CONCENTRATIONS OF THIAMINE AND ITS PHOSPHATE
ESTERS IN RAT TISSUES DETERMINED BY HIGH-
PERFORMANCE LIQUID CHROMATOGRAPHY

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Summary Thiamine and its phosphate esters in rat tissues were
determined by high-performance liquid chromatography after conversion
to thiochrome and its phosphate esters as described previously (Ishii, K.
et al. (1979) Anal. Biochem., 97, 191–195). Total thiamine concentrations
determined in the brain, liver, heart and kidney were 6.39, 25.2, 15.7, and
14.9 nmol/g wet weight, respectively. In all the tissues tested, thiamine, its
monophosphate, pyrophosphate, and triphosphate were found to be
present at the rates of 1.2–7.7, 5.9–11.0, 85.7–90.0, and 0.7–1.6%,
respectively. Although these percentages of thiamine phosphates were
similar to those reported previously using different procedures, the
percentage of thiamine triphosphate content in rat tissues was found to be
fairly lower than that reported. The time required for the whole process
of analysis averaged 90 min after isolation of the organs from the animal.

Keywords HPLC, thiamine and its phosphates, rat tissue concentrations

Microquantitative estimation of thiamine and its phosphate derivatives in
biological material is essential for studying not only their metabolisms but also
their functions (1). Of particular interest are the existence and function of thiamine
triphosphate (TTP) and its presence in several animal tissues has been reported
previously (2–6). However, the method of determining TTP itself is still a subject of
controversy in the study of enzymatic TTP synthesis (7).

We have recently presented a report (1) on a new analytical procedure for
separation and determination of thiamine and its phosphate esters by high-
performance liquid chromatography (HPLC). The procedure involves: 1) con-
version of thiamine and its phosphate derivatives to thiochrome and its phosphates
by alkaline oxidation; 2) HPLC of thiochrome derivatives; and 3) spectrofluorom-

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etrical detection. A complete, rapid and quantitative separation of these compounds was successfully made and the minimum amount detected was 1 pmol for each of the compounds.

In the present study, we have developed a method for quantitative assay of thiamine and its phosphates, especially of TTP in rat tissues using of HPLC.

MATERIALS AND METHODS

Chemicals. TMP was purchased from Sigma Chemical Co. TTP, authentic Thc and authentic ThcMP were provided through the courtesy of M. Yamasaki, Central Research Laboratory of Sankyo Co., Ltd., Tokyo, and TPP, TTP, authentic ThcPP and authentic ThcTP, through S. Yurugi, Central Research Laboratory of Takeda Pharmaceutical Industries, Osaka. The other reagents were all of analytical grade.

High-performance liquid chromatography. The HPLC procedure used was completely identical to that described previously (1).

Preparation of tissue extracts. Adult Wistar rats (male, 200-300 g) were killed by decapitation. The brain, liver, heart and kidney were removed after dissection and immediately frozen in liquid N₂. It took less than 30 sec from killing the animal to freezing the organs. Each of the tissues was weighed (approximately 200 mg) and homogenized in 3 volumes of 5% trichloroacetic acid at 4°C. After centrifugation for 15 min at 16,000 × g at 4°C, trichloroacetic acid was removed by extraction three or four times with the same volume of ethyl ether. The extract (water layer) thus obtained, 0.4 ml in volume, was mixed with 0.05 ml of 0.3 M BrCN and shaken for 1 min, followed by the addition of 0.05 ml of 1 M NaOH. The mixture was agitated for 1 min and an aliquot (5-40 μl) of the oxidized sample was injected onto the column. A blank for the sample was prepared by the addition of 0.05 ml of 1 M NaOH to 0.4 ml of the extract and by the subsequent addition of 0.05 ml of 0.3 M BrCN.

Standard solutions were prepared from solutions of thiamine and its phosphate derivatives (1-10 μM) in exactly the same manner as the tissue extract.

Calculation of thiamine and its phosphate esters in the tissue. An aliquot of the sample and the same volume of the blank were developed by HPLC. The blank areas, which are represented by the shaded areas in Figs. 1 and 2, were subtracted from each peak area of thiochrome derivatives. Calculation of the amount in each peak of the sample was made by each corresponding internal standard peak area detected under the same HPLC conditions.

RESULTS

Authentic ThcTP was not available in the previous experiments (1), and its retention time was not, therefore, compared with that of ThcTP prepared from TTP. In the present study, retention time and molar fluorescence intensity at pH
8.5 of authentic ThcTP were confirmed to be identical to those of ThcTP prepared from TTP (data not shown).

Analytical patterns of thiamine and its phosphate esters in the rat liver by HPLC are shown in Fig. 1. When 5 μl of the sample was applied onto the column, 3 peaks corresponding to a blank peak, ThcMP and ThcPP from the right were detected, but the two peaks of Thc and ThcTP were not demonstrated (Fig. 1a). However, when 40 μl of the sample was injected onto the column, two small peaks that corresponded to the retention times of Thc and ThcTP were detected at the extreme right and extreme left sides, respectively (Fig. 1b). In order to confirm the presence of TTP in this tissue, 2 pmol of ThcTP prepared from TTP was added to the oxidized sample and then chromatographed. The small peak corresponding to ThcTP was enlarged by this addition (Fig. 1c), and recovery of the added TTP was calculated to be 93.0% after measurement of 5 different samples. When TPP and TTP were added to the liver samples before extraction and then analyzed by
HPLC, the average recovery rates of added TPP and TTP were 98.0 and 90.0%, respectively, though they are not illustrated in Fig. 1. In these experiments, the amounts of TPP and TTP added were adjusted to give 10 pmol and 2 pmol, respectively, in aliquots of the final oxidized solution injected onto the column. Similar results were obtained for the recovery of TPP and TTP added to the brain, heart and kidney samples before extraction. This result confirms the presence of TTP in the rat liver and indicates that the tissue TTP content was determined accurately.

Thiamine and its phosphate esters in the rat brain could be analyzed by HPLC by the same procedure as for the liver and the patterns obtained are shown in Fig. 2. The peak of ThcTP was detected when 40 µl of the extract was applied (Fig. 2a) and the recovery of 2 pmol TTP added to the oxidized sample was 90.6% (Fig. 2b).

Fig. 2. HPLC of thiamine and its phosphate esters in the rat brain. 2a, 40 µl of the sample; 2b, 40 µl of the sample plus 2 pmol of ThcTP. Shaded areas represent fluorescences derived from the blanks.

The samples obtained from other tissues, that is, the heart and kidney, were analyzed by HPLC by the same procedure as for the liver and patterns similar to those of the liver and brain extracts were obtained (data not shown). The recovery
of 2 pmol TTP added to the samples was 92.3 and 84.6%, respectively, in the heart and kidney. These recovery values were obtained as averages of 5 different samples analyzed.

The contents of thiamine and its phosphate derivatives in rat tissues determined by the HPLC method are summarized in Table 1. Values are expressed as nmol of each compound/g wet weight of tissues and as averages of 5 different samples.

| Table 1. Contents of thiamine and its phosphate esters in rat tissues. |
|-------------------------|----------------|----------------|----------------|
| Brain                   | Liver          | Heart          | Kidney         |
| Thiamine                | (nmol/g wet weight) | (nmol/g wet weight) | (nmol/g wet weight) |
| Thiamine                | 0.16 ± 0.01* (2.5) | 0.47 ± 0.04 (1.9) | 0.19 ± 0.02 (1.2) | 1.15 ± 0.12 (7.7) |
| TMP                     | 0.38 ± 0.03 (3.9) | 2.78 ± 0.43 (11.0) | 1.31 ± 0.05 (8.4) | 0.89 ± 0.17 (6.0) |
| TPP                     | 5.75 ± 0.14 (90.0) | 21.7 ± 1.92 (86.1) | 14.0 ± 0.33 (89.3) | 12.8 ± 1.82 (85.7) |
| TTP                     | 0.10 ± 0.01 (1.6) | 0.25 ± 0.04 (1.0) | 0.18 ± 0.02 (1.1) | 0.10 ± 0.01 (0.7) |
| Total thiamine          | 6.39            | 25.2           | 15.7           | 14.9           |

* Values represent the mean ± S.D. for 5 rats. The numbers in parentheses refer to percentages of total thiamine.

The most abundant thiamine compound in rat tissues was TPP, which is consistent with the data previously reported (2), and represents an average of 85–90% of the total thiamine present. The contents of thiamine and TMP ranged from 1.2 to 7.7 and from 5.9 to 11.0%, respectively. The TTP levels shown in Table 1 are several-fold lower both in actual amounts and in percentages of the total thiamine than those described by Rindi and De Giuseppe (2). Our results indicate that TTP contents in rat tissues range from 0.10 to 0.25 nmol/g wet weight of the tissue (0.7–1.6% of the total thiamine).

DISCUSSION

Thiamine and its phosphate derivatives in rat tissues could be satisfactorily determined by HPLC after conversion to their corresponding thiochrome derivatives as described previously (1). Complete separation of these derivatives was obtained when a small volume (5 µl) of the oxidized sample was applied, but ThcTP was not observed due to its small quantity (Fig. 1a). A large volume of the sample (40 µl) should be therefore injected onto the column in order to analyze Thc and ThcTP (Fig. 1b). Under this condition the fluorescence peak corresponding to
the Thc area appeared to be divided into two peaks (Fig. 1b) and the second peak from the right was derived from the blank, which is non-thiochrome fluorescent substance(s) in the sample. The blank fluorescences corresponding to the retention times of Thc, ThcMP, ThcPP and ThcTP, respectively, were therefore subtracted from those obtained with the sample extract.

The data shown in Table 1 indicate that thiamine and its phosphate derivatives in rat tissues could be estimated without large variations and the total thiamine contents shown in Table 1 are similar to those reported previously (2), though not identical. The TTP contents listed in Table 1 are, however, lower than those of Rindi and De Giuseppe (2), who showed the following TTP contents in nmol/g wet weight (%): brain, 0.38 (5.0); liver, 1.82 (9.0); heart, 1.13 (5.6); and kidney, 0.54 (5.2). Since in our experiments at least 85% of the ThcTP added to the sample extracts was recovered after HPLC (Figs. 1c and 2b), the results we obtained appear to be fairly reliable. One problem in the determination of TTP in tissues is that approximately 20% of the samples analyzed did not show TTP peaks under the best conditions, using a freshly prepared column. These undetected samples of TTP were involved in the calculation of the thiamine phosphates contents listed in Table 1. If column conditions were lowered after analysis of many samples, the failure rate of TTP detection from the tissues was increased.

The whole process of HPLC analysis required 1.5 hr, while that of the method of Rindi and De Giuseppe needed about 12 hr (2).

Recently a procedure for separation of thiamine phosphate esters by a Sephadex cation exchanger was reported (6), in which estimation of TMP, TPP and TTP in the rat liver by this method was presented. Although complete separation of these phosphate esters was achieved in about 24 hr, both TPP and TTP contents seem to be 4- to 5-fold higher than those shown by Rindi and De Giuseppe (2) when these contents are roughly calculated from the figure given in the paper (6) (TPP, 60.2; TTP, 9.21 nmol/g wet weight).

HPLC of thiamine and its phosphate esters was also reported by Gubler and Hemming (8), in which eluates were monitored first by a UV (254 nm) detector and then subsequently monitored by a fluorescence detector after converting thiamine phosphates to thiochrome derivatives. However, separation of these phosphate esters was still incomplete, when compared with the results described in the previous paper (1) as well as in the present paper. This is probably due to a large flow cell volume, as was pointed out by the authors (8).

The results presented in this paper clearly indicate that HPLC of thiamine and its phosphate esters after conversion to the corresponding thiochrome derivatives is quantitatively applicable for analyzing the content of thiamine phosphates in animal tissues and the procedure requires only 90 min for completion.

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