Journal of Chromatography, 564 (1991) 127–136
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5667

Concomitant determination of thiamin and its phosphate esters in human blood and serum by high-performance liquid chromatography

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(First received June 18th, 1990; revised manuscript received October 2nd, 1990)

ABSTRACT

A high-performance liquid chromatographic method for the simultaneous determination of thiamin and thiamin phosphate esters in human blood or serum has been developed. The eluent consists of acetonitrile and phosphate buffer, in the ratios 90:10 (v/v) for the elution of thiamin and 60:40 (v/v) for the phosphate esters. The four compounds are eluted within 15 min. The detection limit is 13–16 fmol. Between-assay variation is 5–11%. Samples of whole blood and serum from 30 healthy adults were analysed. The following reference values were obtained for 15 females/15 males (nM, mean ± S.D.). In serum: thiamin, 10.9 ± 2.9/16.9 ± 3.3; thiamin monophosphate, 8.3 ± 1.5/3.7 ± 1.5. In whole blood: thiamin, 29.6 ± 10.0/33.4 ± 10.4; thiamin monophosphate, 9.7 ± 2.3/10.9 ± 5.1; thiamin diphosphate, 121 ± 29.6/165 ± 40.4.

INTRODUCTION

Thiamin is present in the human body as free thiamin (T), thiamin monophosphate (TP), thiamin diphosphate (TPP) and thiamin triphosphate (TPPP) [1–3]. T and TP have been detected in human serum and cerebrospinal fluid, T, TP and TPP in erythrocytes and TPPP in nervous tissue. However, the relative importance of each compound and their kinetics have not been studied, owing to the limitations and the complexity of the methods used for their determination.

High-performance liquid chromatography (HPLC) has become the method of choice for the determination of thiamin and its phosphate esters [4–8]. The compounds are measured directly, and it is the only method with an acceptable sensitivity for the low concentrations found in human serum [9,10].

HPLC studies, however, have concentrated on measuring either total thiamin by pretreating the samples with an enzyme to break the phosphate bonds [11–13], or TPP whose biological activity has been clearly described [14,15]. To be able to

0378-4347/91/$03.50

study thiamin metabolism in normal and pathophysiological states, all the thiamin compounds should be assessed at one time, but no routine laboratory method has been established for that purpose, as far as we know.

This paper presents an HPLC method by which all four compounds are measured simultaneously. The method uses mild chromatographic conditions with near neutrale pH for the eluent and pre-column derivatization. We have tested this method by assessing T, TP, TPP and TPPP in whole blood and serum in healthy volunteers who have fasted overnight.

EXPERIMENTAL

Chromatography

The HPLC system comprised a Perkin-Elmer Series 400 solvent-delivery system coupled to a Perkin-Elmer LS1 fluorescence detector (excitation wavelength set at 375 nm and emission measured at 450 nm). A Perkin-Elmer LC1 100 laboratory computer integrator was used to record and measure peak heights and areas.

A Supelcosil NH_2 column (250 mm × 4.6 mm I.D.) from Supelco (Supelco Park, Bellefonte, PA, U.S.A.) was used, protected by a guard column (Supelco LC NH, 20 mm × 4.6 mm I.D.).

The mobile phase consisted of acetonitrile and phosphate buffer (85 m*M*, pH 7.5) in the ratios 90:10 (v/v) for the elution of thiamin and 60:40 (v/v) for the elution of the phosphate esters. The flow-rate was 1.5 ml/min, resulting in a pressure of 55–110 bar owing to the use of a gradient. Prior to its use, the buffer was filtered through a Millipore filter (0.22 μm), and the mobile phase was degassed for 20 min with helium before analysis. A 100-μl injection loop was used.

A 1219 Rackbeta liquid scintillation counter (LKB Wallac) was used to measure [^{14}C]thiamine in the recovery study.

Procedure

Venous blood was collected from the cubital vein in heparin tubes for whole blood samples and in serum tubes (containing a clot activator) for serum samples. Whole blood samples were hemolysed by freezing at −20°C. Serum samples were stored at −20°C until further treatment. For sample preparation, 1 ml of hemolysed blood was diluted with 1.5 ml of saline water.

Deproteinization was done by addition of 250 μl of 2.44 *M* (40%) trichloracetic acid (TCA) to the 2.0-ml serum samples and 315 μl to the diluted whole blood samples, so that the final concentration of TCA in the samples was 5%. After thorough mixing, the samples were left for 1 h in darkness. After centrifugation (20 min, 2000 *g*), 1 ml of supernatant of serum or hemolysate was transferred to fresh glass tubes, and TCA was extracted twice with five volumes of water-saturated diethyl ether. A 400-μl aliquot was subsequently filtered through Millipore Ultrafri MC-10 000 NMWL filter units (Millipore Products Division, Bedford,

MA, U.S.A.) in a Sorvall centrifuge at 5000 *g* for 20 min. The samples were then stored at −20°C for subsequent analysis. All samples were analysed within six months after collection.

Derivatization

Prior to injection on the column, 200-μl samples were derivatized by addition of 20 μl of a 0.3 *M* solution of cyanogen bromide (prepared freshly each day). After mixing they were alkalinized by addition of 20 μl of 1 *M* sodium hydroxide. A 100-μl aliquot of the derivatized sample was injected on the column.

Reagents

Thiamin hydrochloride, thiamin monophosphate chloride and cocarboxylase (thiamin disphosphate) were purchased from Sigma (St. Louis, MO, U.S.A.). Thiamin triphosphate was a gift from Takeda Chemicals (Osaka, Japan). Cyanogen bromide, 97%, was obtained from Aldrich (Steinheim, F.R.G.). Thiazole-[2-^{14}C]thiamin (specific activity 918 mBq/mmol) was purchased from Amersham International (Amersham, U.K.). All the other reagents were from local commercial sources.

Standards

Standard stock solutions of thiamin and thiamin phosphate esters (10^{-3} *M*) were prepared in 0.01 *M* hydrochloric acid and stored at −20°C. Working solutions were prepared freshly by diluting the stock solutions to the required concentrations with deionized and filtered water. The currently used standard concentration was $2 \cdot 10^{-9}$ *M* for each compound.

Subjects

Fifteen healthy males and fifteen healthy females, all hospital staff members, constituted our reference population. The median age for males was 43 years (range 32–54) and for females 47 (23–60). Blood samples were collected after overnight fasting.

Calculations and statistical treatment of data

Means and standard deviations were used for descriptive purposes. The concentrations of T, TP, TPP and TPPP in the erythrocytes were calculated from the measurements in serum and whole blood and the subjects' hematocrit.

Wilcoxon's rank sum test was used for comparisons between mean values, and the method of least squares for calibration of the standards.

RESULTS

Chromatographic separation

Standards were separated with acetonitrile–phosphate buffer (60:40, v/v).

However, the thiamin in biological samples was not separated from the front and therefore could not be measured. A modification of the eluent was therefore made in order to elute thiamin free from interferences.

The following step gradient was programmed: initial elution with acetonitrile–phosphate buffer (90:10, v/v) for 7 min, followed by acetonitrile–phosphate buffer (60:40, v/v) for 13 min, after which the initial conditions were restored (Fig. 1). The sample was injected after 4 min of equilibration (time 0 in Fig. 1). In this system, each compound was eluted separately and with no interference from the matrix (Fig. 1).

The retention times for the four compounds at a flow-rate of 1.5 ml/min showed virtually no variation within a day, and very moderate variations from day to day. T, TP, TPP and TPPP were eluted after 3, 10, 12 and 14 min, respectively.

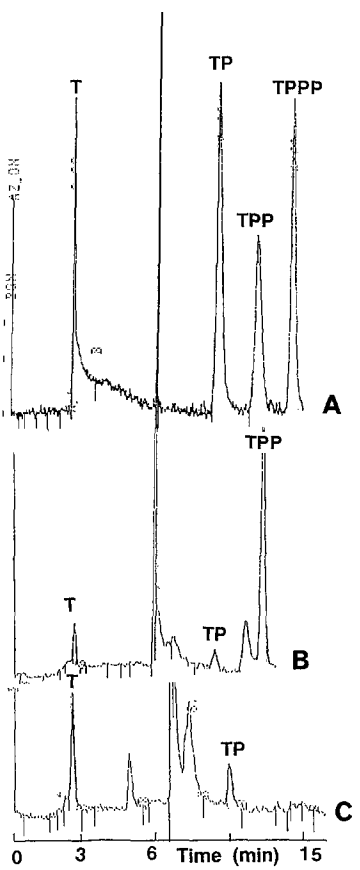

Fig. 1. Elution profile for a standard solution (A), a whole blood sample (B) and a serum sample (C). Peaks: T = thiamin; TP = thiamin monophosphate; TPP = thiamin diphosphate; TPPP = thiamin triphosphate. Injection at time 0.

Whole blood and serum samples were spiked with each standard separately and with the standards combined to verify the identities of the peaks (results not shown).

Calibration curves

The linearity was the same for peak areas and peak heights for both T and TP, but peak areas gave a better correlation coefficient than peak heights for TPP and TPPP (results not shown), and were subsequently used for all measurements. The fluorescence intensity, expressed in terms of area, was proportional to the concentrations of the standard solutions within the physiological range of values. The correlation coefficients were calculated on the basis of five independent analyses for each standard amount. The linear regression lines and the coefficients of correlation for T, TP, TPP and TPPP were: $y = 1.08x + 34.8$ ($r = 0.993$); $y = 2.07x + 121.3$ ($r = 0.996$); $y = 1.52x + 52.0$ ($r = 0.993$); and $y = 1.62x + 92.3$ ($r = 0.994$), respectively. For all four standards, $p < 0.01$.

Recovery

Recovery tests were performed with [^{14}C]thiamin, which was added to serum samples before deproteinization (0.1 nmol, 5000 cpm). The recovery was calculated after deproteinization and extraction with diethyl ether. The mean recovery for twelve assays in triplicate was 87.4%.

Detection limit and reproducibility

Based on a signal-to-noise ratio of 3 as the detection limit, the whole blood or serum sample should contain at least 2 n*M* T, TP, TPP or TPPP to be analysed. The minimal absolute amounts in the analysed aliquot were 13–16 fmol for the different compounds.

The intra-assay coefficient of variation, calculated on the basis of ten analyses of the same sample on one day, was 5.4, 4.8, 6.2 and 11.6% for T, TP, TPP and TPPP, respectively.

Reference values of T, TP, TPP and TPPP in serum, whole blood and erythrocytes

T and TP were detected in serum in low concentrations, but TPP was detected only in whole blood (Table I). Trace amounts of TPPP were found in a few whole blood samples only. In whole blood, T and TP were present in concentrations comparable with those found in serum, whereas TPP was present in concentrations ten to fifteen times higher (Table I). TPP represented the quantitatively most important compound in blood and erythrocytes (Table I). All results had a limited range, with the mean value close to the median, indicating that the distributions were not highly skewed.

In serum, there was significantly a higher concentration of T in males than in females ($p < 0.05$), but a significantly lower concentration of TP ($p < 0.05$) (Table I). There was, however, no difference in the total amount of thiamin in serum (T

TABLE I

CONCENTRATIONS OF T, TP, TPP AND TPPP IN SERUM, WHOLE BLOOD AND ERYTHROCYTES AND THE RATIOS BETWEEN ERYTHROCYTES AND SERUM CONCENTRATIONS

Values are mean ± S.D., from fifteen females and fifteen males.

Subjects	Serum (n*M*)				Whole blood (n*M*)				Erythrocytes (n*M*)			Ratio erythrocytes/serum		
	T	TP	TPP	TPPP	T	TP	TPP	TPPP	T	TP	TPP	T	TP	TPP
Females														
Mean	10.9	8.3	<2	<2	29.6	9.7	121	<2	23.4	4.1	299	2.1	0.5	150
S.D.	2.9	1.5			10.0	2.3	29.6		10.2	2.0	69.7			
Males														
Mean	16.9[a]	3.7[a]	<2	<2	33.4	10.9	165[a]	<2	25.2	9.0[a]	351[a]	1.5	2.4	175
S.D.	3.3	1.5			10.4	5.1	40.4		10.1	4.9	84.2			

[a] Statistically significant difference between males and females ($p < 0.05$).

+ TP) in males and females. In whole blood, T and TP had the same concentration in males and females, but TPP was significantly higher in males ($p < 0.05$) (Table I). There was also a higher TPP concentration in males than in females in the erythrocytes ($p < 0.05$) (Table I).

DISCUSSION

Chromatography

In our system, thiamin and thiamin phosphate esters were easily separated isocratically with acetonitrile–buffer (60:40, v/v), but because of matrix interferences, biological samples could not be analysed. Bontemps *et al.* [16] used a linear gradient but applied their system only to standards. When we tried linear and step gradients in our laboratory, the step gradient proved to be better for column stability. The difference in the amounts of acetonitrile–buffer in the two steps leads to a decrease in pressure of *ca.* 40% over 2 min. In order to obtain stable conditions, the sample was injected after 4 min equilibration time. However, assays with shorter pre-injection time have shown that this delay can be reduced to 2.5 min without alteration of the chromatographic conditions.

Buffer

The molarity of the phosphate buffer was particularly important because of interaction with acetonitrile. At a higher molarity (> 85 m*M*), acetonitrile and the buffer did not mix homogeneously at 90:10 (v/v) and the column could not be stabilized. At a lower molarity, peaks were broader, retention times higher and

the column unstable. The optimal molarity for the phosphate buffer was 82–85 m*M*.

Bettendorf *et al.* [9] analysed the four compounds but changed the mobile phase from buffer–tetrahydrofuran for the elution of T, to buffer–methanol for the elution of TP, TPP and TPPP. Our system has the advantage of using the same components in the mobile phase for the elution of all four compounds.

Vanderslice and Huang [8] have analysed the four compounds simultaneously, eluting thiamin and the thiamin compounds at pH 2.6–5.5; they also reported the successful use of amprolium as an internal standard. However, their detection limit was 380 fmol. Kimura and Itokawas [17] had a detection limit of 30 fmol, which is closer to ours (13–16 fmol).

Derivatization

Vanderslice and Huang [8] and Kimura and Itokawas [17] used post-column derivatization, which is a more complicated procedure than the pre-column derivatization used in our method.

Although cyanogen bromide was shown to be the most efficient oxidizing agent for thiamin [18], its use has been criticized by many authors because it is hazardous and unstable. Weighed aliquots of the solid compound could be kept refrigerated in glass tubes for several months, ready to be dissolved in acetonitrile on the day of the analysis. The solution was perfectly stable for at least 8 h at room temperature. Derivatized samples were stable frozen overnight at −20°C or at least for 6 h at room temperature, after which time a reduction in peak size was observed.

It is essential to add cyanogen bromide to the sample before alkalinization [18] as thiamin is destroyed in alkaline solutions. We used samples treated with sodium hydroxide before cyanogen bromide as blanks, as done earlier by Iwata *et al.* [19].

Recovery

Ideally, recovery should be performed separately for each thiamin compound. However, labelled phosphate esters of thiamin are not commercially available and we chose therefore to use labelled free thiamin. Moreover, the validity of necessary studies for TPP and TPPP is dubious when the added TPP or TPPP, radioactive or not, will mix with the intracellular TPP or TPPP only after deproteinization.

Blood samples

Sample preparation was simpler and faster for whole blood than for packed erythrocytes. Therefore, we calculated the erythrocytes values from the whole blood and serum values and the hematocrit. The error entailed by ignoring the amount of thiamin in other blood cells (particularly leucocytes) is negligible because of the much higher number of erythrocytes.

Reference range

There was no difference between the total thiamin concentrations in males and females in serum. The total thiamin concentration in whole blood was slightly higher in males than in females ($p < 0.05$) owing to the higher concentration of TPP in erythrocytes in males ($p < 0.05$). TP is the intermediate compound between T and TPP, and its concentration will consequently depend on the T and TPP concentrations. In erythrocytes, TPP values are slightly lower in females and so are TP values, as would be expected. Such a difference between males and females has been reported previously only by Wielders and Mink [13], who found a higher TPP concentration in males than in females, and attributed part of the difference to the males' higher hematocrit.

The inverse ratio of T and TP in females and males in serum is of uncertain physiological importance.

In our study, the lower reference limit [mean $-(2 \times$ S.D.)] for total thiamin in serum was 12 nM for both males and females, while in erythrocytes it was 207 and 184 nM for males and females, respectively. Table II lists the concentration ranges previously published by other authors: our results are similar to those of Bötticher and Bötticher [20]. We found higher concentrations in serum than reported

TABLE II

CONCENTRATION OF TOTAL THIAMIN IN SERUM/PLASMA OR WHOLE BLOOD, AND OF TPP IN ERYTHROCYTES, BY VARIOUS HPLC METHODS

Values are mean ± S.D. (or the range in parentheses).

Reference	n	Total thiamin (nM)			TPP in erythrocytes (nM)
		Serum	Plasma	Whole blood	
Schrijver *et al.* [12]	98			115 (70–185)	
Warnock [15]	17				268 ± 58.6
Wielders and Mink [13]	529			113 (81–144)	
	56			117 (88–157)	
Weber and Kewitz [10]	91		11.6 (6.6–43)		
Baines [14]	48				223.5 (132–284)
Bettendorf *et al.* [9]	42	14.1 ± 4.5		–	–
Bötticher and Bötticher [20]	20	19 ± 7.0		184 ± 71.7	–
This study	30	19.8 ± 3.8 (12.9–28.6)		185.2 ± 35.3 (112–313)	325 ± 71.9 (217–528)

by Bettendorf *et al.* [9]. Our concentrations in whole blood are also higher than those reported by Wielders and Mink [13]. We also found higher TPP concentration in erythrocytes than did Baines [14] (Table II). These variations may partly be explained by the difference in sample preparation and a lower recovery when using enzymic dephosphorylation of the phosphate esters.

Thom *et al.* [21] have shown that TPP is rapidly dephosphorylated by fresh plasma when it is not protein-bound, and this may explain that only T and TP are found in normal serum. The fact that only traces of TPPP are found in some blood samples may be due to a similar phenomenon. The dephosphorylation of TPPP goes via TPP, and evidence for such a conversion was found by Vanderslice and Huang [8]. It was, however, prevented by the addition during sample preparation of trichloroacetic acid, which we used in our study. A transformation of TPPP into TPP is unlikely, but cannot be fully excluded.

CONCLUSIONS

As long as the respective roles of thiamin and its phosphate esters and their kinetics are not clearly established, it is important to analyse all thiamin compounds concomitantly in samples from different tissues and body fluids to determine the complete thiamin status of a patient. With our method, a full thiamin status is available within 20 min, and the whole analysis can be automated and used in a routine laboratory. The method has high specificity and sensitivity, and a precision that is sufficient for clinical purposes.

ACKNOWLEDGEMENTS

We thank Berit Falch and Elisabeth Try Valø for their valuable technical assistance. We are also grateful for the financial support provided by Vinmonopolet A.S., the Randi and Hans Arnet Foundation and the Gertrude and Jack Nelson Foundation.

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