels increased slowly.

We had previously reported two methods for post-column derivatization in separating and measuring thiamine and its phosphate esters by HPLC. In the first method[13] we used an ion-exchange column (Shimadzu ISA-07/S2504) and in the second[20] a reversed-phase column (μBondapak C_{18}). With the ion-exchange column, thiamine phosphates were eluted in the order thiamine, TMP, TPP and TTP. The elution peak of TTP was, accordingly, broad and difficult to quantify. In the second method, TTP was eluted first, clearly separated from other, non-specified eluted substances. Therefore, we used this method and column to determine the concentration of TTP in blood. The sensitivity for determination of thiamine phosphates is such that they can be measured in 0.1 ml of human or other animal blood. Although the presence of TTP and TMP in human blood has never been reported previously, we could have confirmed with this method that they are present, and these facts suggest that TMP or TTP may have important biological roles, unknown until now. This method will be useful in studying the clarification of the mechanism of action and metabolism of thiamine and its phosphate esters.

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