Journal of Chromatography, 567 (1991) 71–80
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5857

Determination of thiamine and its phosphate esters by gradient-elution high-performance liquid chromatography

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(First received December 6th, 1990; revised manuscript received January 18th, 1991)

ABSTRACT

A high-performance liquid chromatographic method for the determination of thiamine and its phosphate esters using gradient elution is presented. Chromatography was performed on an octadecyl C_{18} column with post-column derivatization using potassium ferricyanide: thiamine was oxidized to thiochrome, which was detected fluorimetrically. The gradient system avoids the use of a modifier and an ion-pairing reagent. The proposed method enables the separation of thiamine triphosphate, thiamine pyrophosphate, thiamine monophosphate and free thiamine, at concentrations of *ca.* 2 n*M*, within 10 min. The within-assay precision for blood and serum total thiamine was less than 2 and 5%, respectively, and the between-day variation was less than 3 and 5%, respectively. The method is simple and rapid, and it can be used for screening of thiamine deficiency and for clinical study of various diseases related to vitamin B_1 deficiency.

INTRODUCTION

The concentrations of thiamine (vitamin B_1) and its phosphate esters in blood and plasma are important indicators of the nutritional status of thiamine in humans [1]. Clinical aspects of thiamine deficiency, such as Wernike–Korsakoff's psychosis, have been extensively described [2]. Alcohol abuse leading to alcoholic beriberi is also accompanied by thiamine deficiency [3]. Traditionally, thiamine deficiency is determined by the level of erythrocyte transketolase (ETKA). However, a number of pathological conditions may cause abnormalities in the level of ETKA, and its measurement may not be correlated with symptoms of thiamine deficiency [4], as has recently been revealed among the victims of sudden infant death (SID) syndrome [5]. For post-mortem cases, high serum thiamine levels were observed with no abnormalities of ETKA. Determinations of blood and serum thiamine and its phosphate esters are thus essential.

Sudden unexpected death syndrome (SUDS) among South East Asians has been given much publicity recently [6]. The cause of death is not known but dietary factors (especially thiamine deficiency) are the most cited hypothesis [7–9]. It is, therefore, important to have a reliable and sensitive method for the direct determination of individual thiamine vitamers in blood and serum.

0378-4347/91/$03.50

Recent developments in high-performance liquid chromatography (HPLC) suggested that derivatization of thiamine to yield fluorescent thiochrome could be used for the determination of low concentrations of vitamin B_1. This can be done either by a post-column or a pre-column derivatization technique. One of the more sensitive methods, which enabled the detection of thiamine at *ca.* 5 fmol, was recently described by Weber and Kewitz [10]. This technique, however, could measure only free thiamine, not the phosphate esters, which are normally found in the blood.

Thus far, the most sensitive HPLC method, which is able to detect thiochrome and its phosphate esters at the 5–10 fmol level, was described by Bettendorff *et al.* [11]. However, one of the major setbacks of this method is that the analysis must be carried out in two different isocratic elution modes. It is tedious and limited in use to the direct measurement of samples containing high amounts of both thiamine and thiamine phosphate esters, without converting them into free thiamine by hydrolysis.

This paper describes a sensitive and reliable method for the determination of thiamine and its phosphate esters using gradient-elution HPLC and post-column derivatization. The use of a gradient system avoids the use of an ion-pairing reagent and modifier, which are known to cause instability [12]. The proposed method enables the separation and detection of thiamine triphosphate (TTP), thiamine pyrophosphate (TPP), thiamine monophosphate (TMP) and free thiamine (T), at concentrations of *ca.* 2 n*M*, within 10 min. This method has been succesfully used for screening of thiamine deficiency among a group of workers from North-eastern Thailand, believed to have low dietary thiamine intake.

EXPERIMENTAL

Instrumentation

The apparatus was a Gilson programmable gradient HPLC system, consisting of a master pump as a system controller (Model 305), a slave pump (Model 302), a dynamic mixer (Model 811) and an auto-injector (Model 231-401) (Gilson, Villiers-le-Bel, France). The analytical column was a 110 mm × 4.7 mm I.D. Partisphere 5 C_{18} cartridge protected by a guard cartridge system (Whatman, Clifton, NJ, U.S.A.). The detection and quantitation were carried out with a Shimadzu Model RF-535 fluorescence detector (excitation 365 nm, emission 435 nm) and a CR-5A integrator (Shimadzu, Kyoto, Japan), for peak-height measurement against external standards.

A Gilson peristaltic pump (Model miniplus 2) delivered the oxidizing reagent at 0.2 ml/min through a Whatman 0.45-μm pore size filter device to the tee piece (0.5-mm hole, Upchurch Scientific, Oak Harbor, WA, U.S.A.), which was connected to the analytical column and the post-column reactor at ambient temperature. The post-column reactor consisted of a Teflon capillary (90 cm × 0.5 mm I.D.) wound on a tubular rod of 15 mm O.D.

Gradient elution profile

The water used for the preparation of all standards and reagents was distilled and deionized. All chemicals and reagents used were from E. Merck (Darmstadt, Germany). The two mobile phases used for gradient separation of TTP, TPP, TMP and T were: buffer A, 15 m*M* citric acid with pH adjusted to 4.2 with 50% (v/v) ammonium hydroxide solution; buffer B, 0.1 *M* formic acid containing 4% (v/v) diethylamine with pH at 3.2. The gradient elution profile is shown in Table I. The flow-rate was 1.0 ml/min.

Oxidizing reagent

The oxidizing reagent was prepared daily by dissolving 100 mg of potassium ferricyanide in 120 ml of 3 *M* sodium hydroxide. This reagent was protected from light in an amber plastic bottle.

Sample preparation

Heparinized blood was haemolysed by storage at −20°C for 20 min after collection. After thawing and homogenizing, 0.2 ml of sample was mixed vigorously with 0.2 ml of chilled 10% (w/v) perchloric acid (PCA) in a microtube. Serum specimens were treated the same way. The deproteinized sample was allowed to stand at below 4°C for 15 min and then centrifuged for 1 min at 10 000 *g*. A 0.2-ml aliquot of supernatant was introduced with 0.1 ml of buffer solution (1.8 *M* sodium acetate in 0.6 *M* sodium hydroxide) into a costar centrifuge filter unit and centrifuged for 30 s. The filtrate was then transferred into an amber sampler vial, and a 20-μl sample was injected into the HPLC system.

Quality control

A pooled blank and spiked samples of blood and serum from a healthy blood donor was treated according to the same procedures and stored at −20°C in amber sampler vials. These two samples were included in each batch of analyses for day-to-day quality control purposes.

TABLE I

GRADIENT ELUTION PROFILE FOR THE SEPARATION OF THIAMINE AND ITS PHOSPHATE ESTERS

Time (min)	Buffer A (%)	Buffer B (%)
0.00	90	10
0.01	Inject sample	
2.50	50	50
3.00	5	95
6.50	5	95
7.00	95	5
10.00	95	5

Standard preparation

TPP, TMP and T were obtained from Sigma (St. Louis, MO, U.S.A.) TTP was purchased from Wako (Osaka, Japan). The stock solutions of 5 m*M* of each standard were prepared by dissolving each standard in 5 ml of 10% (w/v) PCA, and stored at −20°C. It was observed that the deproteinized sample volume (sample plus PCA) was affected by protein precipitation, and the dilution factor was not the same as for aqueous standards. In order to ensure accuracy of quantitation, the standard addition method was used for standard calibration. The stock standards were further diluted with PCA and used in spiking deproteinized samples, to give a range of concentrations from 2 to 20 n*M* in serum and 2 to 250 n*M* in blood.

RESULTS AND DISCUSSION

Mobile phase for gradient separation on ODS column

Earlier chromatographic experience with HPLC methods suggested that pre-

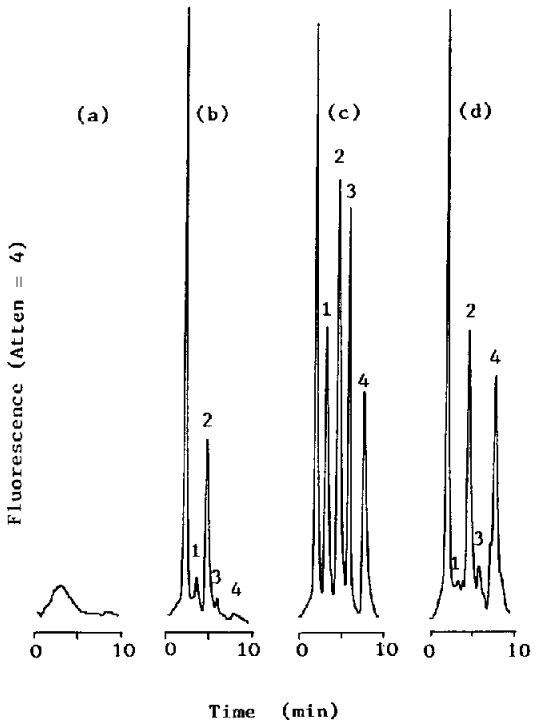

Fig. 1. Chromatograms of (a) a baseline of gradient elution, (b) a blood sample from a healthy subject, (c) the same sample supplemented with 100 n*M* thiamine compound and (d) a post-mortem blood sample. Peaks: 1 = TTP; 2 = TPP; 3 = TMP; 4 = thiamine.

column derivatization is not suitable for analysis using an ODS column [13,14]. This is because the silica-based gels will rapidly deteriorate by the alkalinity of the mobile phase that was needed for thiochrome to be fluorescent [15,16]. It was thus decided to use post-column derivatization.

An oxidizing agent concentration of 100 mg of potassium ferricyanide in 3 *M* sodium hydroxide, at a flow-rate of 0.2 ml/min, was found to be the best for the maximum fluorescence yield of thiochrome and its phosphate esters. The use of 15 m*M* citric acid as buffer A with pH adjustment using ammonium hydroxide instead of sodium hydroxide, and diethylamine instead of methanol as a modifier for buffer B (0.1 *M* formic acid), produced a pronounced effect on gradient separation of TTP, TPP, TMP and T (Figs. 1 and 2).

The change of the composition of gradient eluent does not appear to affect the

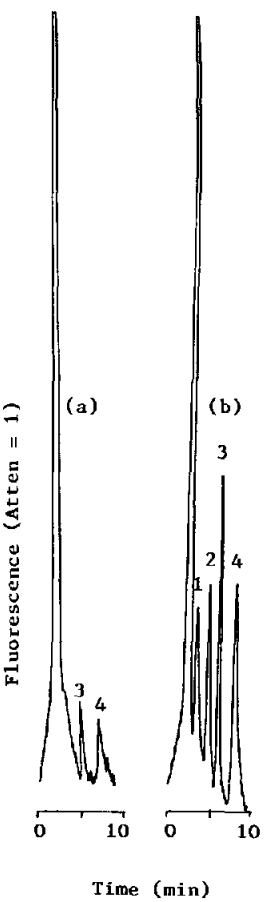

Fig. 2. Chromatograms of (a) a serum sample collected from the same subject as in Fig. 1b, and (b) a serum sample spiked with TTP (1), TPP (2), TMP (3) and T (4), each at a concentration of 20 n*M*.

chromatographic baseline (Fig. 1a). On the contrary, it drifted significantly when sodium hydroxide and methanol were used. Moreover, a lower detection limit was achieved with the present method. The use of methanol in the mobile phase to enhance the thiochrome fluorescence, as suggested by Wielders and Mink [17], requires the use of octanesulphonic acid as an ion-pairing reagent to improve the resolution, and is not necessary. Furthermore, the use of octanesulphonic acid entails lengthy column equilibration and stabilization procedures [12]. The present HPLC conditions allow for analysis of samples immediately after three cycles of gradient elution.

Chromatographic analysis

The chromatogram of a blood sample is shown in Fig. 1b. Fig. 1c shows the same sample spiked with 100 n*M* thiamine vitamers standard. TTP and TPP were eluted with 90% buffer A at 3.2 and 4.5 min, respectively. Thiamine was detected at 7.6 min when the composition of the eluent was changed to 95% buffer B. TMP eluted between TPP and T, and was detected at 5.8 min when the column was equilibrated with *ca.* 50% each of buffers A and B.

Fig. 2a shows the chromatographic analysis of a serum sample collected from the same subject. A chromatogam obtained from a serum sample spiked with 20 n*M* TTP, TPP and TMP, as well as thiamine, is shown in Fig. 2b. The peaks had the same retention times as those in the blood sample.

Analysis time and sensitivity

The peaks of TTP, TPP, TMP and T are sharp, well separated and easy to quantify within 10 min. The detection limit, at a signal-to-noise ratio of 3, is 2 n*M* for blood or serum with an injection volume of 20 μl. When a 100-μl sample is injected to enhance the detection, 0.4 n*M* serum or plasma thiamine and TMP can be detected. The earlier method of Bettendorff *et al.* [11] had a detection limit of 5–10 fmol, which corresponds to a plasma concentration of 0.5 n*M* with an injection volume of 100 μl. Unfortunately, the analysis must be carried out by two different isocratic elution modes with a total analysis time of more than 22 min. This is due to the much higher retention of thiochrome from its phosphate esters [13], and the poor reproducibility of gradient elution on the styrene–divinylbenzene copolymer column with pre-column derivatization procedures [14]. The method is also found to be unsuitable for the analysis of post-mortem blood samples. Autopsy blood samples were usually found to have high concentrations of TPP, TMP as well as thiamine, as shown in Fig. 1d. Weber and Kewitz [10] reported a value of 5 fmol for thiamine, but their method is not appropriate for the analysis of thiamine phosphate esters. Kimura *et al.* [18] achieved 30 fmol with a post-column derivatization technique within 12–15 min, but their mobile phase contained a high concentration of phosphate, which is usually not recommended for chromatographic work. The high salt content of the phosphate buffer, if it is not thoroughly removed when not in use, could result in a significant

drop in the column efficiency [12,17,19–23]. The present method can be used for the direct measurement of individual thiamine vitamers, as well as total thiamine in blood and serum. Prior analysis, hydrolysis with takadiastase or acid phosphatase to convert thiamine phosphate esters into free thiamine [17,24] are all unnecessary.

Stability of standard solutions

It was observed that when TMP and thiamine standards were prepared in 10% (w/v) PCA and stored in the dark at −20°C they were stable for several weeks. On the other hand, TTP and TPP were found to be less stable even for a short period of storage. TTP was found to show a 50% loss by the second day; TPP and TMP appeared in the one-day-old TTP standards. More than 30% of TPP had converted into TMP after five days of storage at 4°C. This observation is in agreement with the earlier report of Warnock [25]. When the standard mixtures were prepared in 0.1 *M* HCl and stored at 4°C, only 80% of them could be detected on the fifth day of analysis.

When using the standard addition method, it is recommended to spike standards into deproteinized samples after centrifugation. This is because thiamine phosphate esters are easily interconverted by various enzyme systems in the blood [24]. TMP in particular was found to disappear rapidly in blood. However, the thiamine vitamers were found to be stable for a month at −20°C with the addition of a half volume of buffer solution (1.8 *M* sodium acetate containing 0.6 *M* sodium hydroxide) after treatment with PCA. This procedure not only minimizes quantitation errors but also reduces the day-to-day variation of calibration and saves time in the preparation of standards.

An extensive study of the stability of thiamine and its phosphate esters under the storage conditions of blood and serum was also carried out, and the results will be presented in another paper.

Reproducibility

We have analysed over 500 samples using this method during the last six months. No significant changes on the chromatograms were observed. It was noted that storage of buffer A may lead to a baseline peak at *ca.* 8.3 min, which is close to the thiamine peak and may affects its detection. Therefore, it is recommended that buffer A should be prepared fresh on a daily basis. A variation of retention time of less than 10%, owing to the variation and sensitivity of pH of mobile phase for daily analysis, has been observed. This can be overcome by the use of pooled samples as internal quality control to reduce the variability of the between-day analysis.

The calibration graphs of the standard addition method were linear for concentrations of TTP, TPP, TMP and T in the range 2–20 n*M* for serum and 2–250 n*M* for blood. The linearity of the calibration and the day-to-day coefficients of variation (C.V.) are shown in Table II. The within-assay precision of the method

TABLE II

LINEARITY AND DAY-TO-DAY VARIATION

Compound	Mean linear regression[a]	Mean coefficient of correlation	Coefficient of variation (n = 4) (%)	
			Linear regression	Correlation coefficient
Blood (2–250 nM)				
TTP	$y = -3.3 + 0.0641x$	0.99	5.0	0.04
TPP	$y = -38.3 + 0.0730x$	0.99	1.7	0.01
TMP	$y = -0.5 + 0.0558x$	0.99	1.5	0.01
T	$y = 0.2 + 0.0995x$	0.99	1.8	0.01
Serum (2–20 nM)				
TMP	$y = -3.4 + 0.0782x$	0.99	5.2	0.05
T	$y = -6.7 + 0.1185x$	0.99	4.6	0.28

[a] y = concentration (nM); x = peak height (μV).

for blood total thiamine was less than 2 and 5% for the serum specimens (Table III). The data in Table III also indicate that the between-day variation of total thiamine in blood and serum samples, treated and stored in buffer media, were less than 3 and 5%, respectively.

Clinical screening

Fresh samples of blood, serum and plasma from healthy blood donors were analysed by the present method. The average total thiamine of eight blood sam-

TABLE III

PRECISION OF THE ASSAY

Compound	Within-day assay		Between-day assay	
	Mean (nM)	C.V. (%)	Mean (nM)	C.V. (%)
Blood				
TTP	6.4	9.7	6.2	6.8
TPP	61.4	2.7	62.9	2.0
TMP	4.5	7.1	4.7	2.4
T	3.6	6.8	2.4	17.7
Total thiamine	76.2	1.4	75.7	3.0
Serum				
TMP	6.3	11.2	6.9	4.2
T	5.1	3.6	4.7	15.0
Total thiamine	11.4	4.9	11.6	5.1

ples was 95.8 n*M* (range 68.7–122.1), where more than 70% of it was present as TPP, and the concentration of TMP was lower than that of TTP and the thiamine level was so low that it could hardly be detected. However, neither TTP nor TPP was detected in either serum or plasma specimens ($n = 5$). The mean values for total thiamine (TMP + T) is 12.9 n*M* (range 5.7–23.3 n*M*) for serum and 13.0 n*M* (range 7.3–21.3 n*M*) for plasma. TMP was present in plasma at a higher concentration than thiamine. However, it is not stable and is partially converted into thiamine or else slowly disappears at 4°C after collection. The values presented here are in close agreement with those reported earlier [11,17,18]. The proposed method is currently being used to study workers from North-eastern Thailand who are believed to have low thiamine intake [6–9].

CONCLUSION

This investigation has shown that the used of citric and formic acids with addition of ammonium hydroxide and diethylamine as mobile phases, for gradient separation on ODS column, combined with pre-column derivatization, provides a useful and convenient HPLC method for the direct analysis of TTP, TPP, TMP and T. It can be used to screen the total thiamine in human blood or serum, and also to quantify different thiamine phosphate esters in a clinical assay for thiamine deficiency or for post-mortem examination of various thiamine-related diseases.

ACKNOWLEDGEMENTS

We are grateful to the IDRC and National University of Singapore for their support (RP 900351). We thank the SUDS Task Force, Singapore, and the SUDS Study Team, NUS, for their assistance.

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