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Note

Determination of thiamine and its phosphate esters in human blood serum at femtomole levels

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The concentration of thiamine (vitamin B_1) in blood plasma is an important indicator of the nutritional status of thiamine in humans. It is therefore important to have a rapid and sensitive method for the determination of thiamine as a clinical test for thiamine deficiency. Until recently, the main tests available were microbiological assays [1, 2], or the determination of erythrocyte transketolase activity [3, 4]. Both methods are indirect, time-consuming and not free of interferences.

Direct assay of thiamine in human blood plasma was not possible owing to the low concentration of vitamin B_1 (ca. 10 nmol/l). Nowadays, the most sensitive method for the determination of thiamine is high-performance liquid chromatography (HPLC), combined with a derivatization procedure of thiamine in order to yield fluorescent thiochrome. This can be done either by a post-column [5—7] or a pre-column derivatization technique [8—15].

In this paper, we propose an extremely sensitive method, compared with existing ones, for the determination of thiamine (T) and its phosphate esters (thiamine monophosphate, TMP; thiamine pyrophosphate, TPP; thiamine triphosphate, TTP) by a pre-column derivatization technique on a reversed-phase HPLC column. This method was used as a tool for the analysis of thiamine as well as of its phosphate esters in human blood serum. It was successfully applied as a clinical assay for the detection of thiamine deficiency in humans.

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EXPERIMENTAL

Instrumentation

The HPLC instrument was an Altex Model 334-50 equipped with an LS-4 fluorescence spectrometer (Perkin Elmer, U.S.A.) and a Chromatopac C-R1A integrator (Shimadzu, Japan). The volume of the flow-cell was 3 μl. The excitation wavelength was set at 365 nm and the emission wavelength at 433 nm. A PRP-1 reversed-phase analytical column (250 × 4.1 mm I.D., 10 μm) from Hamilton (U.S.A.) was used, protected by a pre-column (32 × 4.2 mm I.D.) dry-packed with Vydac-201 RP (30—44 μm) from Macherey-Nagel (Düren, F.R.G.).

The mobile phase was sodium phosphate buffer (15 m*M*, pH 8.5) and either 10% methanol for the elution of thiamine phosphate esters, or 10% tetrahydrofuran (THF) for thiamine. The flow-rate was 0.5 ml/min. Either a 20-μl or a 100-μl injection loop was used.

Preparation of samples and derivatization procedure

Sample preparation and derivatization were essentially the same as described by Bontemps et al. [13].

Fresh blood samples are kept at room temperature until coagulation is complete (not more than 60 min) and centrifuged (10 min, 1000 *g*). The supernatant is saved, and 100 μl of a 30% trichloroacetic acid (TCA) solution are added to 500 μl of supernatant. This solution is centrifuged at 5000 *g* for 15 min, and the TCA is extracted with three volumes of water-saturated diethyl ether.

Prior to injection on the column, samples are derivatized by addition of 50 μl of a daily prepared alkaline ferricyanide solution ($4.3 \cdot 10^{-3}$ *M* potassium ferricyanide in 15% sodium hydroxide) to 80 μl of sample, followed by brief mixing. The concentration of ferricyanide is greater than the values given in ref. 13 in order to have a large excess of oxidant over reducing compounds present in the serum.

Reagents

Thiamine, TMP and TPP were obtained from Sigma (St. Louis, MO, U.S.A.). TTP was a gift from Sankyo (Japan). Aqueous solutions were prepared with water delivered by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.) and filtered on 0.45-μm pore ultrafilters. Organic solvents and other chemicals were of p.a. grade (Merck, Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Reversed-phase HPLC is the method of choice for the analysis of thiamine derivatives. As discussed by Bontemps et al. [14], PRP-1 reversed-phase columns have several advantages over ODS columns for the determination of thiamine derivatives by pre-column derivatization. This is mainly because the pH of the mobile phase must be above 8.0 [9] for the thiochrome derivatives to be fluorescent. At alkaline pH, however, silica-based gels rapidly deteriorate. PRP-1 reversed-phase resin is a styrene—divinylbenzene copolymer

which, because of its chemical stability, can be used within the pH range 1—13. Furthermore, peak-tailing can be considerably reduced at low methanol concentrations on the PRP-1 column compared with ODS columns. In order to improve the reproducibility of the analysis, we decided to switch to an isocratic elution mode rather than gradient elution [13]. Because of the much higher retention of thiochrome than its phosphate esters [14], the analysis must be carried out in two different modes: the mobile phase contains 10% methanol for the determination of the phosphate esters and 10% THF for thiochrome. Fig. 1 shows two chromatograms, one obtained for TTP, TPP and TMP (Fig. 1A), the other for thiamine (Fig. 1B). As expected on a reversed-phase column, TTP is eluted first, followed by TPP and TMP. This elution order is of advantage when analysing samples in which there is less TTP than is the case in many mammalian tissues for example [10].

Fig. 2 shows a calibration graph for standard solutions of thiamine, TMP, TPP and TTP. Linearity was tested up to 6.0 pmol for each derivative. The detection limit (i.e. the minimum amount injected still reproducibly detected) for thiochrome phosphate esters is 10 fmol and for thiochrome 5 fmol. This makes it the most sensitive method described for the analysis of thiamine compounds. Recent methods [10, 13] give a detection limit of 50 fmol for thiamine phosphate esters. Kimura and Itokawa [7] achieved 30 fmol with a post-column derivatization technique. Weber and Kewitz [15] reported a value of 5 fmol for thiamine, but their method is not appropriate for the analysis of thiamine phosphate esters.

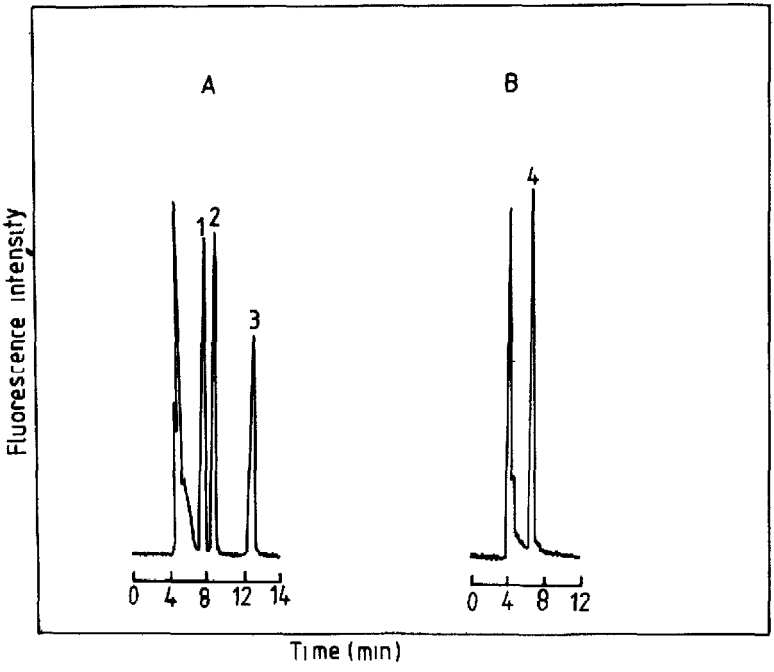
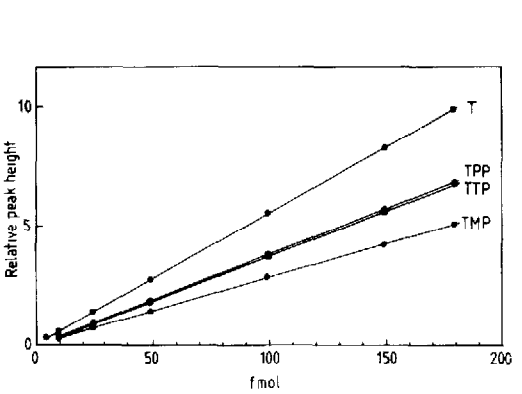


Fig. 1. Chromatograms of standard solutions of thiamine phosphate esters (A) and thiamine (B). The amount of each of the phosphate esters injected was 180 fmol and that of thiamine was 120 fmol. A 20-μl sample loop was used. Peaks: 1 = TTP, 2 = TPP; 3 = TMP; 4 = thiamine.



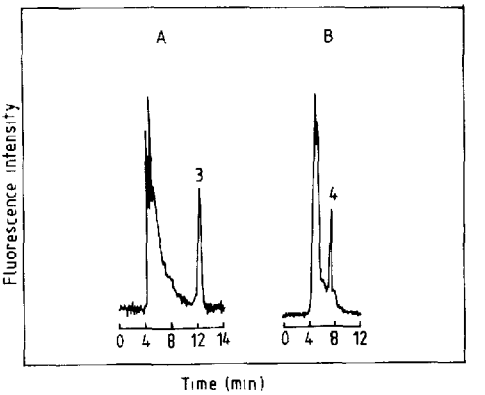


Fig. 2. Calibration curve for standard solutions of TTP, TPP, TMP and thiamine (T).

Fig. 3. Chromatograms of a human blood sample for the analysis of thiamine phosphate esters (A) and thiamine (B). The amounts of TMP and thiamine were calculated to be 103 and 77 fmol, respectively. A 100-µl sample loop was used in the case of (A) and a 20-µl loop for (B). Peaks. 3 = TMP; 4 = thiamine.

For the determination of thiamine in blood serum, a high sensitivity is necessary, as the total content is ca. 10 nmol/l [2, 15]. Such a low concentration has long been a limit to the detection of thiamine in human blood plasma. The detection limit of the assay presented here is ca. 5—10 fmol, which corresponds to a plasma concentration of 0.5 nmol/l. Thus the method is sensitive enough to assay thiamine even in patients suffering from a severe deficiency of vitamin B_1.

The analytical recovery of 0.1 pmol of thiamine and TMP in samples of serum is calculated to be 95 and 96%, respectively. Some control experiments were performed in which sodium hydroxide was added to the sample 4 h prior to the ferricyanide. Thiamine compounds are unstable in alkaline solution, and when the sample is injected on the column, thiamine peaks have disappeared. This test can be performed in order to obtain blank values.

In serum, the thiamine peak is easily detected and eluted without interferences (Fig. 3). As far as thiamine phosphate esters are concerned, TTP and TPP could not be detected in any serum, but small amounts of TMP were apparent. In several cases a 100-µl sample loop was used instead of the 20-µl loop in order to enhance detection. A total of 42 samples was analysed from healthy adults (26 males and 16 females) aged between 24 and 64 years. The thiamine content did not vary with the age or the sex of the people. The results are summarized in Table I. The total thiamine content (thiamine + TMP) of human blood serum was found to be 14.1 ± 4.5 nmol/l with a range of 5.9—20.7 nmol/l. This is in close agreement with the values given by other authors using the HPLC technique. Weber and Kewitz [15] reported plasma concentrations ranging from 6.6 to 42 nmol/l with a median of 11.6 nmol/l. Baker et al. [2] gave a range of 3—15 nmol/l using a microbiological assay. Thiamine accounts for ca. 84% and TMP for 16% of total thiamine according to our results. We applied our assay to the blood serum of subjects suspected to suffer from a vitamin B_1 deficiency. As can be seen from Table I (B and C), the

TABLE I

CONTENTS OF THIAMINE AND ITS PHOSPHATE DERIVATIVES IN BLOOD SERUM FROM HEALTHY (A) AND VITAMIN B_1 DEFICIENT (B, C) SUBJECTS

Subject	Contents (mean ± S.D.) (nmol/l)				
	Thiamine	TMP	TPP	TTP	Total
A	11.9 ± 4.1	2.2 ± 1.0	—	—	14.1 ± 4.5
B	2.3	—	—	—	2.3
C	0.7	—	—	—	0.7

thiamine content was well below the normal values, i.e. 2.3 and 0.7 instead of 14.1 nmol/l, and thus confirms the previous diagnosis. In these cases, thiamine could be detected but neither of the phosphate esters. The intra-assay variability of the method was calculated to be 3.5% for thiamine and 4.5% for TMP in five independent assays on the same serum. The same standard solutions (12.5 n*M* either in TMP or thiamine, kept at −20°C) were used for over two weeks. Within this period, the variability coefficient was found to be 6.7% for thiamine or 8.4% for TMP.

Kimura et al. [16] treated human whole blood with takadiastase (EC 3.4.23.6) and gave a value of 46.2 μg/l for total thiamine. This corresponds to a concentration of ca. 130 nmol/l. This value is in good agreement with those found by Wielders and Mink [6] and Burch et al. [17]: 117 nmol/l and 128—154 nmol/l, respectively. Burch et al. reported that human plasma contains 10% of total blood thiamine, or ca. 13 nmol/l, which is close to the value we obtained. The disadvantage of these methods [6, 10, 17] is that they permit the detection of thiamine but not of its derivatives, thus the phosphate esters have to be hydrolysed prior to analysis for the determination of total thiamine.

Recently Kimura and Itokawa [7] described a very sensitive method that allows the determination of thiamine and its phosphate esters by a post-column derivatization procedure. They applied their method to human whole blood and plasma samples. In blood taken from healthy subjects they found essentially TPP and TTP, while the concentrations of TMP and thiamine were low. In plasma, however, they could detect neither TTP nor TPP, but only TMP and thiamine, which is agreement with our results.

The method presented here is based on a pre-column derivatization procedure of thiamine and its phosphate derivatives. The detection limit is the lowest, i.e. 5 fmol for thiamine and 10 fmol for TMP, TPP and TTP, so far reported [7, 10, 15]. Such a high sensitivity allows the determination of thiamine, as well as its phosphate esters, in human blood serum and plasma. Thus pretreatment of the samples with takadiastase or acid phosphatase (EC 31.3.2) is not necessary.

As we have shown, this technique can provide a simple and rapid tool for the determination of thiamine and its phosphate esters in human blood serum or other body fluids as a clinical test for the detection of thiamine deficiency. Furthermore, it can be used for the assay of thiamine and its derivatives during metabolic studies and thus contribute to our understanding of the role of thiamine and in particular that of TTP in the nervous system.

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REFERENCES

1 O. Mickelsen and R.S. Yamamoto, Methods Biochem. Anal., 6 (1958) 191.
2 H. Baker, O. Frank, I. Pasher, H. Sobotka and S.H. Hunter, Nature, 191 (1961) 78.
3 M. Brin, M. Tai, A.S. Ostashever and H. Kalinsky, J. Nutr., 71 (1960) 273.
4 L.G. Warnock, J. Nutr., 100 (1970) 1057.
5 B.C. Hemming and C.J. Gubler, J. Liq. Chromatogr., 3 (1980) 1697.
6 J.P.M. Wielders and Chr. J.K. Mink, J. Chromatogr., 277 (1983) 145.
7 M. Kimura and Y. Itokawa, J. Chromatogr., 332 (1985) 181.
8 R.L. Roser, A.H. Andrist, W.H. Harrington, R.K. Naito and D. Lonsdale, J. Chromatogr., 146 (1978) 43.
9 K. Ishii, K. Sarai, H. Sanemori and T. Kawasaki, Anal. Biochem., 97 (1979) 191.
10 H. Sanemori, H. Ueki and T. Kawasaki, Anal. Biochem., 107 (1980) 451.
11 L.G. Warnock, Anal. Biochem., 126 (1982) 394.
12 A. Floridi, M. Pupita, C.A. Palmerini, C. Fini and A.A. Fidanza, Int. J. Vit. Nutr. Res., 54 (1984) 165.
13 J. Bontemps, P. Philippe, L. Bettendorff, J. Lombet, G Dandrifosse, E. Schoffeniels and J. Crommen., J. Chromatogr., 307 (1984) 283.
14 J. Bontemps, L. Bettendorff, J. Lombet, Chr. Grandfils, G. Dandrifosse, E. Schoffeniels, F. Nevejans and J. Crommen., J. Chromatogr., 295 (1984) 486.
15 W. Weber and H. Kewitz, Eur. J. Clin. Pharmacol., 28 (1985) 213.
16 M. Kimura, T. Fujita and Y. Itokawa, Clin. Chem. 28 (1982) 29.
17 H.B. Burch, O.A. Bessey, R.H. Love and O.H. Lowry, J. Biol. Chem., 198 (1952) 476.