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OPTIMIZED DETERMINATION OF THIOCHROME DERIVATIVES OF THIAMINE AND THIAMINE PHOSPHATES IN WHOLE BLOOD BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH PRECOLUMN DERIVATIZATION

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SUMMARY

A liquid chromatographic method involving precolumn derivatization for determining thiamine and its phosphate esters in human blood has been optimized. Blood samples stored at −20°C were haemolysed and deproteinized by perchloric acid. The supernatants of the samples were oxidized by addition of potassium ferricyanide–sodium hydroxide, and phosphoric acid was added to obtain a neutral pH in order to extend the column life. The samples were stable after derivatization for at least 24 h, if protected from light and kept at room temperature. Gradient separation with 140 mmol phosphate buffer (pH 7.0), and methanol, *tert.*-butylammonium hydroxide and dimethylformamide as modifiers, on a 3-μm Chromsphere octadecylsilica column gave an analysis time of 15 min. The method was found to be very suitable for the determination of thiamine components in whole blood. The minimal detectable amount is 0.5 nmol/l and the method is linear to at least 1000 nmol/l. The recovery (98 ± 3%) and precision are very good.

INTRODUCTION

Thiamine is present in the blood in four different forms: thiamine, thiamine monophosphate (TMP), thiamine pyrophosphate (TPP) and thiamine triphosphate (TTP). The role of TPP as a coenzyme in carbohydrate metabolism and the role of some phosphorylated form of thiamine in the nerve conduction are well established [1].

Several methods for the determination of thiamine and its phosphate esters have been published [2–10]. These methods can be divided into pre- and post-

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column derivatization techniques with or without hydrolysis of thiamine phosphate esters. All these methods are cumbersome, and in some cases poisonous chemicals are used.

This paper describes a simple, inexpensive and sensitive method with pre-column derivatization of thiamine and its phosphate esters. The method is optimized for the simultaneous determination of thiamine and its phosphate esters. The thiochrome phosphate esters are stable and the column life is extended.

EXPERIMENTAL

Chemicals

Thiamine chloride·HCl, perchloric acid (PCA), $K_3Fe(CN)_6$, NaOH and phosphoric acid were obtained from Merck (Darmstadt, F.R.G.). TMP and TPP were obtained from Sigma (St. Louis, MO., U.S.A.). TTP was donated by Dr. Schrijver (CIVO-TNO, Zeist, The Netherlands). Methanol, dimethylformamide (DMF) and *tert.*-butylammonium hydroxide (TBAH) were obtained from FSA (Leicester, U.K.).

Stock solutions and working standard solutions

Stock solutions of thiamine chloride·HCl, TMP, TPP and TTP were prepared in 0.1 *M* HCl, in concentrations of 3.0 m*M* and stored at 4 °C except for TTP, which was prepared freshly before use. The purity of TTP was evaluated as 83% by high-performance liquid chromatography with UV detection.

In our hands the fluorescence response factors of TTP, TPP and TMP were almost the same. Thus it is possible to use the response factor of TPP to calculate the TTP concentration of the sample, since it is very difficult to obtain TTP. The concentrations of these stock solutions were checked every month by the method of Penttinen [11]. Our results fully agreed with those of Penttinen. Working standard solutions were prepared by diluting the stock solution 1·2000 with distilled water, and were kept at −20 °C. These solutions were stable for at least two months.

The oxidation reagent was a freshly prepared aqueous solution of 12.14 m*M* $K_3Fe(Cn)_6$ and 3.35 *M* NaOH.

Two solutions of PCA were prepared: PCA-1, 7.2%; and PCA-2, 7.2% in 0.25 *M* NaOH.

Mobile phases

Solvent A was prepared as follows: 31.9 g of $K_2HPO_4 \cdot 3H_2O$ were dissolved in 800 ml of distilled water, and 120 ml of methanol, 0.6 ml of 0.5 *M* TBAH and 15 ml of DMF were added. Distilled water was added, and concentrated H_3PO_4 was added to a final pH of 7.0; the final volume was 1 l. The solvent

was filtered through 0.45-μm membrane filters (RC 55, Schleicher/Schuell) before use.

Solvent B was prepared as follows: methanol (70%) was filtered through 0.45-μm membrane filters (RC 55, Schleicher/Schuell) before use.

Sample preparation and derivatization procedure

Venous blood samples were collected in evacuated tubes, containing sodium heparine as anticoagulant. The samples were immediately stored at $-20°C$ in polystyrene tubes (55 mm$\times$11 mm). After thawing the working standard and the blood samples, 1 ml of PCA-1 (4°C) was added to 1 ml of blood and 0.1 ml of distilled water; 1 ml of PCA-2 (4°C) was added to 1 ml of distilled water and 0.1 ml of standard solution.

For recovery samples, 100 μl of standard solution was added instead of distilled water.

After vigorous mixing, the tubes were placed on ice, protected from light. After 10 min the tubes were again vigorously mixed and replaced on ice for another 5 min. Then the mixtures were centrifuged for 15 min at 200 *g* at 4°C. The supernatants were filtered through a Seraclear filter (Technicon 500-4055-01). The filtrates were pipetted in 1-ml portions into polystyrene tubes. When room temperature was reached, 100 μl of methanol were added to all tubes. During the mixing, 200 μl of oxidation reagent were added (pH > 12). After ca. 30 s, 100 μl of 1.43 *M* phosphoric acid were added and the contents of the tubes were mixed. The final pH of the prepared samples and standards was 6.9 ± 0.2. The samples and standards were stable for at least 24 h at room temperature if protected from light.

Chromatographic analysis

Before injection the samples were filtered through 0.2-μm syringe filters (Dynagard 200-2-200 ME, Microgon, Laguna Hills, CA, U.S.A.). A 50-μl sample was injected with a Rheodyne valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.). A Chromspher C_{18} analytical column (3 μm particle size, 100 mm$\times$4.6 mm I.D.) and a corresponding guard column were used (Chrompack, Middelburg, The Netherlands). The mobile phase was pumped at 1.0 ml/min by a low-pressure gradient pump (Model 600, Waters Assoc., Milford, MA, U.S.A.). With the help of a system controller (Model 600 E, Waters Assoc.) a gradient elution (Table I) was set up.

The total analysis time was 15 min, including the stabilization of the column. Detection was performed by a spectrofluorimeter, Model RF 535 (Shimadzu, Tokyo, Japan), equipped with a 12-μl flow-through cell. The excitation wavelength was 367 nm, and the emission was detected at 435 nm. The resulting signal was displayed and computed by an integrator DATA module 745 (Water Assoc.).

TABLE I

GRADIENT ELUTION PATTERN

Time (min)	Flow-rate (ml/min)	Percentage of solvent A	Percentage of solvent B	Curve shape
0	1.0	100	0	Linear
2	1.0	80	20	Linear
6	1.0	80	20	Linear
7	1.0	100	0	Linear

The concentrations of thiamine and its phosphate esters were calculated by external calibration and checked by recovery results.

A sample of frozen donor blood was included in each batch of analysis for internal quality-control purposes.

RESULTS AND DISCUSSION

Chromatographic experiences

Bontemps et al. [8] have shown that thiamine, TMP, TPP and TTP can be separated by gradient elution in the reversed-phase mode using 25 m*M* phosphate buffer (pH 8.4) and methanol as mobile phase. This method is suitable for the measurement of thiamine compounds in excitable tissues.

Our aim was to develop a simple method for the simultaneous determination of the four different forms of thiamine in whole blood. First we developed an isocratic method, but this was cumbersome due to the long column stabilization time required, caused by aspecific peaks eluting at the end of the analysis. Thus we set up a new gradient method, which is a modification of the method of Bontemps et al. [8].

We carried out some experiments with different concentrations of $K_3Fe(CN)_6$ and NaOH; our results were similar to those of Bontemps et al. [8] (results not shown). Addition of methanol as a modifier increased the formation of the thiochromes, which resulted in higher fluorescence.

Penttinen [11] suggested that the different fluorescence intensities of thiochromes were due to the different amounts of non-fluorescent oxidation products, especially the disulphide derivative.

We varied the methanol concentration in order to get a stable, reproducible signal. Too much methanol produced asymmetric peaks. Our final concentration of methanol was 12%; this resulted in well shaped peaks and reproducible retention times: $k' = 1.7$, 2.11, 3.26 and 6.88 for ThcTP, ThcPP, ThcMP and Thc, respectively (Thc = thiochrome).

The stability of the chromatographic system was shown by repeated injections of a standard sample (Fig. 1). The corresponding data are shown in Table

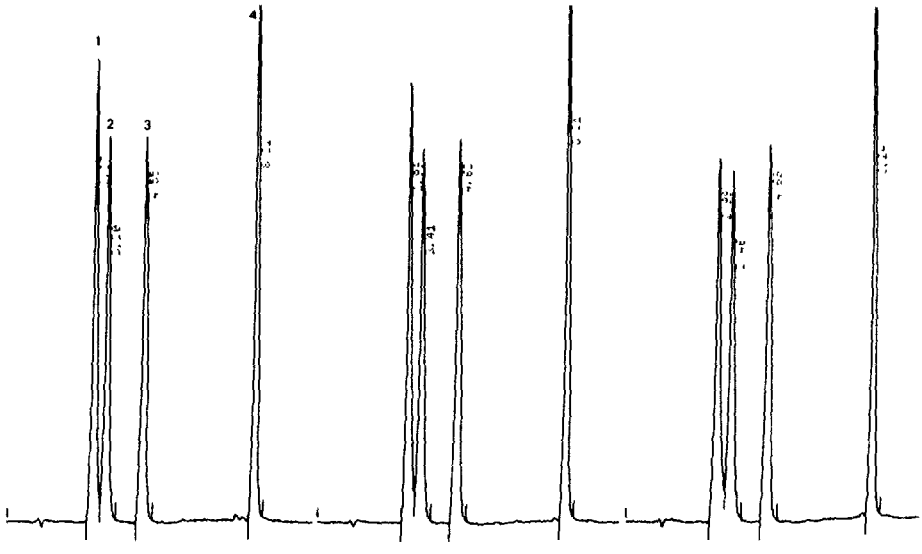

Fig. 1. Elution profile for a standard solution injected three times. Peaks: 1 = TTP; 2 = TPP; 3 = TMP; 4 = thiamine.

TABLE II

CHROMATOGRAPHIC PARAMETERS: PEAK QUALITY

$N_{\frac{1}{2}} = 5.53\ (t_R/W_{\frac{1}{2}})^2$. $N_{4.4\%} = 25\ (t_R/W_{4.4\%})^2$. $R_s = (t_{R1} - t_{R2})/\ (W_{\frac{1}{2}(1)} + W_{\frac{1}{2}(2)})$ t_R = retention time. $W_{4.4\%}$ = peak width at 4.4% of peak height. R_s = resolution factor.

Compound	$N_{\frac{1}{2}}$	$N_{4.4\%}$	R_s
Thiamine	$13.1 \cdot 10^3$	$11.3 \cdot 10^3$	
TMP	$3.3 \cdot 10^3$	$2.2 \cdot 10^3$	
TDP	$2.0 \cdot 10^3$	$1.3 \cdot 10^3$	TDP-TMP: 3.4
TTP	$1.7 \cdot 10^3$	$1.3 \cdot 10^3$	TTP-TDP: 1.2

II. Fig. 2 shows a typical chromatogram of a blood sample injected three times. Addition of TBAH as modifier resulted in longer retention times for ThcTP, ThcPP, ThcMP and a shorter retention time for Thc. DMF addition resulted in better separation of the thiochrome esters.

Column lifetime and the pH influence on the stability of the samples

Although the fluorescence intensity is pH-dependent and reaches a plateau at pH > 8 according to Ishii et al. [7], we used a buffer of pH 7.0. At this pH, our signal was 20% lower than that of Ishii et al. [7]. Post-column addition of NaOH increased the signal, but we omitted this step because of dilution of the sample and peak broadening. The column life at pH 7.0 was increased, and

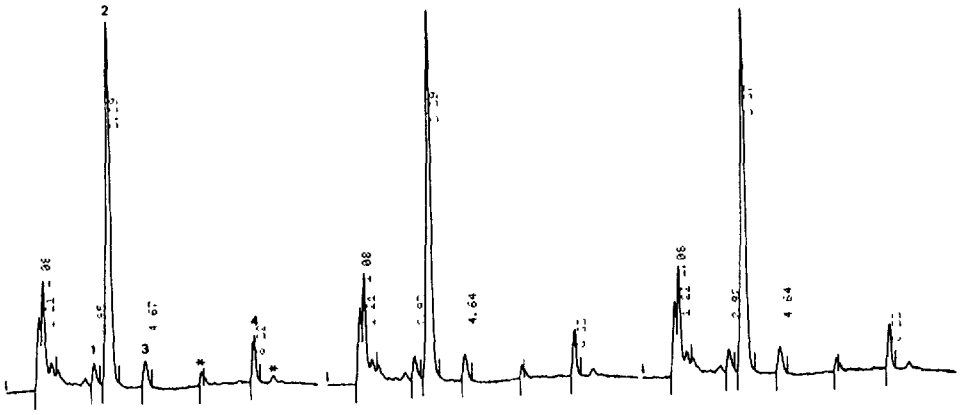

Fig. 2 Elution profile for a human blood sample injected three times. Peaks: 1 = TTP; 2 = TPP; 3 = TMP; 4 = thiamine. Unknown impurities are indicated by an asterisk.

better reproducibility was possible. Also the oxidized thiamines are stable at this pH for at least 24 h at room temperature. At higher pH values the oxidized thiamines began to deteriorate at once.

Temperature influence on the stability of the samples

After venapuncture, samples should be stored at $-20\,^{\circ}C$ as soon as possible. At this temperature no decrease in TTP, TPP, TMP or thiamine was observed after six months. At $4\,^{\circ}C$ the concentration of TTP decreased by ca. 50% after 24 h.

The whole procedure of sample derivatization should be carried out at constant temperature since the efficiency of thiochrome formation varies with temperature.

After the oxidation step phosphoric acid was added after ca. 30 s. The stability of the samples with a final pH of 6.9 ± 0.2 at room temperature and protected from light is at least 24 h.

Precision, accuracy and linearity

The intra-assay coefficients of variation (C.V.), calculated from the analysis of ten whole blood samples and a spiked sample, are shown in Table III. The inter-assay C.V., based on the analysis of one patient sample in ten batches of analysis, is shown in Table IV.

The accuracy of the method was investigated by recovery experiments in different patient samples (Table V). The method is linear for TPP, TMP and thiamine concentrations up to 1000 nmol/l. Taking a signal-to-noise ratio of 5 as the detection limit, the sample injected should contain at least 0.5 nmol/l thiamine and phosphate esters each. This corresponds to an absolute amount

TABLE III

PRECISION RESULTS: INTRA-ASSAY ($n=10$)

Compound	Sample		Spiked sample	
	Concentration (mean ± S.D.) (nmol/l)	C.V. (%)	Concentration (mean ± S.D.) (nmol/l)	C.V. (%)
TTP	1.54 ± 0.24	16	95.0 ± 0.36	0.4
TPP	119.8 ± 0 76	0.6	261.1 ± 1.54	0.6
TMP	4.1 ± 0.76	12	135.7 ± 0.94	0.7
Thiamine	5.2 ± 0.27	5	123.4 ± 1.24	1.0

TABLE IV

PRECISION RESULTS: INTER-ASSAY ($n=10$)

Compound	Concentration (mean ± S.D.) (nmol/l)	C.V. (%)
TTP	2.9 ± 0.7	24
TPP	178 ± 5.4	3.0
TMP	7.4 ± 1.2	16
Thiamine	6.8 ± 0.8	12

TABLE V

RECOVERY RESULTS

Compound	n	Recovery (mean ± S.D) (%)	C.V (%)
TTP	4	101 ± 7.4	7.3
TPP	10	99 ± 4.2	4.2
TMP	10	102 ± 2.5	2.4
Thiamine	10	90 ± 7.5	8.3

of ca. 7 fmol for each component injected. The calculation of the final results of the samples was based on external calibration.

Reference values

Samples (65) were obtained from healthy volunteers. Reference values were calculated from the mean ± 2 S.D. The mean (± S.D.) of TPP was 120 ± 17.5 nmol/l, 4.1 ± 1.6 nmol/l for TMP and 4.3 ± 1.9 nmol/l for thiamine. For TTP all measurements were below 4.0 nmol/l.

CONCLUSION

These results show that our method is simple, cheap and easy to carry out. The parameters that affect oxidation have been optimized. The chromatographic conditions have been optimized with respect to stability of the signal, column life, time of analysis, reproducibility of the results and separation of the oxidized thiamines. The clinical importance of TTP is not yet clear [1].

We encountered a few patients with low TPP and a relatively high TTP. In most cases these patients were alcoholics. The precise role of TTP in the clinical setting still remains to be elucidated.

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