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Short Communication

Determination of thiamine and its phosphate esters in human erythrocytes by high-performance liquid chromatography with isocratic elution

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Abstract

A high-performance liquid chromatographic method for the simultaneous determination of thiamine and its phosphate esters in human erythrocytes, using postcolumn derivatization, is presented. The sample preparation and the choice of the analytical column avoid the use of an elution gradient. The four thiamine compounds (thiamine and thiamine monophosphate, diphosphate and triphosphate) are eluted within less than 15 min with a detection limit of *ca.* 20 fmol. The reproducibility and accuracy of the assay are satisfactory. Normal physiological red blood cell concentrations of the four thiamine compounds are included.

1. Introduction

Thiamine diphosphate (TDP) is known to play a key role like coenzymes in many biological reactions involved in carbohydrate metabolism. Recently, Bettendorff and co-workers [1,2] showed that thiamine triphosphate (TTP) was involved in the regulation of chloride permeability in rat brain. Furthermore, some workers have reported modifications in the repartition and metabolism of the four thiamine compounds [thiamine (T), thiamine monophosphate (TMP), TDP and TTP] in rat liver, kidney, heart and small intestine [3] and in human blood [4] following alcohol intake.

Several HPLC methods using fluorescence of thiochromes obtained by oxidation of the thiamine compounds have been reported. Precolumn derivatization [5–10] is more convenient than postcolumn derivatization [8,11–15]. However, the silica-based gels used for separating thiamine derivatives can deteriorate rapidly owing to the alkalinity of the mobile phase needed for optimum fluorescence (pH > 8). Most of the methods propose time-consuming separations with an elution gradient [7,8,12,15]. Only Kimura and Itokawa [11] separated the four thiamine compounds from erythrocytes using isocratic elution with a postcolumn derivatization technique. However they used a mobile phase containing too high a concentration of phosphate according to Lee *et al.* [12] and Wielders and Mink [14].

This paper describes a rapid method for the determination of thiamine and its phosphate esters using isocratic elution with postcolumn

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derivatization. The method permits the separation and detection of the four thiamine compounds within less than 15 min and has been successfully applied to the determination of thiamine compound levels in human erythrocytes, which contain at least 90% of blood total thiamine [16].

2. Experimental

2.1. Instrumentation

HPLC analysis was performed using a Waters Model 600E pump and gradient controller (Waters Chromatography Division, Millipore, Milford, MA, USA) and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 20-μl fixed sample loop. The analytical column was μBondapak C_{18} (10-μm particles; 300 mm × 3.9 mm I.D.) (Waters) protected by an RP-18 New Brownlee guard cartridge system (7-μm particles; 15 mm × 3.2 mm I.D.) (Applied Biosystems, San Jose, CA, USA). Separations were achieved by isocratic elution at a flow-rate of 1.0 ml/min. Detection and quantification were carried out with a Waters Model 470 fluorescence detector and a Servotrace (Sefram, Paris, France) for peak-height measurement against external standards. The maximum excitation (365 nm) and emission (435 nm) wavelengths were determined by recording fluorescence spectra during the analysis using a stop-flow technique. The post-column reactor consisted of a zero-dead-volume tee-piece (0.5-mm hole) (Upchurch Scientific, Oak Harbor, WA, USA) and a PEEK capillary (120 cm × 0.5 mm I.D.) shielded from light. A Gilson (Villiers-le-Bel, France) Minipuls 2 peristaltic pump delivered the oxidizing reagent at 0.15 ml/min. Its pressure pulses were damped by a 100-cm PEEK capillary between this pump and the tee-piece. The column and postcolumn reactors were at ambient temperature.

2.2. Reagents

Thiamine hydrochloride, TMP chloride and TDP chloride were purchased from Sigma (St. Louis, MO, USA) and TTP from Wako (Osaka, Japan). Diethyl ether (puriss.) stabilized with 2,6-di-*tert.*-butylmethylphenol and all other chemicals were obtained from Merck (Darmstadt, Germany).

The mobile phase was ammonium citrate buffer (15 m*M* citric acid, pH adjusted to 4.2 with ammonia solution)–0.4% (v/v) diethylamine in 0.1 *M* formic acid in the ratio (90:10, v/v). This mobile phase was freshly prepared on the day of use and filtered through a 0.45-μm Millipore filter.

The oxidizing reagent was an aqueous solution of 0.01% (w/v) potassium hexaferrocyanate(III) in 3 *M* sodium hydroxide.

2.3. Sample preparation

Millimolar stock standard solutions of each standard were prepared in 0.01 *M* hydrochloric acid and stored at −20°C. These solutions were stable for 3 months. Because we observed that dilution up to 10^{-6} *M* in water, 0.01 or 0.1 *M* hydrochloric acid, 5% (w/v) trichloroacetic acid (TCA) or 0.6 *M* perchloric acid led to the hydrolysis of thiamine phosphate esters, working standard solutions were freshly prepared by spiking a deproteinized sample with standard solutions (final concentration of T, TMP, TDP, TTP = 10 or 100 n*M*).

Venous blood samples were collected in tubes containing lithium heparinate as anticoagulant. The samples were centrifuged quickly after collection (3500 *g*, 15 min, 4°C). Erythrocytes were haemolysed by rapid freezing at −20°C for at least 30 min. Deproteinization was achieved by addition of 0.5 ml of 10% (w/v) TCA to 0.5 ml of haemolysate. After centrifugation (3500 *g*, 15 min, 4°C), the supernatant was transferred into a glass tube. TCA was removed with five volumes of diethyl ether and a final purification of the sample was performed with five volumes of *n*-hexane. A 20-μl volume of filtered (0.22-μm Millipore filter) sample was injected onto the column.

2.4. Subjects

Twenty-eight healthy females (average age 35.8 years, range 22–65 years) and 24 healthy

males (average age 37.0 years, range 21–60 years) took part in the study. Samples were collected after overnight fasting.

3. Results and discussion

The retention times of TTP, TDP, TMP and T were 4.4, 5.2, 6.8 and 12.0 min, respectively. No additional peak could be detected as long as 15 min after the elution of T. The baseline was stable during the elution. A typical chromatographic analysis of erythrocytes before and after spiking with 100 n*M* TTP, TDP, TMP and T is shown in Fig. 1. Our mobile phase corresponds to the initial period of the gradient developed by Lee *et al.* [12]. However, the sample preparation and the choice of a stationary phase that interacted less with thiamine allowed us to avoid the use of a gradient.

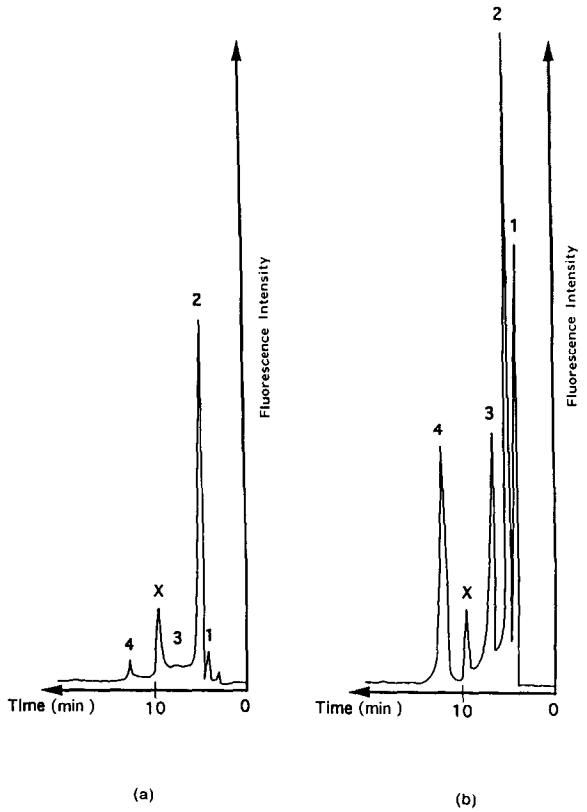

Fig. 1. Chromatograms of (a) erythrocytes and (b) erythrocytes spiked with 100 n*M* thiamine compounds. Peaks: 1 = TTP; 2 = TDP; 3 = TMP; 4 = T; X = unknown compound.

Recovery tests, performed in triplicate by spiking the sample before deproteinization with 10 or 100 n*M* T, TMP, TDP and TTP, gave results not significantly different from 100% [110 ± 6.1, 104 ± 1.6, 110 ± 5.7 and 95 ± 2.8% (mean ± S.D.), respectively].

The intra- and inter-assay coefficients of variation (C.V.s) measured ($n = 20$) on a sample spiked with TMP and T were respectively 2.6 and 6.5% for T, 5.0 and 6.1% for TMP, 3.5 and 3.8% for TDP, 8.8 and 11.5% for TTP and 2.8 and 2.8% for total T.

Linearity and detection limits were determined by the analysis of standard solutions containing from 0 to 1000 n*M* of each compound. The fluorescence intensities (peak heights) were proportional to concentration up to 1000 n*M*. With a signal-to-noise ratio of 3 and an injection volume of 20 μl, the detection limit was less than 1 n*M* for TTP and TDP and 1 n*M* for the other thiamine compounds.

In human erythrocytes, phosphorylated forms of thiamine, mainly TDP, were detected (Table 1). Trace amounts of TMP were found in only a few samples. Our TDP results are lower than those reported by Warnock [17] and Tallaksen *et al.* [5] but are close to those found by Baines [9] and Floridi *et al.* [10]. To our knowledge, the concentration of the other forms of thiamine in erythrocytes has not been reported. Finally, no significant differences were observed with regard to age and sex. This finding is in contrast with the conclusions in previous studies [5,14]. However, the use of whole blood instead of erythrocytes in these studies together with the presence of higher haematocrits in males may account for the observed discrepancy.

4. Conclusion

The proposed HPLC technique with isocratic elution allows the accurate and reproducible determination of thiamine and its phosphate esters in human erythrocytes within less than 15 min. This simple procedure should be useful in characterizing the relationship between bio-

Table 1
Thiamine and its phosphate esters in human erythrocytes

Parameter	Females	Males	Females and males
n	28	24	52
TTP (n*M*)	8.0 ± 8.0	6.0 ± 5.0	7.0 ± 6.0
TDP (n*M*)	177 ± 27.0	175 ± 29.0	176 ± 28.0
TMP (n*M*)	<2	<2	<2
T (n*M*)	3.0 ± 2.5	4.0 ± 2.2	4.0 ± 2.0
Total T (n*M*)	188 ± 27.0	185 ± 31.0	186 ± 30.0
Percentage of phosphorylation[a]	98.3 ± 1.2	98.0 ± 1.5	98.1 ± 1.3
TDP/total T (%)	94.0 ± 4.8	94.7 ± 3.1	94.3 ± 4.1

Results are means ± S.D.
[a] Percentage of phosphorylation = [(TTP + TDP + TMP)/(total T)] · 100.

chemical status and clinical features in various neurological diseases.

5. Acknowledgements

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