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Comparative studies on the high-performance liquid chromatographic determination of thiamine and its phosphate esters with chloroethylthiamine as an internal standard using pre- and post-column derivatization procedures

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ABSTRACT

Two improved reversed-phase high-performance liquid chromatographic procedures for the rapid separation and sensitive fluorimetric quantification of thiamine and its phosphate esters are presented using pre-column and post-column derivatiziation. Further, for the first time chloroethylthiamine has been introduced into thiamine determination as an internal standard which allows the analytical procedure to be controlled. Complete separation and sensitive detection can be achieved by both methods within 15 min. The pre-column derivatization technique is easier to perform but is sometimes accompanied by technical problems caused by the derivatization reagent. In the post-column derivatization procedure the chromatographic system was not attacked but a chemically inert derivatization pump was essential. Analysis of rat intestinal tissue by both methods including a simplified extraction scheme yielded the same results, indicating that the techniques are interchangeable.

INTRODUCTION

Early methods for the analysis of thiamine derivatives such as animal experiments [1] or microbiological assays [2,3] were based on the biological function of the vitamin. These tests are often sensitive but time-consuming, expensive and difficult to standardize and therefore sometimes less reliable. Further, they are not specific for single derivatives and therefore do not allow differentiation between the different forms of thiamine which is necessary in metabolic studies.

The most common procedure in thiamine analysis is the alkaline oxidation of thiamine and its phosphate esters to highly fluorescent thiochromes [4]. This reaction has frequently been combined with conventional column chromatography [5–7] as well as with high-performance liquid chromatography (HPLC) [8–25] to separate the thiochromes from other fluorescent compounds present in the sample.

Some of the HPLC procedures described are suitable for determining thiamine only [8–14], *i.e.* the phosphate esters must be hydrolyzed prior to chromatography.

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Thus they do not allow different derivatives to be quantified. Of the systems capable of separating all thiamine derivatives, some have only been applied to standard solutions [15–17]. Therefore their suitability for the analysis of biological materials is not proven.

Moreover, only one successful attempt to integrate an internal standard has been described using amprolium [25], although several thiamine derivatives such as chloroethylthiamine might be suitable for this purpose.

The present work was aimed at establishing two rapid HPLC procedures for the quantification of thiamine and its phosphate esters as well as chloroethylthiamine as an internal standard. The systems are based either on chromatography of the intact thiamines and post-column derivatization to thiochromes or on pre-column oxidation of the vitamers to thiochromes with subsequent chromatography of the latter. A simplified scheme for the extraction of thiamine compounds from biological materials will be shown. Both methods were applied to rat intestinal tissue.

EXPERIMENTAL

Chemicals

Thiamine-chloride-hydrochloride (T), thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) were purchased from Sigma (Deisenhofen, Germany), thiamine triphosphate (TTP) from Wako (Osaka, Japan) and 5-chloroethylthiamine (CET) was provided by courtesy of Dr. Saeki (Kyoto, Japan). All substances were diluted in 0.01 *N* hydrochloric acid and kept at −80°C. All other chemicals and solvents were obtained from Merck (Darmstadt, Germany) and were of the highest purity available. Water was purified with a Millipore Q system (Waters, Eschborn, Germany).

Chromatography

Separations were carried out on a Merck–Hitachi HPLC system consisting of an L-5000 gradient former, a 655A-11 solvent metering pump, an F-1000 fluorescence detector, a D-2000 integrator and a loop injector (Rheodyne, Model 7125) with a 20-μl syringe. All analyses were performed at room temperature. Excitation and emission maxima of the different thiochromes were determined by recording fluorescence spectra during the analysis using the stop-flow technique. Quantification was performed on the basis of peak area. Calibration graphs were constructed for each compound by plotting the peak area *versus* the amount injected, and calculations were made by least-square regression analysis.

The analytical columns were filled by the upward slurry technique [26] using 2-propanol for preparing the slurry. In both methods the analytical columns were connected with a C-135 B pre-column kit (Upchurch Scientific, Oak Harbour, WA, USA) containing dry-packed Shandon ODS Hypersil (10 μm). All the solvents were freshly prepared on the day of use, filtered through 0.45-μm filters (Schleicher & Schüll, Dassel, Germany) and degassed ultrasonically under vacuum.

Pre-column derivatization technique

Oxidation of thiamine and its derivatives to fluorescent thiochromes was performed with the help of a 0.1% solution of potassium ferricyanide in 15% sodium

hydroxide. The solution was freshly prepared from an aqueous stock solution (1%) of potassium ferricyanide every 2 h. For derivatization 80 μl of the samples were mixed with 50 μl of the reagent. A 20-μl sample of this solution was injected exactly 1 min after adding the reagent.

Thiochrome derivatives were separated on a 5-μm ODS Hypersil (Shandon) analytical column (250 × 4.6 mm I.D.). The mobile phase consisted of a gradient of 25 m*M* potassium hydrogenphosphate and methanol (Table Ia), which was pumped at a flow-rate of 1.2 ml/min. The pH of the buffer was adjusted to 8.4 with orthophosphoric acid. Fluorescence was recorded at excitation and emission wavelengths of 365 and 450 nm, respectively.

TABLE I

GRADIENT COMPOSITION DURING THE HPLC ANALYSIS OF THIAMINE DERIVATIVES APPLYING PRE- OR POST-COLUMN DERIVATIZATION PROCEDURES

(a) Pre-column derivatization technique				(b) Post-column derivatization technique				
Component[a]	time (min)			Component[b]	time (min)			
	0	6	11		0	2	5	8
A (%)	85	50	50	C (%)	100	100	0	0
B (%)	15	50	50	D (%)	0	0	100	100

[a] A = 25 m*M* potassium phosphate (pH 8.4); B = methanol.
[b] C = 50 m*M* sodium citrate (pH 4.0)–5 m*M* sodium hexane sulfate–5% methanol; D = 50 m*M* sodium citrate (pH 4.0)–5 m*M* sodium hexane sulfate–50% methanol.

Post-column derivatization technique

Thiamine and its derivatives were separated on a 3-μm ODS Hypersil (Shandon) column (125 × 4.6 mm I.D.) with a gradient of methanol and 50 m*M* sodium dihydrogenphosphate (Table Ib) whose pH had been adjusted to 4.0 with orthophosphoric acid. The flow-rate was 0.8 ml/min.

The derivatization reagent (0.02% postassium ferricyanide in 10% sodium hydroxide) was pumped into the eluent stream leaving the column through a T-junction with a laboratory-constructed perfusor pump at a flow-rate of 0.4 ml/min. The solution was replaced every 2 h. After a reaction zone consisting of a 2-m PTFE tube (0.8 mm I.D.), three-dimensionally coiled to minimize peak broadening [27], the fluorescence of the resulting thiochromes was measured at the wavelengths mentioned above.

Extraction procedure

To extract thiamine and its derivatives from biological materials, tissue was removed from the animals and immediately frozen in liquid nitrogen. About 1 g of tissue was homogenized in a glass–PTFE homogenizer in ice-cold 10% trichloroacetic acid (TCA) at 4°C, after 10 nmol of CET had been added. The homogenate was

brought to an end volume of 3 ml and was centrifuged for 20 min at 10 000 *g*. The resulting pellet was re-extracted with 10% TCA twice more, and the combined supernatants were brought to a pH of 4.5 by adding solid sodium acetate. This solution was either directly injected (post-column derivatization method) or used for derivatization (pre-column derivatization technique).

Extraction efficiency was determined by an endogenous labeling technique: Rat intestinal tissue was incubated *in vivo* for 1 h using the "ligated loop" technique [28] with 1 ml of Krebs' bicarbonate solution containing 5 m*M* glucose and 2 μM (thiazole-2-[^{14}C])thiamine-chloride-hydrochloride ([^{14}C]T, specific activity 0.85 GBq/mmol, Amersham, Braunschweig, Germany). The radiochemical purity of thiamine was examined by HPLC and found to be 98%. When incubations were terminated the segments were removed from the animals and extracted as described above. The radioactivity in the supernatant was measured by liquid scintillation counting and compared with that in the untreated homogenate. Pretests revealed that no measurable interconversion or loss of different thiamine derivatives resulted from the TCA treatment.

Application of the method to biological materials

The thiamine content of rat intestinal tissue was determined by both methods. Quantification was performed using an external standard graph and results were corrected for the incomplete extraction as determined in extraction efficiency studies.

RESULTS AND DISCUSSION

Pre-column derivatization technique

Fig. 1A shows a typical chromatogram of thiochromes using pre-column conversion of the thiamine derivatives. All substances were completely separated within 14 min and the next sample could be injected after approximately 15 min as the initial elution conditions can be restored at 11 min, *i.e.* before the analysis is terminated. Table IIa gives the quantitative chromatographic parameters for thiamine analysis by reversed-phase HPLC after pre-column conversion of the analyte. Only a few of the various methods for thiamine analysis using pre-column derivatization are capable of separating all vitamers [17–19,22], which is essential for metabolic studies. One of these methods was only used to separate standard solutions [17].

Although the chromatographic system described here is the first pre-column technique to integrate an internal standard, the separation is as fast as in other pre-column techniques capable only of separating natural thiamine derivatives [17,22]. In one case applying post-column derivatization, amprolium was used as an internal standard [25]. We prefer to use CET which has a greater structural similarity to thiamine and its derivatives than amprolium. We propose introducing an internal standard in thiamine analysis when the vitamers are quantified by the thiochrome reaction. Pretests revealed that the pre-column derivatization may produce varying results if it is not exactly standardized.

In contrast to other authors [22] who have reported that derivatized samples can be stored in the dark for three days, we observed that reproducibility decreased even 1 h after adding the derivatization solution. Therefore oxidation was performed just before HPLC analysis, taking into account the fact that complete oxidation is achieved within 15 s [10].

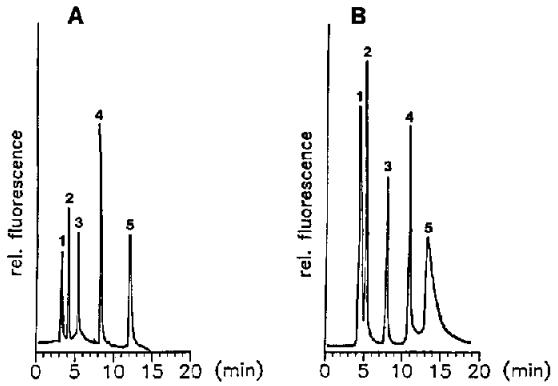

Fig. 1. Chromatographic separation of thiochromes and thiamine derivatives. (A) Separation of thiochromes after pre-column conversion of different thiamine derivatives. Conditions: column, Shandon Hypersil ODS (5 μm, 250 × 4.6 mm I.D.); mobile phase, gradient (Table Ia) consisting of 25 m*M* potassium phosphate (pH 8.4) and methanol at a flow-rate of 1.2 ml/min; ambient temperature; fluorimetric detection at excitation and emission wavelengths of 365 and 450 nm, respectively; 20–30 pmol of each compound were injected. Peaks: 1 = TTP-thiochrome; 2 = TPP-thiochrome; 3 = TMP-thiochrome; 4 = T-thiochrome; 5 = CET-thiochrome. (B) Separation of intact thiamines and subsequent derivatization to the corresponding thiochrome derivatives by a post-column derivatization arrangement. Conditions: column, Shandon Hypersil ODS (3 μm, 125 × 4.6 mm I.D.); mobile phase, gradient (Table Ib) consisting of 50 m*M* sodium citrate (pH 4.0)–5 m*M* sodium hexane sulfate–5% methanol and 50 m*M* sodium citrate (pH 4.0)–5 m*M* sodium hexane sulfate–50% methanol at a flow-rate of 0.8 ml/min; derivatization reagent (0.02% potassium ferricyanide in 10% sodium hydroxide) was pumped into the eluent stream leaving the column at a flow-rate of 0.4 ml/min; detection and peaks as described under (A).

TABLE II

QUANTITATIVE CHROMATOGRAPHIC PARAMETERS FOR HPLC ANALYSIS OF THIAMINE DERIVATIVES

Compound	Retention time (min)	Range of linearity (pmol)	Detection limit (pmol)	Standard curve parameters[a]			Precision[b]
				a	*b*	*r*	
(a) After pre-column derivatization procedure to the corresponding thiochromes							
TTP	2.85	0.5 – 200	0.1	21.3	−11.3	0.994	6.8
TPP	4.11	0.2 – 200	0.05	23.6	−10.8	0.995	4.2
TMP	5.20	0.2 – 200	0.05	27.6	−19.8	0.996	5.9
T	9.01	0.1 – 200	0.02	59.6	−19.8	0.998	2.7
CET	12.3	0.1 – 200	0.02	6.1	5.3	0.993	3.3
(b) After separation of intact thiamines and subsequent derivatization to the corresponding thiochrome derivatives							
TTP	3.99	0.4 – 200	0.1	24.4	−58.0	0.996	5.4
TPP	4.95	0.2 – 200	0.05	36.0	−33.1	0.998	5.5
TMP	6.33	0.2 – 200	0.05	35.1	−42.1	0.999	3.5
T	10.64	0.1 – 200	0.03	44.6	16.6	0.998	3.4
CET	13.54	0.6 – 200	0.1	15.2	12.6	0.994	2.6

[a] Parameters were determined using the equation $y = ax + b$, with x = pmol injected and y = peak area.
[b] Precision is the percentage deviation of the mean as it has been obtained by repeated analysis of the same sample ten times; sample contained 62.5 pmol per injection of each compound.

Nevertheless, the addition of a constant amount of an internal standard like CET to each sample makes it possible to control the reproducibility of the analytical procedure and allows possible deviations within the derivatization step to be corrected. One group analyzing thiamine with a post-column derivatization technique reported the use of salicylamide as an internal standard [10], but it is evident that this substance, which is structurally dissimilar to the thiamine molecule, is not suitable for this purpose.

As Table IIa indicates, the pre-column technique is highly reproducible and enables us to detect subpicomolar quantities of thiamine and its derivatives. The detection limits, which were determined at a signal-to-noise ratio of 3:1, are in the same range as those observed by other authors [20–24].

A disadvantage of the pre-column derivatization technique is the fact that aggressive solutions have to be applied to the chromatographic system. We noted that parts of the loop's injector were rapidly destroyed. This can only be prevented by using special, *i.e.* chemically inert, materials. Moreover, in spite of using a pre-column the analytical column was attacked within several days during routine analysis, leading to worse resolution and peak symmetry. This could be prevented when the system was flushed with distilled water for 5 min after each analysis.

Post-column derivatization technique

As Fig. 1B and Table IIb clearly indicate, chromatographic separation of intact thiamine derivatives on reversed-phase HPLC with post-column conversion of the vitamers to the corresponding thiochromes also allows the fast and sensitive quantification of these substances. Separation was terminated after 16 min and the next sample could be injected immediately because the gradient program was terminated after 8 min and therefore the column was re-equilibrated by the time the analysis was finished. Detection limits were comparable to the pre-column derivatization technique and thus to other methods proposed [20–24]. Concerning the reproducibility of both chromatographic procedures presented here, it should be mentioned that we injected very low quantities of substrate. As can be seen from the results of others [29], reproducibility increases when higher amounts are chromatographed.

Several techniques for the HPLC separation of thiamine with post-column derivatization to thiochromes have been proposed [10–12,15,16,21,23,25], but some authors separated thiamine itself but not its phosphate esters [10–12]. The application of post-column HPLC analysis of thiamine and its derivatives to biological material was shown in two cases only [23,25]. As mentioned, one of these techniques integrated amprolium as an internal standard [25].

In contrast to the pre-column procedure described above, separation of intact thiamine derivatives obviously did not present any technical problems, but delivery of the oxidation reagent by a conventional HPLC system attacked the equipment. Therefore we used a laboratory-constructed perfusor-like pump which was chemically inert and guaranteed a continuous flow without pulsation. Unlike the commercially available derivatization systems this pump was cheap to acquire.

Extraction procedure

As can be seen from Fig. 2, practically no impurities were found in the chromatograms of rat intestine, indicating that extraction is highly specific. This could also

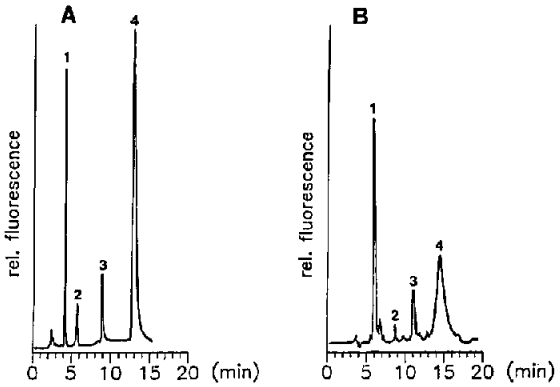

Fig. 2. Typical chromatograms of thiamine derivatives from rat intestinal tissue; separation conditions as in Fig. 1. Peaks: 1 = TPP-thiochrome; 2 = TMP-thiochrome; 3 = T-thiochrome; 4 = CET-thiochrome.

be confirmed by recording fluorescence spectra of the different peaks, which were compared with those of standards and found to be identical. Compared with other extraction methods using TCA with subsequent removal of the acid by extraction with ethyl ether [19,22,24], the procedure described here is much easier and faster to perform.

The extraction efficiency is one important factor responsible for the precision of a method, but it is difficult to determine [30]. We chose an endogenous labeling technique to evaluate the extraction efficiency using intestinal tissue as a model matrix. Chromatographic studies revealed that the relation of radiolabeled thiamine derivatives after incubation (Fig. 3) was identical to the endogenous substrates (Table III), thus indicating that [^{14}C]T had equilibrated with the endogenous metabolites. Extraction efficiency as judged by this technique was 86.4 ± 3.7% (n = 12).

We favor the endogeneous labeling technique over the generally given "recovery rates", which are easier to determine but which involve several problems. For example they do not reflect the efficiency with which protein bonds are cracked because substrates given to tissue homogenate just before the extraction cannot be expected to behave like the endogenous substances. Our results confirm this assumption. For comparison we also determined the recovery rates, which were found to be about 95–100% and thus in the same range as those found by several authors [8,14,23–25], whereas extraction efficiency was lower.

Analysis of rat intestinal tissue

Table III gives the amount of different thiamine metabolites as determined by the two methods, and Fig. 2 shows typical chromatograms. Both procedures led to similar results concerning the total content of thiamine as well as the relation between the different metabolites. Differences were within the deviation of the thiamine contents and not statistically significant. TTP could not be detected in rat intestinal tissue, which is not surprising considering that there is probably no function for this derivative in the gut wall. On the other hand, most of the thiamine was found as TPP,

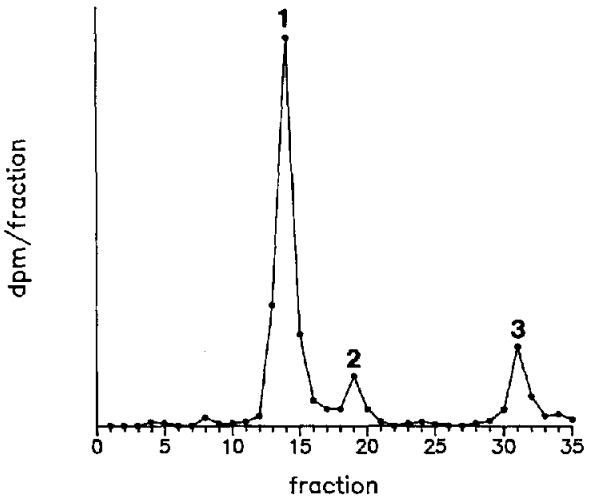

Fig. 3. Separation of radiolabeled thiamine derivatives from "ligated loops" of rat small intestine. Intestinal tissue was incubated with 2 μM [^{14}C]T and extracted as described under Experimental; separation conditions as in Fig. 1B; the solvent stream was collected in fractions of 0.3 ml and assayed by liquid scintillation counting. Peaks were identified by comparison with standard chromatograms. Peaks and percentage relation of total thiamine: 1 = TPP-thiochrome (74.3%); 2 = TMP-thiochrome (7.6%); 3 = T-thiochrome (18.1%).

which is because of its biological role and the mechanism of intestinal absorption of thiamine, which involves rapid conversion to TPP as can ben seen from the incubation experiments (Fig. 3) as well as from the results of others [31,32].

Table IV shows the reproducibility of the overall methods as determined by five-fold analysis of the same samples emphasizing that the technique is easily reproducible. When interpreting these small variations it should not be overlooked that these discrepancies might even be due to an inhomogeneity of the sample itself because five small pieces of gut were analyzed separately.

The results clearly indicate that both methods presented in this paper allow the

TABLE III

THIAMINE CONTENT AND THIAMINE DERIVATIVES OF RAT INTESTINAL TISSUE AS DETERMINED BY PRE-COLUMN DERIVATIZATION AND POST-COLUMN DERIVATIZATION TECHNIQUE, RESPECTIVELY (nmol/g ± S.D.); PERCENTAGE OF TOTAL THIAMINE DERIVATIVES

	T	Percentage	TMP	Percentage	TPP	Percentage	Total
Pre-column[a]	2.31 ± 0.87	16.5	1.25 ± 0.63	8.9	10.46 ± 2.96	74.6	14.02
Post-column[b]	2.05 ± 0.96	16.6	0.79 ± 0.71	6.4	9.52 ± 0.97	77.0	12.36

[a] Pre-column derivatization of thiamine derivatives to the corresponding thiochromes with subsequent separation of these compounds as shown in Fig. 1A and Table Ia.

[b] Separation of intact thiamines with post-column derivatization to thiochromes as shown in Fig. 1B and Table Ib.

TABLE IV

PRECISION OF THE OVERALL METHOD AS DETERMINED BY FIVE-FOLD ANALYSIS OF THE SAME GUT SAMPLE

Extraction and HPLC analysis were carried out with five small intestinal segments obtained from the same rat. Values given are the means ± S.D. (nmol/g tissue)

	T	TMP	TPP
Pre-column[a]	2.15 ± 0.16	1.08 ± 0.09	10.38 ± 1.06
Post-column[b]	2.23 ± 0.13	0.77 ± 0.08	9.66 ± 1.03

[a] Pre-column derivatization of thiamine derivatives to the corresponding thiochromes with subsequent separation of these compounds as shown in Fig. 1A and Table Ia.

[b] Separation of intact thiamines with post-column derivatization to thiochromes as shown in Fig. 1B and Table Ib.

fast separation and quantification of thiamine and its phosphate esters from biological samples. There can be no general answer to the question of which method is preferable, this must be decided individually depending on the purpose and technical possibilities of each laboratory. For routine analysis we propose post-column derivatization with a perfusor-like derivatization pump which is less expensive, whereas determination of single samples is easier to perform by the pre-column technique.

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