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Ion-pair reversed-phase high-performance liquid chromatographic method for the separation of a set of unphosphorylated thiamine-related compounds

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ABSTRACT

A set of unphosphorylated thiamine-related compounds including thiamine derivatives [thiamine disulphide, D,L-2-(1-hydroxyethyl)thiamine, 0-benxoylthiamine and 2,3,4,5-tetrahydrothiamine], antagonists (oxythiamine, pyrithiamine and amprolium) and thiamine degradation products [4-amino-5-hydroxymethyl-2-methylpyrimidine and 4-methyl-5-(2-hydroxyethyl)thiazole] were successfully separated by ion-pair reversed-phase high-performance liquid chromatography on a standard analytical octadecylsilica column with an aqueous mobile phase containing sodium n-octanesulphonate (0.88%) and trifluoroacetic acid (0.88%) in a carefully profiled gradient of tetrahydrofuran. It is suggested that such chromatographic runs may be useful as a homogeneity criterion for synthetic preparations of thiamine-like substances and for following some thiamine reactions in solutions like, e.g., in alkali.

INTRODUCTION

Recent applications of high-performance liquid chromatography (HPLC) in the analytical biochemistry of thiamine (vitamin B,) were predominantly focused on simultaneous determinations of thiamine and its mono- , di- and triphosphate esters in body fluids [1–4] and various animal tissues [5–9]. Necessary for these purposes, ultrasensitive fluorimetric detection (down to low femtomole levels) is based on either precolumn [1–3,6–13] or postcolumn [4,5,8,14–16] derivatization of thiamine and its esters to the corresponding highly fluorescent thiochromes. Most frequently, the separations of intact thiamine phosphates and thiamine were performed by reverse-phase chromatography including anion pairing by alkyl sulphonates [8,16,17]. Only occasionally have some alternative separation principles been exploited, such as reversed-phase partitioning with cation pairing [17], reversed-phase partitioning with no ion pairing [4], normal-phase partitioning [5] and anion exchange [17].

The ubiquitous natural occurrence and major biological roles of thiamine phosphates has resulted in less attention being paid to analyses for other compounds of structural similarity to thiamine. There are several experimental lines, however, where a biochemist may lack a powerful method suitable for separating unphosphorylated, structural analogues of vitamin B, from its major natural forms. Some compounds with vitamin B_1 activity such as thiamine disulphide [18,19] may be present in various extracts of biological origin. Many thiamine antagonists have been used for metabolic studies [20]. The detection and determination of potential thiamine metabolites are also of primary

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importance [21]. An aim of this work corresponds to applications of thiamine-related compounds as chemical probes of binding-site topography in thiamine transport/storage proteins [22] and perhaps also in some thiamine-consuming enzymes such as thiamine pyrophosphokinase [23] or thiaminase [24]. Preparations of such the chemical probes should be ultrapure as any traces of native ligand can lead to false determinations of affinity to a binding protein. HPLC can be given consideration in order to provide necessary, powerful criteria for probe homogeneity.

Organic compounds of structural similarity to unphosphorylated thiamine have only occasionally been the subject of HPLC separations. Two reports were published on the successful separation of oxythiamine from thiamine in some ion-pair reversed-phase systems in which pyrithiamine, another thiamine antagonist, co-eluted with the vitamin, however [17,25]. A third well established vitamin B_1 antagonist, amprolium, was used as an internal standard in thiamine determinations [16] and more recently chloroethylthiamine found a similar application [8].

In this paper we present an ion-pair reversed-phase HPLC method optimized for the separation and simultaneous determination of a range of unphosphorylated thiamine-related compounds including major thiamine antagonists, simple thiamine derivatives and some thiamine degradation products.

EXPERIMENTAL

Thiamine-related compounds

Thiamine hydrochloride (T), amprolium hydrochloride (AMPR), pyrithiamine hydrobromide (PYT), thiamine monophosphate chloride (TP) and thiochrome (TC) were purchased from Sigma (St. Louis, MO, USA), thiamine disulphide trihydrate (TS2) from Aldrich (Milwaukee, WI, USA) and 4-methyl-5-(2-hydroxyethyl)thiazole (TH) from Fluka (Buchs, Switzerland). Other thiamine derivatives were synthesized by published methods: 0-benzoylthiamine (BT) by treating T with benzoyl chloride [26], D,L-2-(1-hydroxyethyl)thiamine hydrochloride (NET) by treatment of T with acetaldehyde [26], oxythiamine chloride (OT) from T by refluxing in HCl [26], 4-amino-5-aminomethyl-2-methylpyrimidine dihydrochloride (PYNH2) by acid degradation of TS2 [27], 4-amino-5-aminomethyl-2-methylpyrimidine hydrochloride (PYOH) from PYNH2 by treatment with nitrous acid [28] and 2,3,4,5-tetrahydrothiamine (H4T) by reduction of T with sodium borohydride [29]. The synthesized compounds were identified by their melting points and *W* spectra and their purity was routinely checked by thin-layer chromatography on silica gel 60 precoated plates with pyridine-acetic acid-water (10: 1:40).

Other chemicals

Sodium 1-pentanesulphonate and 1,4-dioxane of spectrophotometric grade were obtained from Aldrich, trifluoroacetic acid (TFA) (HPLC/Spectro grade) from Pierce (Rockford, IL, USA), aluminium-precoated plates (silica gel 60) for thin-layer chromatography and N,N-dimethylformamide (DMF) for UV-spectrophotometry from Fluka, sodium hexane-1-sulphonate (LiChropur) , sodium octane- 1-sulphonate (LiChropur), methanol (LiChrosolv, gradient grade), acetonitrile (LiChrosolv, gradient grade), tetrahydrofuran (THF) (LiChrosolv) and other chemicals (ACS grade) from Merck (Darmstadt, Germany). Water was distilled twice from glass and then further purified with a Milli-Q Plus system (Millipore).

Mobile phases

In the procedure finally adopted, an aqueous solution containing TFA (0.16%) and sodium octanesulphonate (0.16%) was diluted twice by volume with either water to give solvent A or with THF to give solvent B. Details on other solvent systems used are given under Results and Discussion. All mobile phases were filtered through 0.45-μm nylon 66 membranes (Supelco, Gland, Switzerland) and then degassed by bubbling helium through for 10 min in a Pharmacia–LKB (Uppsala, Sweden) solvent conditioner.

Samples

Thiamine-related compounds were dissolved in solvent A to a concentration of 1 mg/ml and

were stored refrigerated. Samples were diluted with solvent A as needed and filtered through 0.45-μm disposable filter units (Supelco).

Chromatography

Separations were performed on a Pharmacia–LKB HPLC system consisting of a Model 2249 gradient pump capable of low-pressure mixing of up to three solvents, a Model 2141 dual-channel variable-wavelength UV-Vis monitor, a Rheodyne Model 7125 manual injector equipped with a 50-μl sample loop, a SuperPac cartridge column with a 5-μm Spherisorb ODS-2 cartridge (250 mm x 4 mm I.D.) and a 5-μm Spherisorb ODS-2 guard cartridge (10 mm x 4 mm I.D.). IBM-PC-AT dedicated software HPLC Manager (ver. 2.01) was used for system control and data acquisition and PE Nelson 2600 (ver. 5.0) evaluation software for peak integration. The chromatography was carried out at ambient temperature and a flow-rate of 1 ml/min with simultaneous monitoring of absorbances at 270 and 360 nm.

RESULTS AND DISCUSSION

Optimization of mobile phase composition for isocratic ion-pair reversed-phase HPLC of test thiamine analogues

This work was aimed at the separation of compounds that are either permanently cationic (because of a quaternary nitrogen of a thiazolium ring) or capable of accepting protons in acidic media (amino groups, nitrogen-containing heterocycles). They were expected to interact with an octadecylsilica (ODS) column if paired with suitable anions such as alkyl sulphonates. Because of the marked structural differences between the substances to be separated, solvent systems capable of resolving thiamine from other water-soluble vitamins rather than from thiamine phosphates were chosen as the starting point. Numerous methods have been published on the chromatographic determination of B vitamins in foods and pharmaceuticals [18,30,31, and references therein] on reversed-phase columns (ODS) in mobile phases based on water-methanol mixtures containing acetic acid or an acidic buffer (acetate, citrate, phosphate) and alkyl sulphonates with 5-8 carbon atoms.

In searching for the optimum method for the separation of tmphosphorylated thiamine analogues, isocratic elution on a Pharmacia-LKB ODS-2 analytical column was first applied with spectrophotometric detection at 270 nm. The test samples contained thiamine-like compounds of closest structural similarity (T, PYT, HET, OT) and additionally TP (for comparative purposes). To optimize the mobile phase composition, we first followed and then markedly extended its variations reported in the literature. The following factors were tested, as shown schematically in Fig. 1: (i) length of hydrocarbon chain of the ion-pairing reagent; (ii) concentration of alkyl sulphonate in the mobile phase; (iii) nature of organic solvent (methanol, acetonitrile, DMF–methanol, 1,4-dioxane, THF); (iv) organic solvent concentration; and (v) substitution of TFA for acetic acid in the mobile phase.

Finally, the optimum separations of thiamine analogues were obtained with a mobile phase containing a subtle balance of TFA (0.06-0.08%), n-octanesulphonate (0.08%) and THF (10–12%). A representative chromatogram is shown in Fig. 2. Using this chromatographic system, PYT and T could be completely separated for the first time by an ion-pair reversed-phase HPLC method.

Recommended gradient elution method for ion-pair reversed-phase HPLC of a series of thiamine-related compounds

When the samples contained some thiamine analogues that interact strongly with the ODS phase (TS2, AMPR, BT), a gradient of organic solvent in the mobile phase had to be applied for their elution. The gradient elution mode had. additional positive effects on the overall separations as most peaks were sharpened and any tailing was markedly reduced.

The method finally adopted that meets the aim of work presented here is based on an aqueous mobile phase containing 0.08% of TFA and 0.08% of n-octanesulphonate and a biphasic gradient of THF. As shown in Fig. 3, twelve unphosphorylated thiamine-related compounds with very different structures, including thiamine

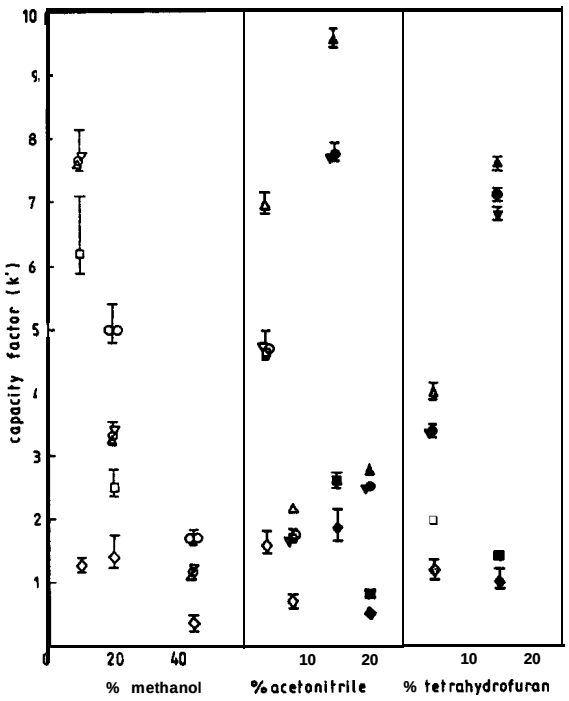

Fig. 1. Schematic representation of the elution behaviour of thiamine derivatives in isocratic ion-pair reversed-phase HPLC on an LKB ODS analytical column. Separations were performed at a flow-rate of 1 **ml/min**, with an aqueous mobile phase containing (i) an ion-pairing reagent: 0.1% of n-pentanesulphonate (white symbols), 0.05% of ***n*-hexane**-sulphonate (shaded symbols) or 0.05% of n-octanesulphonate (black symbols) and (ii) an acid: either 1% acetic acid (when methanol was used) or 0.1% TFA. ◇,◆ = TP; ❑ ,W = OT; ▽,▼ = **PYT**; ○,● = T; △,▲ = HET; ○○,●● = TS2. Capacity factors *[k' = (t, − t0)/t0*, where t_R and t_0 are retention times of an analyte and of an **unretained** substance, respectively] are plotted together with peak half-widths, represented by bars.

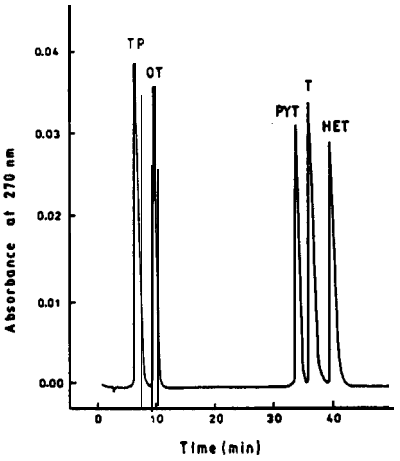

Fig. 2. Representative chromatogram of **optimized** isocratic ion-pair reversed-phase HPLC of some thiamine derivatives. The separation was performed on the analytical ODS column with an aqueous mobile phase containing 0.08% of ***n*-oc**-tanesulphonate, 0.08% of TFA and 12% **(v/v)** of THF at a flow-rate of 1 ml/mm, with spectrophotometric detection. About 2 **μg** of each compound were applied in a total sample volume of 50 **μl**.

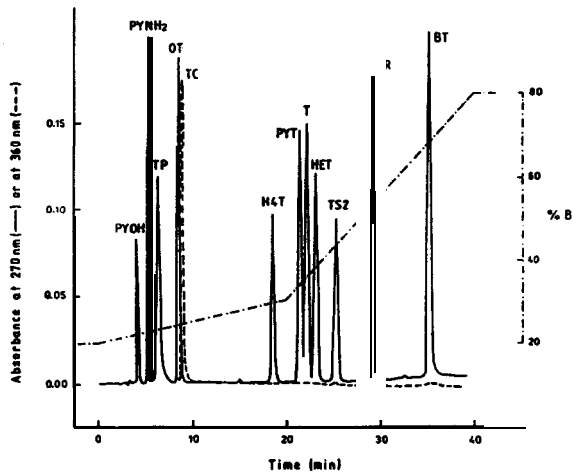

Fig. 3. Separation of a series of thiamine-related compounds by the recommended gradient-elution ion-pair **reversed**-phase HPLC method. A **50-μl** sample containing 2.5 **μg** of each compound was applied to the analytical ODS column and separated in a binary gradient formed between solvents containing 0.08% of n-octanesulphonate and 0.08% of **TFA** either in water (solvent A) or in 50% (v/v) THF (solvent B). Chromatography was performed at ambient temperature and a flow-rate of 1 **ml/min** with simultaneous detection at 270 and 360 mn. The gradient profile shown was formed at proportioning valves before entering the pump. The solvent delay between the proportioning valves and the injector was about 1.5 min.

antagonists, derivatives and degradation products, were separated in a single run within 40 min. Additional chromatographic parameters are given in Table I. As can be seen, a reasonable compromise between the chromatographic behaviour of very different compounds was obtained. The peaks are acceptably sharp and usually do not tail, except perhaps for TP (and TH, not listed). For most pairs of closest neighbours on the chromatogram, complete separation is observed. The overlapping of OT and TC does not matter on an analytical scale as the

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR ION-PAIR REVERSED-PHASE HPLC METHOD RECOMMENDED FOR THE SEPARATION OF THIAMINE-RELATED COMPOUNDS

Separation conditions as described in Fig. 3. Each parameter is given as a mean of three independent runs. Capacity factor: $k' = (t_R - t_0)/t_0$, where t_R = retention time and t_0 = retention time for unretained compounds (t_0 = 2.5 min). $N_{1/2} = 5.53(t_R/W_{1/2})^2$, $N_{4.4\%} = 25(t_R/W_{4.4\%})^2$, where $W_{1/2}$ and $W_{4.4\%}$ *are the* peak widths at half or 4.4% of the peak height, respectively. Resolution factor: $R_s = (t_{R_1} - t_{R_2})/[W_{1/2(1)} + W_{1/2(2)}]$.

Compound	k'	$N_{1/2}$ ($\times 10^{-3}$)	$N_{4.4\%}$ ($\times 10^{-3}$)	R_s
PYOH	0.52	4.73	4.95	*2.8*
PYNH2	0.90	2.74	*3.10*	1.5
TP	1.21	1.66	1.09	3.5
OT	1.98	5.75	5.06	0.6
TC	2.10	3.59	3.14	16
H4T	6.05	18.3	16.3	4.5
PYT	7.29	17.7	17.2	0.9
T	7.54	22.2	20.4	2.0
HET	8.02	27.5	25.7	3.0
TS2	8.92	17.5	21.2	5.4
AMPR	10.5	63.7	72.0	11
BT	13.3	44.0	39.3	

latter compound is selectively detected (at 360 nm). For better separation of PYT and T, the isocratic mode is suitable, as shown in Fig. 2.

Simultaneous determination of unphosphorylated thiamine-related compounds by ion-pair reversed-phase HPLC

This optimized HPLC method for the separation of unphosphorylated thiamine-related compounds could easily be upgraded to a quantitative procedure. Simultaneous determination of all the compounds tested could be performed on the basis of peak areas. The analytical parameters of the method are given in Table II. Calibration graphs (dose–response) were constructed for each thiamine-related compound separately, in the range 0.01-2.5 μg per injection. Within this range, excellent linearity of the detector response was observed (correlation coefficients always >0.995) and the reproducibility was good (relative standard deviations <6%).

Detection limits were determined by sequential dilution of a standard mixture (Fig. 3) of thiamine-related compounds (2.5 μg each). Slight variation of this parameter was observed, from 1.1 ng for BT to 5.4 ng for PYOH. Obviously, for mixtures of T and PYT, the detection limits are much worse if one compound is in a large excess relative to the other. Such mixtures, however, can easily be analysed by switching to the isocratic mode (Fig. 2).

Application 1: detection and determination of impurities in preparations of synthetic thiamine analogues

The gradient-elution HPLC method introduced here is capable of resolving a number of thiamine-related compounds of very diverse structures. Hence we feel justified in recommending chromatographic runs such as that presented in Fig. 3 to provide a serious homogeneity criterion for synthetic preparations of

TABLE II

ANALYTICAL PARAMETERS OF THE ION-PAIR REVERSED-PHASE HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF THIAMINE-RELATED COMPOUNDS

Compound	Calibration graph[a]		Detection limit[b] (ng)	Relative standard deviation[c] (%)
	Slope (10^6 μV s μg^{-1})	Intercept (10^3 μV s)		
PYOH	0.61	3.24	4.9	2.1
PYNH2	1.29	-4.01	2.5	0.9
TP	1.01	-4.95	2.6	4.2
OT	1.19	0.06	2.3	0.8
TC	1.42	14.2	1.8	1.2
H4T	0.93	-12.5	5.0	3.0
PYT	1.18	-6.87	1.9	3.1
T	1.29	-12.4.	1.7	0.9
HET	1.10	8.62	2.8	2.5
TS2	0.97	-0.70	2.4	1.0
AMPR	1.77	-11.0	1.4	0.7
BT	2.01	3.71	1.1	1.1

[a] Determined in the range 0.01-2.5 μg of a compound per injection; peak areas (μV s) were plotted *versus* amount of compound injected (μg); calibration graphs fitted by linear regression.
[b] Injection resulting in a peak three times higher than the noise.
[c] Peak area measurements, repeated five times, for 100 ng of a compound per injection.

this class. Impurities can be detected and, if identified, can be directly determined. Taking into account the detection limits in Table II, one can calculate that as little as 0.1% of potential impurities (of "thiamine-related character") can in principle be determined by HPLC of microgram amounts of a tested compound.

Two examples from our own synthetic practice are presented in Fig. 4.

Application 2: monitoring of thiamine degradation in alkaline media

Since early work by Zima [27], processes of thiamine destruction in alkaline solutions have been studied by many workers and have become representative of the chemical properties of vitamin B_1. Various products and in variable yields are formed depending on pH, temperature, nature of alkalinizing compound and the presence of organic solvents and oxidizing reagents *etc.* [32–34]. We performed preliminary studies to test whether the chromatographic method introduced here could be suitable for following this complex model reaction. A relatively sharp gradient of 1,4-dioxane was applied for this purpose, to shorten the separation time and to demonstrate some flexibility of the chromatographic methods introduced here with respect to gradient profile and nature of the organic solvent. Relatively mild degradation media, with no oxidizing agents except dissolved oxygen, were studied. The results are summarized in Fig. 5.

Of potentially expected degradation products [27,32,33], four (PYOH, TH, TS2 and TC) were available as standards. Three of them (PYOH, TH and TS2) were identified in some reaction mixtures in various yields. Two additional compounds were detected although their proper identification was not attempted. That labelled 3 in Fig. 5 absorbed at 360 nm and not at 270 nm and was eluted in a distorted band. The other,

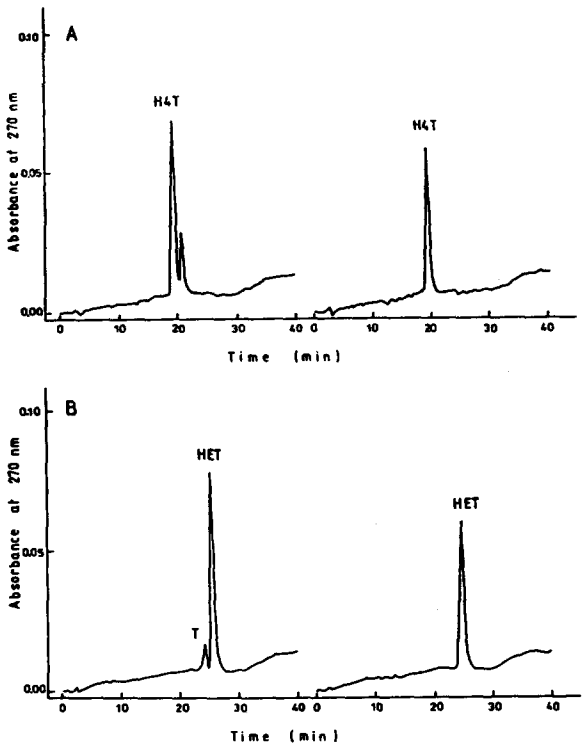

Fig. 4. The recommended gradient-elution ion-pair reversed-phase HPLC method as a homogeneity criterion for selected synthesized preparations of (A) H4T and (B) HET. Left, crude preparations, recrystallized twice; right, preparations after further careful purifications. The impurity in H4T was not identified; that in HET seemed to be T (*ca.* 7%) and could not be easily eliminated.

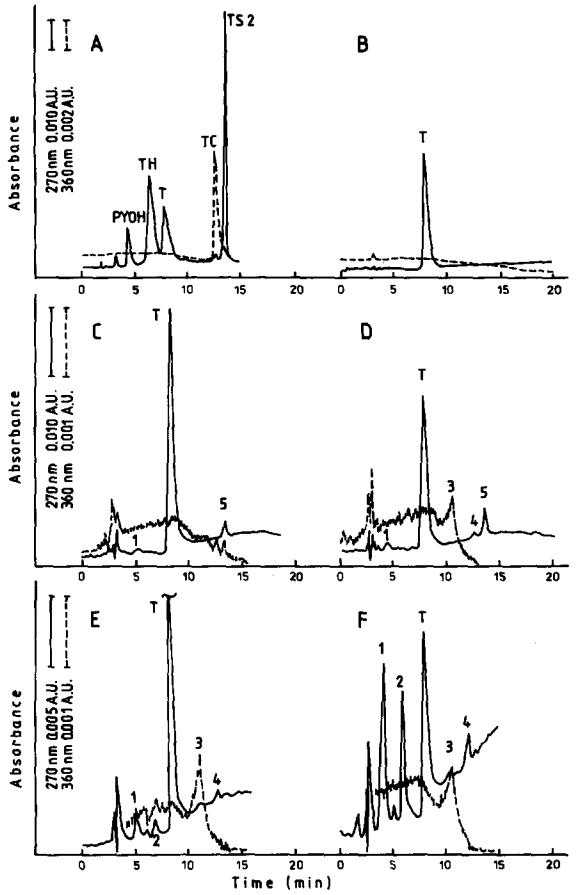

Fig. 5. Degradation of T in mild alkaline media as monitored by ion-pair reversed-phase HPLC. T (0.2 m*M*) was incubated: (B) in 0.1 *M* NaOH at 20°C for 0 min and (C) for 1 h, (D) in 2% Na_2CO_3 at 50°C for 2 h and (E) in 0.1 *M* NaOH at 100°C for 5 min and (F) for 15 min. Samples of 20 μl were applied to the analytical column and separated with an aqueous mobile phase containing 0.08% of *n*-octanesulphonate and 0.08% of TFA in a gradient of 1,4-dioxane (2.5% for 5 min and then to 25% in 10 min) at a flow-rate of 1 ml/min. (A) Some standards were separated under the same conditions.

labelled 4, had a slightly shorter retention time than TC and did not absorb at 360 nm. Whether these unidentified products have any relationship to those originating from the yellow and white thiol intermediates [27,32,34] needs further investigation.

CONCLUSIONS

It is suggested that reversed-phase HPLC on an ODS column with an acid–water mobile phase and with alkyl sulphonates serving as ion-pairing reagents may be of universal suitability for the separation of a wide range of unphosphorylated thiamine-related compounds. *n*-Octanesulphonate (0.08%) for ion-pairing, TFA (0.08%) as the acid component and a carefully profiled gradient of THF content in the mobile phase were found to provide the optimum compromise between different chromatographic properties of the chosen test set of compounds, including well known thiamine analogues, antagonists and degradation products. This and similar separation systems are generally recommended for testing the homogeneity of synthetic preparations of thiamine-related substances. The

suitability of chromatographic monitoring of some chemical (and possibly biochemical) reactions of vitamin B_1 has been opened up as a possibility.

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