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Note

Separation and determination of thiamine, pyrithiamine, pyridine, 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-(β-hydroxyethyl)thiazole by reversed-phase high-performance liquid chromatography

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Thiaminase I and II, found in microorganisms, plants and marine animals, are known to cause thiamine deficiency in both farm animals and humans[1,2]. Thiaminase I breaks down thiamine with the help of a basic co-substrate to give 4-methyl-5-(β-hydroxyethyl)thiazole (Thz) and a substituted pyrimidine moiety of thiaminase. If the other substrate is pyridine, then the substituted pyrimidine product is pyrithiamine. Thiaminase II cleaves thiamine into Thz and 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP). To distinguish between thiaminase I and II, it is important to be able to determine these degradation products.

In a previous paper[3], we reported the separation of thiamine and its common degradation and oxidation products by high-performance liquid chromatography (HPLC). This note deals with the separation of HMP, pyrithiamine, thiamine, pyridine and Thz, products of the degradation of thiamine by thiaminase I and II.

EXPERIMENTAL

Reagents

Thiamine hydrochloride and pyridine were obtained from Wako (Osaka, Japan). Pyrithiamine hydrochloride, HMP and Thz were donated by the Central Research Division of Takeda Chemical Co. (Osaka, Japan). All other chemicals were of the best grade commercially available.

Apparatus

The following were used: LC-3A pump for liquid chromatograph; SIL-1A injector; μBondapak C_{18} column; SPD-2A UV detector; and strip-chart recorder. The μBondapak C_{18} column was purchased from Waters Assoc. (Milford, MA, U.S.A.) and all other equipment from Shimadzu (Kyoto, Japan).

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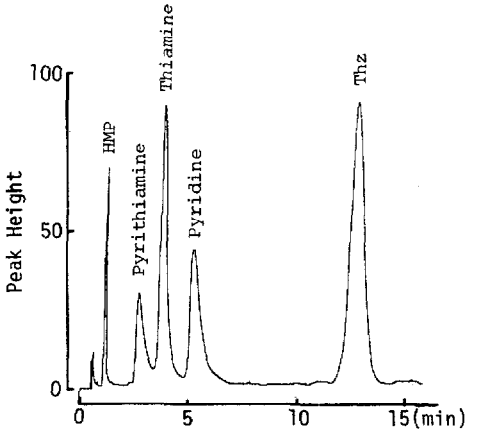

Fig. 1. Elution profiles of HMP, pyrithiamine, thiamine, pyridine and Thz.

Procedure

The mobile phase [0.2 *M* phosphate buffer (pH 5.4) in 4% acetonitrile] was pumped at a flow-rate of 3 ml/min into the HPLC column. A sample (5 μl) was injected on to the column and the absorbance at 250 nm was monitored continuously with a UV detector and recorded graphically.

RESULTS AND DISCUSSION

The separation of HMP, pyrithiamine, thiamine, pyridine and Thz, as shown in Fig. 1, offers the opportunity for the rapid and simultaneous determination of both kind of thiaminases.

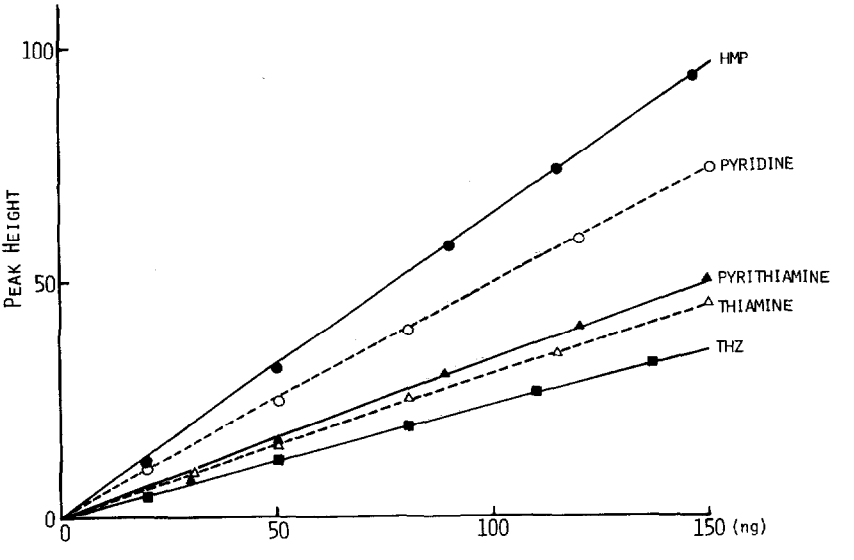

Fig. 2. Calibration graphs obtained for HMP, pyrithiamine, thiamine, pyridine and Thz.

In an incubation mixture containing thiamine, pyrithiamine and biological extracts or microorganisms, an increase in either pyrithiamine or HMP alone indicates the sole presence of thiaminase I or thiaminase II, respectively. An increase in both pyrithiamine and HMP shows the presence of both kinds of enzyme and allows for the determination of their relative activities. The non-thiaminase modification of thiaminase in the system can also be checked by the molar ratio of HMP, pyrithiamine and Thz produced. Kinetic studies of both enzymes can be carried out by this HPLC procedure. The procedure is rapid and accurate and allows the determination of down to 10 ng of each substance.

Fig. 2 shows the linearity between amount and peak height obtained for each compound up to 150 ng. Finally, pyrithiamine and thiamine can be converted into fluorophores by post-column conversion with potassium hexacyanoferrate(III)–sodium hydroxide[4].

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