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Note

Separation and determination of thiamin and its phosphate esters by reversed-phase high-performance liquid chromatography

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In animal tissues four different forms of thiamin, thiamin (T), thiamin monophosphate (TMP), thiamin pyrophosphate (TPP) and thiamin triphosphate (TTP), exist. TTP is of particular interest as it can play an important rôle in the nerve excitation process which is independent of the coenzyme rôle of thiamin.

Recently, techniques of high-performance liquid chromatography (HPLC) have been introduced into thiamin research[1–9] and a great deal of progress in this field can be expected since these methods are sensitive and rapid. Previously, we exploited a differential fluorometric determination of thiamin and its phosphate esters using straight phase HPLC with post labelling[3]. Using this method thiamin phosphates were eluted in the order thiamin, TMP, TPP and TTP. However, for the accurate determination of TTP in animal tissues, a method which can elute TTP first is more suitable.

For this purpose, we have contrived a reversed-phase HPLC method for the differential determination of thiamin and its phosphate esters.

EXPERIMENTAL

Reagents

Thiamin hydrochloride was obtained from Wako (Osaka, Japan), TMP and TPP from Sigma (St. Louis, MO, U.S.A.). TTP was donated by the Central Research Division of Takeda Chemical Co. (Osaka, Japan). All other chemicals were of the best grade commercially available. A 0.2 *M* sodium phosphate–phosphoric acid buffer (pH 4.3) was used for the mobile phase. A solution of 0.01% potassium hexocyanoferrate(III) in 15% sodium hydroxide was used for converting thiamin and its phosphate esters into fluorophores.

Apparatus

The system consists of an LC-3A pump for liquid chromatography, an SIL-1A injector, μBondapak C_{18} column (300 × 4 mm I.D.), CTO-2A column oven (35°C), a PTFE mixing coil (1000 × 0.15 mm I.D.), a PPR-1A proportioning pump (flow-rate

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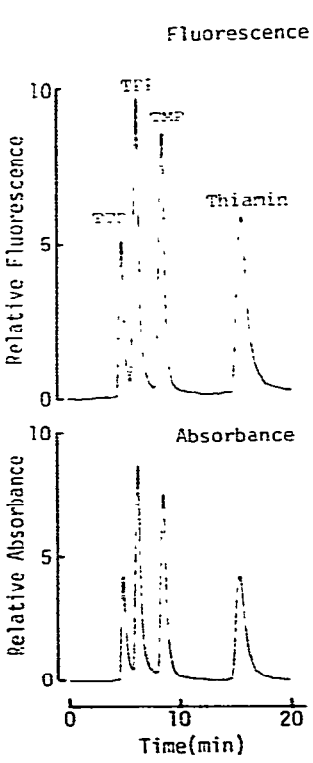
1.0 ml/min), an SPD-2A UV detector (flow cell 10 × 0.1 mm I.D.), an RF500 LCA spectrofluorimetric detector (square-shaped flow cell, 12 μl) and a Chromatopac C-RIA (chart speed 2 mm/min). All of the equipment was purchased from Shimadzu (Kyoto, Japan).

Procedure

The mobile phase was pumped at a flow-rate of 1.0 ml/min. A 10-μl volume of T, TMP, TPP and TTP in the 0.2 *M* sodium phosphate–phosphoric acid buffer (pH 4.3) was loaded on the sample loop and injected on to the column. The column effluent was loaded on the flow cell and TTP, TPP, TMP and T were detected with the UV detector at a wavelength of 280 nm. The potassium hexacyanoferrate(III)–sodium hydroxide solution was then applied at 1.0 ml/min by a proportioning pump and mixed with the effluent to convert TTP, TPP, TMP and T into fluorophores. The fluorophores were measured with the spectrofluorimeter (excitation wavelength, 375 nm; emission maximum, 435 nm) connected to the Chromatopac and recorded graphically. The peak-height method was used for quantitation.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained for a standard solution contain-



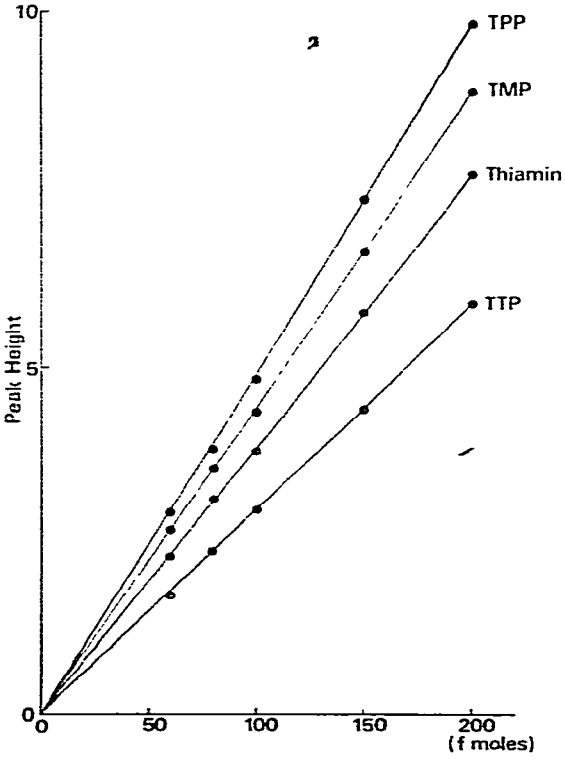


Fig. 1. Chromatogram of TTP, TPP, TMP and thiamin.

Fig. 2. Calibration graph obtained for thiamin, TMP, TPP and TTP.

ing 10 pmoles each of T, TMP, TPP and TTP. These were eluted in the order TTP, TPP, TMP and T. The chromatographic pattern of UV absorbance closely resembles that given by fluorescence.

Fig. 2 shows a standard calibration curve for T, TMP, TPP and TTP. A linear relationship exists between amount and peak hiehgt for each compound over the range 50–200 fmoles.

As compared to the previous method[3], this method has advantages for determination of TTP in the presence of high concentrations of TPP.

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