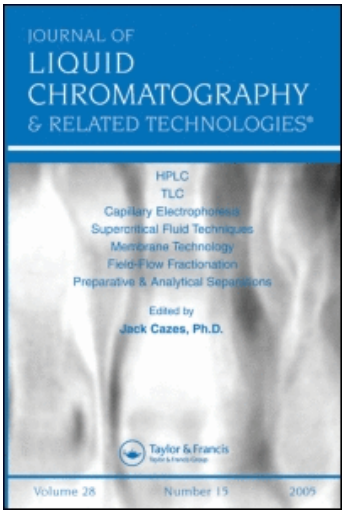

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Separation of Thiamin, Thiamin Antagonists and Their Phosphate Esters by High-Performance Liquid Chromatography

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SEPARATION OF THIAMIN, THIAMIN ANTAGONISTS AND THEIR PHOSPHATE ESTERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Methods are presented for the separation of the phosphate esters of thiamin, oxythiamin or pyrithiamin and the separation of thiamin and oxythiamin by high-performance liquid chromatography. Detection by means of a UV monitor and fluorometer with a post-column oxidation system is described which allows for selective detection of thiochrome, pyrichrome and their respective phosphate esters. Anion exchange and ion-pair reverse phase chromatography are the chromatographic modes of separation.

INTRODUCTION

Since the first report on the occurrence of thiamin triphosphate in rat liver by Rossi-Fanelli et al (1), much interest has been generated in this ester of thiamin, as well as the diphosphate ester, the recognized coenzyme form of the vitamin. The triphosphate ester has also been found in yeast cells and higher plants (2). Of the three thiamin phosphate esters more commonly known to occur in biological material, only the diphosphate and triphosphate esters appear to have biological activity, with free thiamin and thiamin monophosphate considered only as precursors or hydrolysis products of the more phosphorylated forms of the vitamin. Various separation methods have been used to resolve and to estimate thiamin and its phosphates in biological materials. Many paper chromagraphic procedures are available (1,3-5) and

can be quantitated, but usually their wide employment has been for qualitative determinations. A few techniques require prior purification steps, as in the case of some electrophoretic methods (6). Several quantitative methods (7-11) use various types of ion-exchange resins in conventional column chromatography. Simultaneous determination of thiamin, oxythiamin and their phosphate esters from rat tissue (12) has been accomplished by taking advantage of the fact that thiamin phosphate esters give the thiochrome reaction, whereas oxythiamin and its esters do not form the highly fluorescent oxidation products, namely thiochrome and its phosphate esters. Separation of thiamine and its esters after conversion to thiochrome has been achieved with conventional column chromatography (13) and more recently by high-performance liquid chromatography (14). High-performance liquid chromatography has been applied in general to only thiamin determinations, particularly in pharmaceutical preparations (15). Gas chromatography of the trimethylsilyl derivatives of various thiamin metabolites has been attempted (16). The silylation and chromatography of thiamin derivatives containing both the pyrimidine and thiazole moieties were unsuccessful. Acylation (17) has also been tested in the search for stable but volatile derivatives of thiamin.

This paper describes a method for the separation of thiamin and oxythiamin by high-performance liquid chromotography. Two methods are described for the separation of thiamin and its individual phosphate esters. The same methods are also applicable to resolving pyrithiamin and its phosphate esters or oxythiamin and its phosphate esters prior to selective fluorometric detection of those compounds which can be oxidized to form thiochrome, pyrichrome or their phosphate esters. The detection system is similar to that of Van De Weerdhof, Wiersum and Reissenueber (18). The methods presented here have been published previously in an abbreviated form (19). These methods are based on different chromatographic modes; namely, anion exchanges and ion-pair reverse phase chromatography. The advantages offered are the versatility, speed, sensitivity and selectivity of detection made possible

in the analysis of samples of biological origin or of complex reaction mixtures and their products. The recovery of the separated compounds prior to conversion to thiochrome is an option offered by these methods over those methods which rely on the separation of thiochrome and its ester.

MATERIALS

The conditions required for phosphate ester separation necessitate a liquid chromatograph capable of linear gradient formation. The high pressure liquid chromatograph used was a Varian model 4200 series equipped with a model 635 series variable wavelength spectrophotometer (cell volume, 8.0 µl). The effluent oxidizing system was connected as the second detector and consisted of a mixing module (Varian Instruments, part No. 02-0015-75-00) and an oxidant reservoir (Varian Instruments, part No. 02-001673-00). The oxidant flow rate was a function of the hydrostatic pressure developed from a nitrogen line which in turn was controlled by a gas regulator gauge (Hoke, Inc., model No. 5124F4B) and a fine metering valve (Nupro Co., part No. SS-25G). The effluent line of the mixing module was connected to a Turner model 111 fluorometer (Turner Associates, Palo Alto, CA). Initially the fluorometer was used with a 600 µl flow cell attachment (Turner Associates), but it was later reduced to 100 µl by replacement of the commercial flow cell with thick-wall glass capillary tubing. The primary filter was a narrow pass filter with a peak at 360 nm for selection of the excitation wavelength. Two secondary filters--a sharp cut filter (passes longer than 415 nm) and a narrow pass filter (peaks at 436 nm)--were used in monitoring the emission light. Detector responses were recorded by a Rikadenki 2 pen strip chart recorder. A Vydac anion exchange column, 500 mm x 2.0 mm (i.d.), particle size 30-40 µm, a strong anion exchanger supplied in the chloride form (Spectra-Physics, Santa Clara, CA), was the column of choice in the anion exchange mode. For reverse phase chromatography a µBondapak C_{18} column, 300 mm x 4.0 mm (i.d.), particle size 10 µm (Waters Associates, Milford, MA) was used. Guard columns, 50 mm x 2.2 mm

(i.d.), packed with Vydac Anion or Vydac Reverse phase material were used with the anion and reverse phase columns, respectively.

ACS spectralanalyzed methanol (Fisher Scientific Co., Fair Lawn, N.J.) was used in preparation of the solvents. Tetrabutylammonium hydroxide (40% aqueous solution) was obtained from Aldrich Chemical Co. (Milwaukee, WS). Thiamin monophosphoric acid chloride HCl (90-95%), thiamin pyrophosphate chloride (cocarboxylase, 98-100%) and thiamin hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). The antagonists of thiamin, namely pyrithiamin (neo) HBr and oxythiamin chloride HCl, were also purchased from Sigma. The ion-pair reagent, 1-heptane-sulfonic acid sodium salt was obtained from Eastman Organic Chemicals (Rochester, NY).

Reagent grade chemicals were used throughout these studies for preparation of the oxidation reagent, synthesis reactions, etc. Deionized distilled water was used in the preparation of the mobile phases. Reagent grade KH_2PO_4 still contained significant amounts of UV absorbing impurities. Much of this contamination in the buffer was removed in a manner approximating that of Shmukler (20). The oxidant for the fluorometric detection system consisted of 0.6 mM $K_3Fe(CN)_6$ in 15% w/v NaOH.

The phosphate esters of thiamin and its antagonists, oxythiamin and pyrithiamin, were synthesized by the method of Matsukawa et al (21) and stored at -20°C under vacuum.

METHODS

The anion exchnage column separation of the thiamine phosphate esters was initially performed using (Solvent A) 0.005 M KH_2PO_4, pH 6.0 and (Solvent B) 0.5 M KH_2PO_4, pH 6.0 to form an increasing linear gradient at 5% B/min from 0% B to 100% B from the time of injection. The flow rate of 1.0 ml/min developed a pressure drop of 600 psi. The nitrogen regulator and fine metering valve were used to maintain a constant pressure of approximately 20 psi on the oxi-

dation line. The fluorometer slit width was set at maximum sensitivity (30χ) with the 600 µl flow cell and at 10χ sensitivity after replacement of the commercial cell with the 100 µl flow cell described earlier. In order to simultaneously record both the spectrophotometer full scale (0.5 absorbance unit) and 100% fluorescence full scale of the fluorometer on the same chart without overlap, the recorder channels were set to 0.1 V and 2 mV, respectively, voltages twice the full signal output of both the spectrophotometer and fluorometer. UV detection was at 254 nm or 280 nm for thiamin or oxythiamin, respectively. Peaks produced from mixtures were identified by comparison with retention times of purified commercial standards.

The responses of the two detectors were tested for linearity over the concentration range of 0.250 µg to 1.000 µg of thiamin. Peak heights and integrator counts were obtained for six replicates at each concentration. Linearity of response was determined by least squares analysis of variance. Thiamin weighed for standards was dried for one hour at 110°C to constant weight.

The reverse phase µBondapak C_{18} column was tested under isocratic conditions described by Wills, et al (15) for the possible resolution of thiamin from its antagonists. These tests were monitored at 280 nm for increased detectability of the oxythiamin.

The same column was also used to resolve the synthesis mixture of thiamin and its phosphate esters. The solvents used contained the ion-pairing agent, the tetrabutylammonium cation. Solvent A consisted of aqueous 0.05 M tetrabutylammonium hydroxide and 1% v/v acetic acid (final pH 4.3). Solvent B was an 80:20 mixture of methanol and solvent A. The mobile phase composition was held constant at 100% solvent A for 10 min after injection of the mixture. The solvent composition was then changed at a rate of 5% B/min.

RESULTS

Anion exchange chromatography resulted in the separation of the phosphate esters of both thiamin and oxythiamin. Corresponding monophosphate, diphosphate

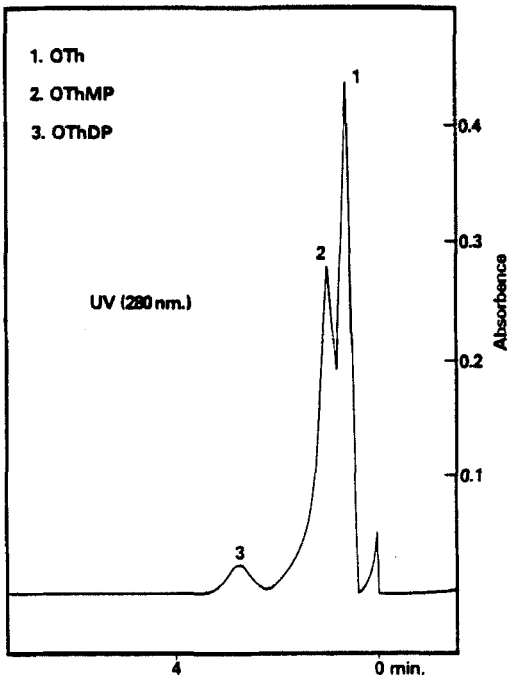

FIGURE 1. Anion exchange chromatography of oxythiamin (OTh), oxythiamin monophosphate (OThMP) and oxythiamin diphosphate (OThDP). Operating conditions: column, Vydac Anion; solvent A, 0.005 M KH_2PO_4 at pH 6.0; solvent B, 0.5 M KH_2PO_4 at pH 6.0; gradient, linear from 0% B at injection to 100% B (5% b/min); flow rate 1.0 ml/min; detection wavelength, 280 nm.

and triphosphate esters of thiamin and oxythiamin were found to elute with identical retention times. Figure 1 shows the separation of the oxythiamin phosphate esters.

A reduction in the concentration of potassium dihydrogen phosphate in the initial solvent from 0.005 M to 0.001 M and a delay in the initiation of the gradient made a substantial improvement in resolution between the monophosphate esters and the diphosphate esters. This improvement is illustrated in the chromatogram produced from a sample of the product of the thiamin triphosphate synthesis reaction (figure 2). This mixture was separated by anion exchange directly after redissolving the crystalline product in water. The product of the thiamin phosphate ester syn-

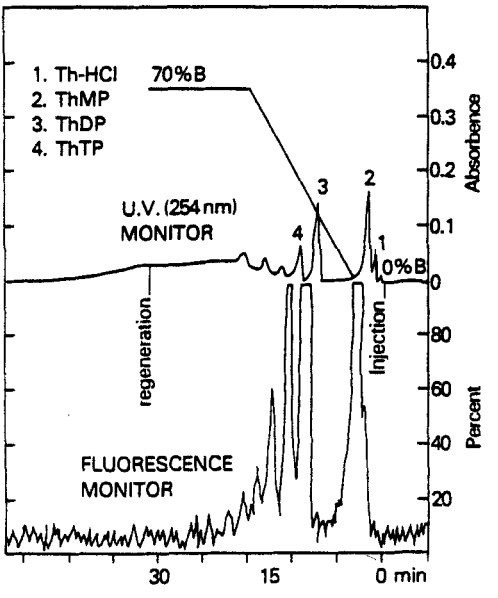

FIGURE 2. UV and fluorescence detection of thiamin HCl (Th-HCl), thiamin monophosphate (ThMP), thiamin diphosphate (ThDP) and thiamin triphosphate (ThTP). Conditions are the same as given in figure 1 with the following exceptions: solvent A, 0.001 M KH_2PO_4; gradient, linear to 70% B after 4.0 min from the time of sample injection; UV wavelenth, 254 nm; Fluorescence detection as described in methods.

thesis reaction is a crude mixture of unreacted thiamin, thiamin monophosphate, thiamin diphosphate, thiamin triphosphate and higher phosphorylated forms, the predominant ester being the diphosphate. A linear gradient was initiated 4 minutes after injection of a 20 µl sample. The rate of change of solvent composition was a 5% increase in B/min from 0% to 70% solvent B. Table 1 gives the chromatographic data for the separation in figure 2.

From previous determinations, the time lapse between detectors was shown to be approximately 1.0 min under the conditions given. Table 1 presents an obvious complication involving the late-eluting peaks observed in the UV trace of figure 2. The last two peaks were detected by the fluorometer within or prior to the 1.0 minute lapse time for flow between the detectors. Applicability of this ion-pair reverse phase chromatographic mode for the

TABLE 1

Chromatographic Data for High Pressure Liquid Chromatography Anion Exchange of a Synthetic Mixture of Thiamin Phosphate Esters*

Compound	UV Retention Time (Min)	Fluorescence Retention Time	Lapse Time
Th-HCl	1.0	2.0	1.0
ThMP	1.8	3.0	1.2
ThDP	8.8	10.0	1.2
ThTP	11.1	12.1	1.0
Th (Tetra-P)	13.6	14.6	1.0
Th (Penta-P)	15.8	16.4	0.6
Th (Hexa-P)	18.9	18.6	-0.3

*These data were obtained from the chromatogram shown in Figure 2. The existence of higher phosphorylated compounds than the possible tetra-phosphate which form a thiochrome species appear doubtful.

separation of B vitamins was shown by Wills, et al (15). Figure 3 shows that the method also separates thiamin from oxythiamin. The phosphorylated species elute at the void volume with the exception of thiamin monophosphate which is only slightly retained in this system. Pyrithiamin is unresolved from thiamin. (not shown).

The reverse phase system was also tested in the separation of the phosphate ester of thiamin by using the tetrabutylammonium action as a counter or pairing ion. A chromatogram produced from a standard mixture of thiamin and two of its esters with this mode of chromatography is presented in figure 4.

The linearity of response for increasing concentrations of thiamin was determined for both the spectrophotometer and fluorometer. The data obtained are given in Table 2. The coefficients of determination for the respective response versus concentration plots have been included in the table.

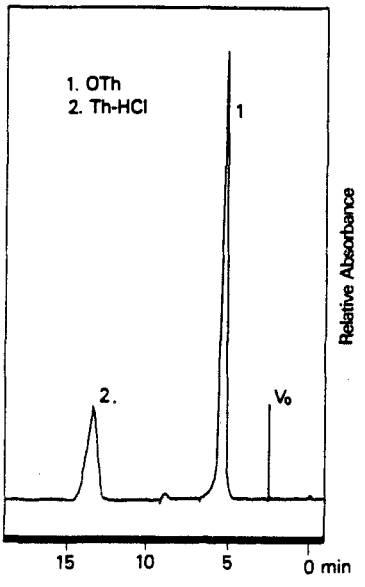

FIGURE 3. Isocratic ion-pair reverse phase chromatography of oxythiamin (OTh) and thiamin HCl (Th-HCl). Operating conditions: column, μBondapak C_{18}; mobile phase, 3:1 water/methanol plus 1% acetic acid and 0.001 M heptane-sulfonic acid; flow rate, 2.0 ml/min; pressure, 2500 psi; detection wavelength, 280 nm.

Separation of the compounds of the thiamin triphosphate ester synthesis mixture by the ion-pair reverse phase method is shown in figure 5. This chromatogram was produced prior to the reduction in the volume of the flow cell and graphically illustrates that, while the thiamin, thiamin monophosphate and thiamin diphosphate are resolved in the UV trace (similar to figure 4) the larger flow cell of the fluorometer produces only one larger unresolved fluorescent peak. Thiamin triphosphate and the higher phosphorylated forms of thiamin begin eluting from the column by use of a linear gradient of increasing methanol composition which commences at a constant rate of 5% B/min solvent change after an initial 10 minute delay. Separations of synthesis reaction mixtures of pyrithiamin and oxythiamin phosphate esters produce similar chromatograms and retention times to those shown in figures 4 and 5. Thiamin, pyrithiamin and oxythiamin elute at the void volume (3 min) under these conditions.

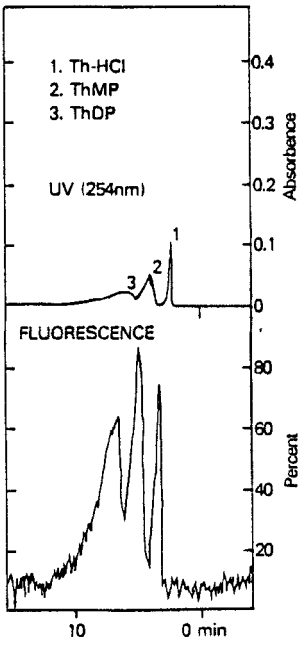

FIGURE 4. Ion-pair reverse phase chromatography of thiamin HCl (Th-HCl), thiamin monophosphate (ThMP) and thiamin diphosphate (ThDP). Operating conditions: column, (μBondapak C_{18}; mobile phase (solvent A), 0.05 M tetrabutylammonium hydroxide and 1% v/v acetic acid (pH 4.3); flow rate, 1.0 ml/min; pressure, .500 psi; detection by UV (254 nm) and the fluorescence system with the 100 μl flow cell at 10χ sensitivity.

DISCUSSION

The inherent sensitivity of fluorescence measurements led us to the construction of the high-performance liquid chromatographic fluorescence monitor and the effluent oxidation system for selective detection of thiamin and its phosphate esters. The conditions for oxidation of thiamin to thiochrome also permit the conversion of pyrithiamin to pyrichrome. Both products of these oxidations are highly fluorescent compounds. Oxythiamin, a second thiamin antagonist, must be monitored by UV detection since it forms no flurorescent compound. This difference allows differential detection of compounds even if they should elute simultaneously.

TABLE 2

Linearity Response Data for Thiamin*

Th Conc (ng)	Integrator Counts(+)	Peak Height UV 254(+) (cm)	Fluorescence Dial Reading(+) (%)	Fluorescence Peak Height(+) (cm)
250	56,282 ± 4652	0.69 ± 0.05	13.4 ± 2.1	1.83 ± 0.19
500	117,697 ± 4648	1.36 ± 0.05	30.7 ± 3.1	3.74 ± 0.46
1000	250,704 ± 5004	2.43 ± 0.12	54.7 ± 2.7	7.05 ± 0.38
Coefficient of Determination	0.9996	0.9966	0.9907	0.9987

* This quantitation was completed under conditions identical to those given in Figure 4.

± Ave. ± S.D. for 6 samples.

For the separation of the thiamin phosphate esters the anion exchange column used provides excellent resolution (figure 2); however, oxythiamin and its phosphate esters do elute with the same retention times as the corresponding esters of thiamin. Such results indicate the absence of significant differential absorption effects due to either the amino or hydroxyl groups of the pyrimidine ring of thiamin or oxythiamin, respectively. The last two peaks produced by the UV monitor shown in figure 2, having retention times of 15.8 and 18.9 min (Table 1), are not the same materials which produce the last two peaks of the fluoresence detector trace. Incompletely removed UV-absorbing buffer contaminants was believed to have been the cause of these spurious peaks. This explains the curious lapse of time between detectors for these late eluting peaks, whereas the late-eluting fluorescent materials are not present in sufficient concentration for detection by the less sensitive UV-monitor. The existance of thiamin

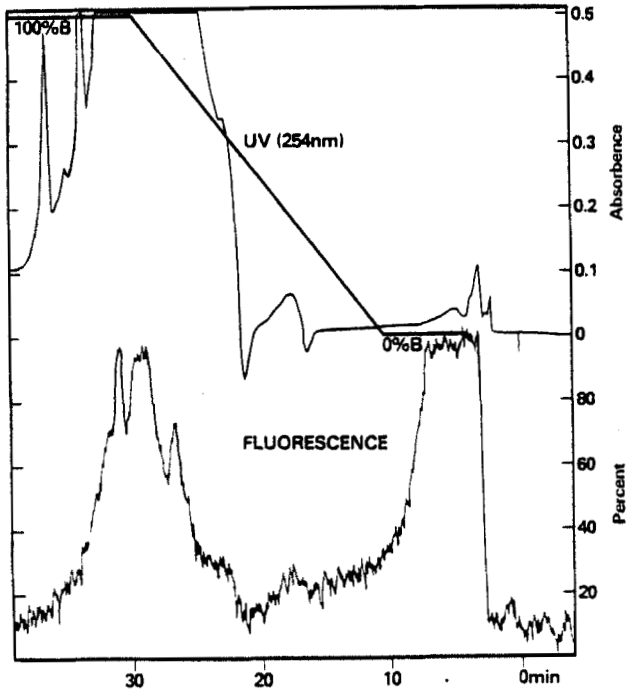

FIGURE 5. Ion-pair reverse phase chromatography of a thiamin triphosphate ester synthesis reaction mixture. Operating conditions are given in figure 4 with the following exceptions: solvent B, 80:20 mixture of methanol/solvent A; gradient, linear at 3.3% B/min to 100% B after 10 min from the time of sample injection; detection by UV (254 nm) and fluorescence system with the 600 µl flow cell at 30χ sensitivity.

pentaphosphate and thiamin hexaphosphate is doubtful, but under the given conditions of the synthesis this reaction cannot be ruled out. Other more highly phosphorylated thiamin compounds than the triphosphate giving rise to peaks may include one in which a phosphoric acid group is attached to the amino group of the pyrimidine moiety.

Ion-pair reverse phase chromatography is applicable to the separation of thiamin-like compounds and their phosphates. Both anionic and cationic pairing agents of counter-ions can be used, dependent upon the separation sought as demonstrated in figures 3 and 4, respectively. The mechanism of retention under the

conditions listed for figure 4 definately involves the phosphate moieties of the parent compounds and the counter-ion, the tetrabutylammonium cation. Thiamin, pyrithiamin, and oxythiamin are not retained under these conditions, nor are any of the phosphorylated compounds retained in the complete absence of the tetrabutylammonium pairing agent, as after a thorough rinsing of the column with methanol. In other works using the same column and solvents (22) the mechanism of retention appears to be predominantly of the ion-exchange type by conversion of the reverse phase column into an anion exchanger by coating the packing material with the ion-pair reagent. The relatively high concentration (0.05m) of pairing agent used would also support this type of mechanism.

The separation of thiamin triphosphate ester synthesis reaction mixture was best accomplished under the conditions given for the anion exchange column (figure 2). In the ion-pair reverse phase mode the separation is possible (figures 4 and 5), but highly phosphorylated forms of the vitamin or its antagonists are strongly retained. Thiamin triphosphate elutes with a retention time of approximately 25 to 30 min (figure 5). It was not determined whether the fluorescent peaks in this region were due to other phosphorylated forms or whether the three peaks represent different counter-ion forms of the triphosphate ester, i.e., thiamin triphosphate paired with one, two, or three alkylammonium counter-ions because of the different ionized forms of the triphosphate moiety. For separation of thiamin triphosphate and other esters the anionic exchange column is recommended.

From the data gathered on the linearity of response for both detectors, it can be seen that the fluorometer dial reading was found to be the least precise method for measurement (Table 2). This corresponds with observations made during the actual chromatography. The electromechanical junction of the particular instrument is sluggish and does not always correlate in magnitude with the electrical response to the recorder. Slow response time for the instrument has been cited by others (23) as a drawback for its application to high-performance liquid chromatography. The need to detect both naturally fluorescent and

selectively derivatized compounds has led to the commercial availability of fluoresence detectors specifically designed for applications as described here. It would therefore be possible with such detectors to greatly enhance the possible qualitative and quantitative aspects of those methods. In regards to the sensitivity of the detector in this study, no less than 50 ng of thiamin hydrochloride was detectable; however, others recently were able to detect 1 pmole of thiochrome, which is equivalent to 0.337 ng of thiamin hydrochloride (14), using a fluorometer equipped with a microflow cell of 16 µl and having better electronic stability.

The methods described here for the separation of thiamin and its antagonists and particularly their respective phosphate esters are a significant improvement over previously existing methods and shold be of significant value in the determination of these compounds in mixtures and in biological material. Separation of thiamin phosphate esters rather than the pre-column oxidation to form thiochrome phosphate esters with subsequent separation is an advantage in studies where isolation and recovery of these compounds is desired. The selectivity afforded by the post-column oxidation system and the sensitivity inherent in the fluorometric detection allows many applications of high-performance liquid chromatography to the analysis of thiamin and other water soluble vitamins. No use of this analytical tool appears to have been made to evaluate the level of thiamin and phosphate esters in tissues, such as liver or blood, in order to assess the status of animals or man with respect to these compounds (24). These methods are very encouraging in regard to this prospective area of investigation.

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