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Note

Thiamin analysis and separation of thiamin phosphate esters by high-performance liquid chromatography

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High-performance liquid chromatography (HPLC) has been proposed for the analysis of water-soluble vitamins. Methods for analyzing thiamin in food [1—3], tissues [4—6], urine [7], and commercial vitamin preparations [8, 9] have been published. In general, either prior to [4, 6, 7] or after [1, 5] HPLC, the methods require reacting thiamin and its phosphate esters with alkaline ferricyanide to form a fluorescent thiochrome which is then measured. Although commercial vitamin-rich preparations have been analyzed for thiamin [8, 9] using UV detection, only one report [2] has described thiamin analysis of a food item using UV detection. While UV detection methods are less sensitive than those employing fluorescence detection of thiochrome, they are nevertheless, much simpler in terms of sample preparation and avoid the danger that naturally occurring compounds such as polyphenols will interfere with the oxidation reaction involved in thiochrome formation [10] and lead to spurious results.

Presented here is a rapid, sensitive, and reproducible method of sample preparation and analysis for thiamin in food (breakfast cereal) and in urine specimens. An extension of the method which allows for separating and quantifying the following reagent standards: thiamin (Th), thiamin disulfide (TDS), thiamin monophosphate (TMP), thiamin diphosphate (TDP) and thiamin triphosphate (TTP) in a single run is also presented.

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EXPERIMENTAL

Chemicals

All reagents were of the highest chemical purity available and all reagents were made up in Milli-Q (Millipore, Bedford, MA, U.S.A.) reagent-grade water. Disposable C_{18} reversed-phase columns (3 ml) were obtained from Analytichem International (Harbor City, CA, U.S.A.). Chemical reagents were obtained from the following sources: acetonitrile (LC grade) from Alltech (Los Altos, CA, U.S.A.); ammonium phosphate (Ultrex grade) from J.T. Baker (Phillipsburg, NJ, U.S.A.); Th, TDS, TMP, and TDP from ICN Pharmaceuticals (Plainview, NY, U.S.A.); TTP was a generous gift from Central Research Laboratories of Sankyo (Tokyo, Japan).

Instruments

The liquid chromatograph was a Varian LC 5060 (Palo Alto, CA, U.S.A.) with a 300 × 4.6 mm, weak anion-exchange column (MicroPak AX-5) with a 5-μm diameter particle size and a diamine functional group. Column effluent was monitored at 245 nm using a Varichrom-5 UV detector and recorded on a Varian strip-chart recorder (4.25 mm/min). The liquid chromatograph was connected in series with low-dead-volume stainless-steel tubing to a Hewlett-Packard 8450 spectrophotometer (Palo Alto, CA, U.S.A.) having an 8-μl flow-cell to permit spectrophotometric scanning of peaks as they emerged from the UV detector flow-cell.

Preparation and HPLC of thiamin reagent standards

A freshly prepared aqueous standard solution of thiamin (0.2 m*M*) was prepared and aliquots of this standard were loaded on the HPLC column and eluted at 30°C isocratically with 5 m*M* $NH_4H_2PO_4$ (pH 2.85) pumped at a flow-rate of 0.5 ml/min. Additional aliquots of the thiamin standard solution were carried through the sample clean-up procedure used for urine and crude homogenates of breakfast cereal.

Preparation and clean-up of cereal and urine samples for thiamin analysis

A breakfast cereal was chosen as the representative food and prepared for thiamin analysis in the following way. The cereal was ground to a fine powder in a mortar. A 1-g sample was suspended in 100 ml of 0.1 *N* hydrochloric acid and autoclaved at 121°C for 30 min. The suspension was centrifuged at 14,000 *g* for 15 min at room temperature. Aliquots of the supernatant were applied to disposable C_{18} reversed-phase columns whose particle size was 10 μm. The columns were washed twice with water and twice with methanol. Thiamin was then eluted with a mixture of 5 m*M* $NH_4H_2PO_4$ (pH 2.85)—acetonitrile—phosphoric acid (3.9:1:0.1, v/v/v) and a 10-μl aliquot of the eluent was applied to the HPLC column. A casual sample of human urine was diluted with 9 volumes of water and an aliquot applied to the C_{18} reversed-phase column. The column was then washed and the thiamin eluted and aliquots were loaded on the HPLC column as described for the cereal sample.

HPLC of cereal and urine extracts for thiamin

Thiamin was eluted isocratically from the HPLC column at 30°C with 5 m*M* $NH_4H_2PO_4$ (pH 2.85), flow-rate 0.5 ml/min, and quantitated by monitoring the UV absorption of the eluting peaks at 245 nm. The identity and purity of the eluent peak containing thiamin was confirmed by spectrophotometrically scanning the leading and trailing edges of the thiamin peak. Peak heights of thiamin from the cereal and urine samples were compared with that of the thiamin reagent (0.2 m*M*) taken through the same clean-up and HPLC procedures.

Preparation and HPLC of thiamin ester reagent standards

A freshly prepared aqueous standard solution containing a mixture of the following forms of thiamin: Th (0.2 m*M*), TDS (0.15 m*M*), TMP (0.2 m*M*), TDP (0.3 m*M*), and TTP (0.3 m*M*) was applied to the HPLC column. The various forms of thiamin were separated using the following buffers: buffer A, 870 ml of acetonitrile plus 130 ml of 5 m*M* $NH_4H_2PO_4$ (pH 2.85); buffer B, 5 m*M* $NH_4H_2PO_4$ (pH 2.85); and buffer C, 750 m*M* $NH_4H_2PO_4$ (pH 4.0). The elution program consisted of 100% buffer A for 15 min followed by a gradient of buffer B from 0—100% at 4%/min. This was followed by a gradient of buffer C from 0—100% at 2.5%/min. The flow-rate was 0.4 ml/min for the first 15 min, then was increased to 1.0 ml/min during the next 2 min and remained at this flow-rate throughout the analysis. At these flow-rates the column pressure ranged from 40—70 atm. The column was maintained at 30°C. The chemical identity of the peaks eluted from the HPLC column was confirmed from their characteristic UV spectra.

RESULTS

Thiamin elutes from the HPLC column in 4 min as is shown in Fig. 1. The peak height was reproducible as shown in Table I and was in linear proportion

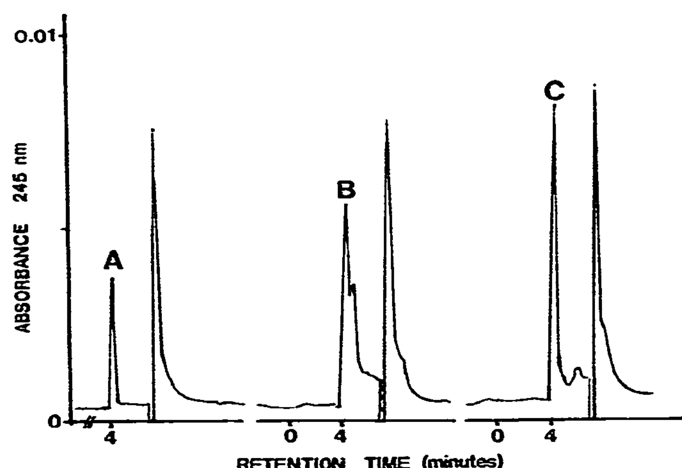

Fig. 1. HPLC of thiamin reagent standard (A), thiamin in breakfast cereal (B) and in urine (C) after clean-up on C_{18} reversed-phase columns. Absorbance range 0.01 with detection at 245 nm. The peak following thiamin was due to the solvent mixture used for eluting the thiamin from C_{18} reversed-phase columns.

TABLE I

REPRODUCIBILITY AND LINEARITY OF PEAK HEIGHT RESPONSE AT THREE CONCENTRATIONS OF REAGENT STANDARD THIAMIN

Run No.	Thiamin concentration (ng per 10 μl)		
	2	3	5
1	5.46*	9.14	12.70
2	4.45	9.40	13.46
3	5.21	9.53	12.19
4	4.45	8.76	12.46
Mean peak height ± S.D.	4.89 ± 0.52	9.21 ± 0.34	12.70 ± 0.55
Coefficient of variation (%)	5.1	3.7	4.3

*Values are peak height in cm.

from 0.5 to 10 ng Th. The HPLC chromatograms for the cereal and urine sample, and also standard thiamin are shown in Fig. 1. Recovery of standard thiamin taken through the cereal and urine sample clean-up procedure was quantitative. The cereal shows a small shoulder peak not completely separated from the thiamin. Other water-soluble vitamins including pyridoxin, riboflavin and niacin coelute with a retention time of 4.8 min. However, these water-soluble vitamins are, for all practical purposes, quantitatively removed from the C_{18} reversed-phase columns with the two water and two methanol washes. Calculation of thiamin in the cereal from the peak height gave a value of 75.5 μg/g cereal compared to the 53.5 μg/g calculated from the information on the cereal carton. The thiamin in urine chromatographed with a sharp peak and was not contaminated with other vitamins. This sample of urine contained 11.4 μg/ml of urine as calculated from the HPLC chromatogram.

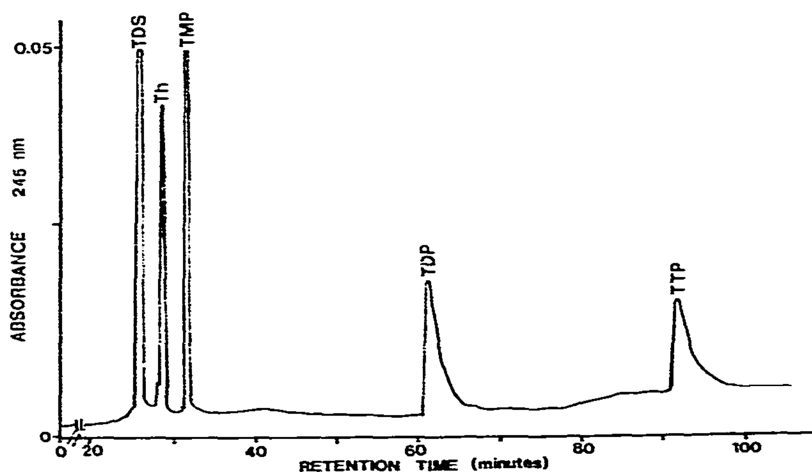

Fig. 2. HPLC chromatogram of TDS, Th, TMP, TDP, and TTP at concentrations of 0.15, 0.2, 0.2, 0.3 and 0.3 mM, respectively. The absorbance range was 0.05 with detection at 245 nm.

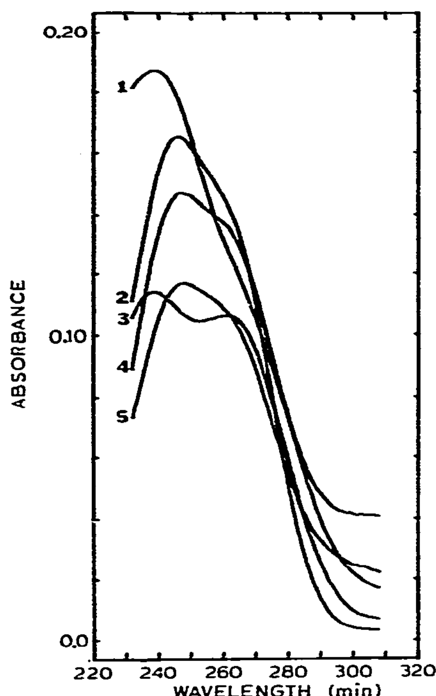

Fig. 3. UV absorption spectra of the various forms of thiamin after HPLC. The absorbance values are relative. Curves: 1 = TDS; 2 = TMP; 3 = Th; 4 = TDP; 5 = TTP.

A HPLC chromatogram showing the separation of Th and TDS from the phosphate esters is shown in Fig. 2. The five compounds were eluted within 100 min. The TDS which does not occur in tissues or blood can be used as an internal standard. The UV spectra for the various forms of thiamin as they came off the HPLC column are shown in Fig. 3. Thiamin has a characteristic UV spectrum with two absorption maxima, one at 245 nm and the other at 265 nm, while the UV spectra of the three phosphate esters are similar with maximum absorption at 248 nm. TDS has one absorption maximum at 245 nm.

DISCUSSION

Previously published methods of thiamin assay by HPLC involve first, the conversion of thiamin to a fluorescent thiochrome compound before or after elution from HPLC and detection with a fluorescent detector. The major advantage of measuring fluorescent thiamin derivatives is the high sensitivity. However, compounds including polyphenols can interfere with the oxidation reaction involved in the thiochrome formation [10]. The advantage of the method described here is that it is fast and simple; it does not require the conversion of thiamin to thiochrome and fluorescence detection; and it is sufficiently sensitive for foods, for urine, and possibly for many common biological tissues. The clean-up procedure is simple and rapid in removing major interfering substances. While no attempt was made in our study to maximise sensitivity, this can be increased by adjusting the absorbance range.

The separation of thiamin and the various phosphate esters along with the practically simultaneous scanning of their absorption spectra in Fig. 3 demonstrate the use of this technique in confirming the identity of the eluted peaks. Since a ternary buffer system was employed for this separation, thiamin elutes at a later time (28 min) than when buffer B was used isocratically (Fig. 1). Another advantage of the described method is that it can be upgraded to separate Th, TDS, TMP, TDP, and TTP in a single run. This capability is extremely useful for kinetic studies on various forms of thiamin. With further modification the method could be adapted for use in the analysis of tissues for these thiamin compounds.

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