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Note

Differential fluorimetric determination of picogram levels of thiamine, thiamine monophosphate, diphosphate and triphosphate using high-performance liquid chromatography

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Free thiamine and thiamine monophosphate (TMP), diphosphate (TDP) and triphosphate (TTP) are found in animal tissues, and column chromatographic[1–3] and electrophoretic[4,5] methods have been used for their determination.

There is evidence for two independent roles of thiamine[6–8], as a cofactor in intermediate carbohydrate metabolism and in a physiological role in the nerve excitation process. The above-mentioned methods have not been utilized to determine thiamine phosphates in small nervous tissues in animals (*e.g.*, pheripheral nerve in the rat). Recently, Roser *et al.*[9] established a sensitive high-performance liquid chromatographic (HPLC) method for the determination of urinary thiamine. We have developed an HPLC method capable of determining 0.05 pmole of thiamine phosphates.

EXPERIMENTAL

Apparatus

The following were used: LC-3A pump for liquid chromatograph; SIL-1A injector; Shimadzu ISA-07/S2504 LC column (25 mm × 0.4 mm I.D.); CTO-2A column oven (35°); stainless-steel mixing coil (100 mm × 0.1 mm I.D.; CRB-1B incubator box (35°); PRR-1A proportioning pump (flow-rate 0.5 ml/min); RF 500-LCA spectrofluorimetric detector (excitation 375 nm, emission 435 nm); square-shaped flow cell (12 μl); and strip-chart recorder (chart speed 2.5 mm/min). All of the equipment was purchased from Shimadzu (Kyoto, Japan).

Reagents

Thiamine was obtained from Wako (Osaka, Japan), TMP and TDP from Sigma (St. Louis, Mo., U.S.A.), and TTP was donated by the Central Research Division of Takeda Chemical Co. (Osaka, Japan). All other chemicals were of the best grade

commercially available. For the mobile phase 0.7 *M* sodium acetate solution was used. To convert thiamine and thiamine phosphates into fluorophores, a mixture of 15% sodium hydroxide and 0.02% potassium hexocyanoferrate(III) solution was used.

Procedure

For HPLC, the mobile phase was pumped at a flow-rate of 0.5 ml/min. A 20-μl volume of a solution containing thiamine and its phosphate esters (10^{-6}–10^{-7} *M*) was loaded on the sample loop and then injected on to the column, the zero time being marked. Potassium hexacyanoferrate(III)–sodium hydroxide solution was applied at 0.5 ml/min by a proportioning pump and mixed with the column effluent to convert thiamine phosphates into fluorophores. The fluorophores were measured with the spectrofluorimeter connected to the chromatograph and recorded graphically. The peak-height method was used for quantitation. Fig. 1 shows a schematic diagram of the method.

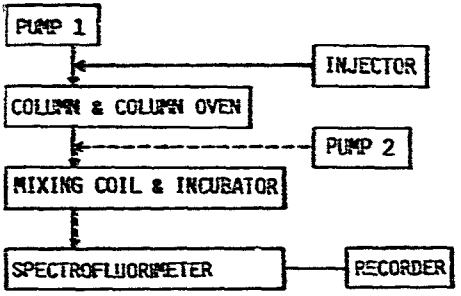

Fig. 1. Schematic diagram of the high-performance liquid chromatograph equipped with a fluorescence detector, and using potassium hexacyanoferrate(III) and sodium hydroxide as reagents.

RESULTS AND DISCUSSION

Using the proposed method, thiamine phosphates were eluted in the order thiamine, TMP, TDP and TTP. A typical chromatogram obtained with a solution containing 1 pmole each of thiamine, TMP, TDP and TTP in 20 μl is shown in Fig. 2.

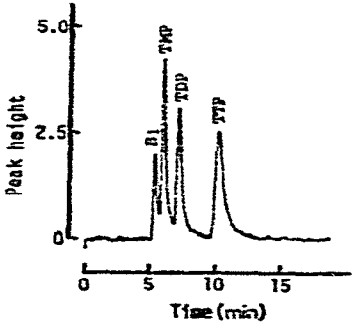

Fig. 2. Separation of thiamine (B_1), TMP, TDP and TTP by HPLC.

Fig. 3 illustrates the relationships between peak heights and concentrations of thiamine, TMP, TDP and TTP. A linear relationship was observed for each compound in the concentration range 0.05–1.5 pmoles.

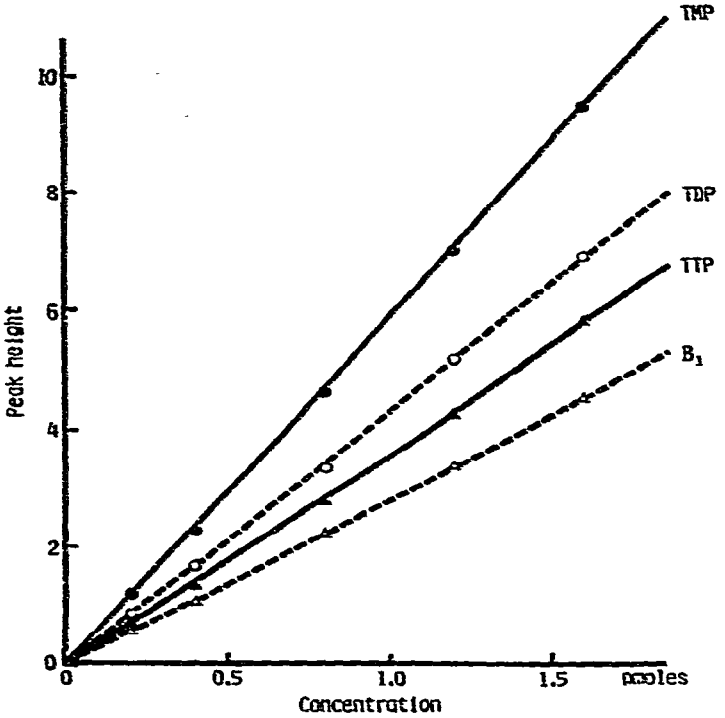

Fig. 3. Calibration graph obtained for thiamine, TMP, TDP and TTP.

This method is simple, reproducible and rapid. The sensitivity is sufficient for application to the determination of thiamine and its phosphate esters in a small amount of nervous tissues in animals.

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