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Journal of Chromatography B, 757 (2001) 301–308

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Comparison of ion-pair and amide-based column reversed-phase liquid chromatography for the separation of thiamine-related compounds

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Received 1 November 2000; received in revised form 2 February 2001; accepted 7 March 2001

Abstract

Two reversed-phase chromatographic methods for the separation of thiamine and related compounds are compared. The first procedure is based on the ion-pair technique using an octadecylsilica column, while the second uses a new amide-based stationary phase, which avoids the need to form ion-pairs, leading to narrower peaks and a simpler mobile phase. Analyses were performed by gradient elution and a photo-diode array was used for detection. Specificity was demonstrated by the retention characteristics, UV spectra and by comparing the peak purity index with commercial standards. The procedures were applied to the determination of thiamine-related compounds in pharmaceutical preparations and urine. No preliminary sample treatment was required.

Keywords: Amide-based stationary phase; Thiamine; Vitamins

1. Introduction

Thiamine (vitamin B_1) was the first member of the B group to be identified. The B complex includes many compounds of differing chemical structure and biological roles [1]. Thiamine containes a pyrimidine nucleus and a thiazole linked by a methylene bridge. The most closely related compounds are their mono- and pyrophosphate esters. However, nonphosphorylated structural analogues of vitamin B_1, including compounds with vitamin B_1 activity, antagonists, metabolites and compounds of similar organic structure are also of primary importance for biochemical purposes. The stability of thiamine depends on pH, temperature, ionic strength and the presence of ions, the enzyme-linked form being less stable than the free form. Decomposition of the vitamin involves fragmentation into thiazole and pyrimidine derivatives. The daily recommended intake of thiamine is generally 8–15 mg [1], although such an amount may easily exceeded by oral consumption. When the intake is lower than the minimum requirement, thiamine is not excreted in urine, while any excess appears in the urine as thiamine or pyrimidine [1] when the intake is higher.

High-performance liquid chromatography (HPLC) is a separation technique which has important advantages in vitamin analysis [2], although the technique is not exempt of problems, as indicated by Nicolson et al. [3]. Chromatographic procedures for thiamine

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00163-3

determination generally produce results similar to those obtained by the fluorimetric method proposed by the Association of Official Analytical Chemists (AOAC) [4], but are more convenient [5]. Thiamine determination has been reviewed by Kawasaki and Samemori [6]. Generally the reversed-phase technique [7,8] has been used, while the addition of an ion-pair reagent to the mobile phase increases retention [9,10]. The ion-pair reagents used are salts of the pentane-, hexane- or heptanesulfonic acids. During recent years, several papers concerning the determination of thiamine in pharmaceutical products have been published [9–18]. In the analytical biochemical field, such studies focus on the determination of thiamine and/or related compounds in different biological fluids [8,19–28].

In the present study, the separation of thiamine (T), thiamine monophosphate (TP), thiamine pyrophosphate (TPP), oxythiamine (OT), *S*-benzoylthiamine (BT), 4-methyl-5-(2-hydroxyethyl)thiazole (TH) and amprolium (AMPR) is optimised using a photo-diode array detector. A comparison of the reversed-phase ion-pair technique using an octadecylsilica column and a reversed-phase technique without ion-pair formation using a new amide-based stationary phase is presented. The procedure could be applied to the determination of thiamine and related compounds in pharmaceutical products and urine. The separation using the amide-based column is advantageous with respect to other existing methods [9,10] because the peaks are considerable narrower and column-life is longer due to the simplicity of the mobile phase.

2. Experimental

2.1. Apparatus

The liquid chromatography (LC) system consisted of a Shimadzu FCV-10ALvp liquid chromatograph operating at room temperature with a flow-rate of 1 ml/min. The spectrophotometric detector was a photo-diode array Shimadzu SPD-M10Avp operating at three wavelengths of 230 nm for T, TP, TPP, TH and BT; 250 nm for AMPR and 264 nm for OT. The software was Class-LC10 (Shimadzu) and the detector was connected to a SPD-MXA integrator. Aliquots of 50 μl were injected manually using a Model 7125-075 Rheodyne injection valve. Two analytical columns were used to compare the results. For the ion-pair technique, a 15×0.4 cm I.D. column (Supelco) made of stainless steel and packed with Spherisorb ODS-2 with a particle size of 5 μm was used. A Supelco guard column packed with the same stationary phase was also used. For the reversed-phase technique without ion-pair formation, a similar column was packed with RP-AmideC_{16} with a particle size of 5 μm (Supelco). A guard column packed with the same stationary phase was also used.

2.2. Reagents

Acetonitrile (ACN; Romil, Loughborough, UK) and methanol (Riedel-de Haën, Seelze, Germany) were of liquid chromatographic grade. Doubly distilled water and 5% acetic acid (Merck, Darmstadt, Germany) were purified using a Milli-Q system (Millipore, Bedford, MA, USA). The solvents were degassed by purging with helium gas. Stock solutions (200 μg/ml) of T, TP, TPP, OT, BT and AMPR were prepared by dissolving 10 mg of the commercial product (Sigma, St. Louis, MO, USA), without previous purification, in 50 ml of water. The solution of TH was prepared by dilution of 5 μl of the commercial product (Sigma) in 25 ml of water. They were kept in dark bottles at 4°C. Working standard solutions were prepared by dilution with water immediately before use.

2.3. Samples

For analysis of the pharmaceuticals, each commercial sample (the whole capsule or tablet) was dissolved with water and diluted in a volumetric flask. An aliquot was filtered through a 0.45-μm nylon Millipore chromatographic filter, diluted when necessary (depending on the analyte level in the sample) and analysed. To study thiamine recovery from urine, samples were obtained from a volunteer, filtered and analysed as soon as possible.

3. Results and discussion

3.1. Optimising the separation of thiamine-related compounds using the ion-pair technique (IPC)

The ion-pair technique was selected because thiamine-related compounds have an ionic character since they are in cationic form. The stationary phase was ODS (C_{18}), which permitted a greater retention when ion pairs were formed with anions such as alkylsulfonates, and the organic solvent selected was acetonitrile. The type and concentration of the ion-pair reagent to be used was studied. The retention of the different compounds was similar with pentane-, hexane- and octanesulfonate, and so sodium pentanesulfonate was chosen. The decrease of the capacity factors of the thiamine-like compounds with increased pentanesulfonate concentration is shown in Fig. 1A. A 0.1% value was selected. Varying the pH by adding different concentrations of acetic acid alters the retention and Fig. 1B shows the decrease in the k' values when the acid percentage increased. A 1% acetic acid percentage was selected. Finally, Fig. 1C shows the results obtained when the influence of the ACN percentage was studied. As expected, retention decreased as the ACN percentage in the mobile phase was higher. Amprolium and thiamine pyrophosphate were retained in the column.

In summary, isocratic elution of thiamine-like compounds was not possible since the optimal mobile phase for separating TP and TH (6% ACN) did not elute the rest of the compounds. When a higher concentration of organic solvent was selected (30–40% ACN), BT and T were separated, while TP and TH eluted at the void time and TPP and AMPR were retained. Although these last compounds could be eluted with a stronger solvent, the other compounds eluted together at the void time. Consequently, a gradient elution technique was tried in an attempt to achieve good peak resolution and to shorten the total analysis time. The system consisted of two solvents: an aqueous phase (solvent A) containing 1% acetic acid and 0.1% sodium pentanesulfonate and an organic phase (solvent B) containing 1% acetic acid, 0.1% sodium pentanesulfonate and 50% ACN. The gradient was started using a mobile phase A–B (88:12, v/v), which allowed the separation of TP and TH from the void time. Then, several gradients with different ranges and profiles were tried. The optimal gradient selected was the following. First, an initial isocratic step with A–B (88:12, v/v) for 7 min followed by a linear gradient to A–B (40:60, v/v) during 1 min, this mixture being held for 5 min. Finally, the initial conditions were re-established in 1 min and held for 15 min. The flow-rate was 1 ml/min. In these conditions TPP and AMPR gave very tailed peaks. The elution order and the retention characteristics

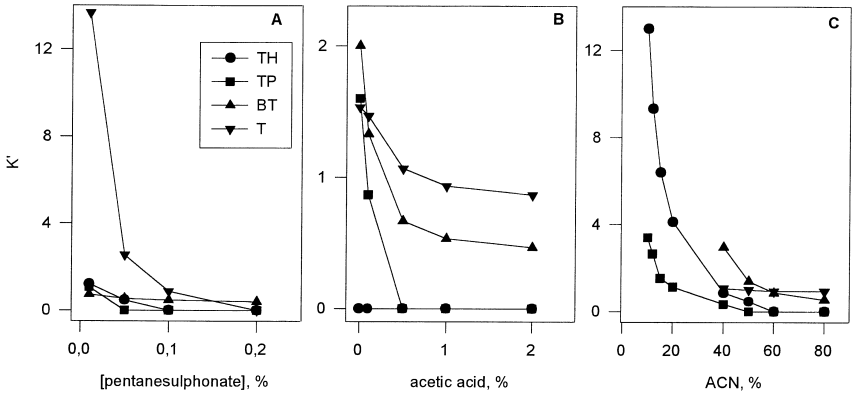

Fig. 1. Variation of the capacity factors of the thiamine-like compounds with (A) the concentration of sodium pentanesulfonate in the presence of 2% acetic acid and 40% ACN; (B) the percentage of acetic acid in the presence of 0.1% pentanesulfonate and 40% ACN; and (C) the percentage of ACN in the presence of 0.1% pentanesulfonate and 1% acetic acid.

were: 1, TP (t_R=5.38 min; k'=2.22); 2, TH (t_R=11.38 min; k'=5.81); 3, BT (t_R=12.1 min; k'=6.24); 4, T (t_R=14.24 min; k'=7.52).

Calibration graphs were performed by plotting concentration (μg/ml) against peak area. Table 1 shows the equations obtained for the calibration graphs and the regression coefficients. The precision of the method was demonstrated by repetitive analyses, calculating the average relative standard deviation (RSD) for 10 replicate injections of the same sample.

3.2. Optimising the separation of the thiamine-related compounds using an amide-based column

For most applications involving ionic samples, separation using reversed-phase (RPC) must also be considered because IPC separation is more complicated and subject to experimental problems. However, RPC using the ODS column provided poor results due to the interaction of the thiamine compounds of a basic nature with the silanol groups of the silica-based columns. Some manufacturers use an endcapping process to achieve greater reaction of the residual silanol groups. Thus, a stationary phase for basic compounds based on a ligand with amide groups (RP-AmideC_{16}) and an endcapping of trimethylsilyl was tested.

The first experiments with the RP-AmideC_{16} column involved studying the influence of pH using a mobile phase 25 m*M* potassium dihydrogenphosphate–ACN (95:5, v/v). A pH of 7 was selected. When the influence of the KH_2PO_4 concentration was studied, retention decreased when the phosphate concentration increased. A 25 m*M* concentration was chosen. Finally, Fig. 2A shows the variation in retention with ACN percentages between 0 and 70%. As can be seen, all the compounds were eluted from the column and, as expected, retention decreased for all the compounds when the proportion of organic solvent was increased. Fig. 2B shows the plot of ln k' vs. ACN percentage and the regression lines for the fit to a quadratic equation. A good fit was only obtained for a restricted range up to about 30%.

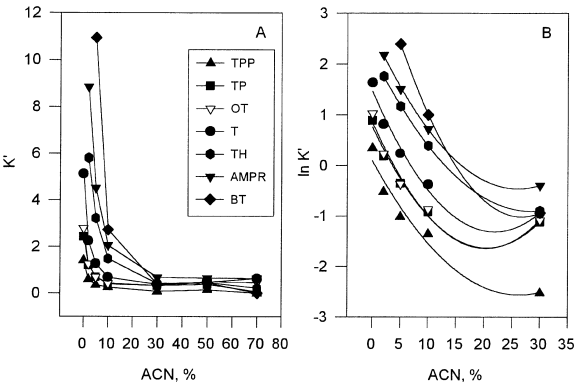

Fig. 2. (A) Variation of the capacity factors with ACN percentage using 25 m*M* KH_2PO_4, pH 7 as aqueous phase; (B) fit curves of ln k' to a quadratic equation.

Once again, peak separation was poor in isocratic conditions and gradient elution was necessary. The optimal gradient found was a first linear gradient from an initial mobile phase 25 m*M* KH_2PO_4, pH 7–ACN (100:0, v/v) to a 25 m*M* KH_2PO_4, pH 7–ACN (90:10, v/v) mixture during 10 min and then an isocratic step with this mobile phase during 10 min; finally, the initial conditions were re-established in 1 min and held for 15 min. The chromatographic profile obtained using this programme is shown in Fig. 3. The elution order and the retention characteristics were: 1, TPP (t_R=5.16 min; k'=1.68); 2, TP (t_R=5.68 min; k'=1.95); 3, OT (t_R=6.07 min; k'=2.16); 4, T (t_R=6.90 min; k'=2.59); 5, TH (t_R=11.00 min; k'=4.72); 6, AMPR (t_R=12.10 min; k'=5.29) and 7, BT (t_R=16.71 min; k'=7.69).

Calibration graphs were performed by plotting

Table 1
Calibration graphs using ion-pair formation

Compound	λ (nm)	Intercept	Slope (ml/μg)	Correlation coefficient	Linearity interval (μg/ml)	RSD (%)
TP	230	−7087	87 010	0.9999	1–12	1.5
TH	230	−1471	92 799	0.9999	0.3–12	1.4
BT	230	411	126 220	0.9994	1–10	1.3
T	230	8961	93 330	0.9993	1–10	1.6

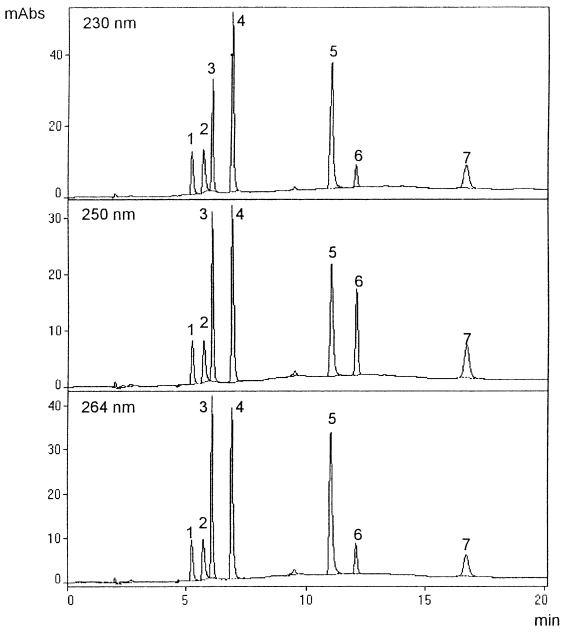

Fig. 3. Chromatographic profile using the amide-based column and gradient elution. Flow-rate, 1 ml/min; injected sample (containing 1 μg/ml of each thiamine-related compound), 50 μl. The peaks correspond to: 1, TPP; 2, TP; 3, OT; 4, T; 5, TH; 6, AMPR and 7, BT.

concentration (μg/ml) against peak area. Table 2 shows the equations obtained. The precision of the method calculated by RSD for 10 replicate determinations of the same sample at 1 μg/ml level is also included. The detection limits were calculated on the basis of 3σ and the quantitation limits on the basis of 10σ, using the regression lines for the standards according to Miller and Miller [29]. Values are also given in Table 2.

3.3. Comparison of both separation techniques

Once the chromatographic conditions for the separation had been optimised, both techniques (ion-pair formation and reversed-phase with an AmideC_{16} column) were compared. The best results, defined by total elution of the mixture components and the obtention of narrow and non-tailed peaks, were obtained using the AmideC_{16} column. In addition, this experimental procedure is simpler because column life is increased and smaller quantities of reagents are necessary.

3.4. Analysis of pharmaceutical products and urine samples

The procedure was applied to the determination of thiamine-related compounds in pharmaceutical preparations. Fig. 4 shows the chromatograms obtained when injecting a sample of the different products. The peaks were identified by: (1) comparing the retention data obtained for the sample, the standards and the sample spiked with the standards under identical conditions and (2) using the photo-diode array detector to continuously measure the UV–visible spectrum while the solute passed through the flow-cell and measuring the absorbance ratio at two wavelengths.

When the absorption spectra of the different peaks obtained for the standards, the pharmaceutical samples and the spiked samples were compared, a good agreement was found. The following criteria were used to confirm the purity of the peaks: (a) peak purity curve, which is based on the similarity between the spectrum at the top of the target peak and the spectra at each point on that peak. If there is any

Table 2
Calibration graphs using the amide-based column

Compound	λ (nm)	Intercept	Slope (ml/μg)	Correlation coefficient	Linearity interval (μg/ml)	DL (μg/ml)	QL (μg/ml)	RSD (%)
TPP	230	−832	73 992	0.9998	0.05–5	0.005	0.02	1.7
TP	230	−5101	71 900	0.9997	0.1–5	0.07	0.2	2.1
OT	264	−4943	2 555 369	0.9998	0.02–5	0.005	0.02	1.7
T	230	−3304	401 900	0.9999	0.02–5	0.01	0.03	1.1
TH	230	−3310	379 768	0.9999	0.02–5	0.02	0.08	1.7
AMPR	250	9514	109 812	0.9998	0.1–5	0.08	0.2	3.1
BT	230	2354	104 239	0.9999	0.02–5	0.01	0.03	4.8

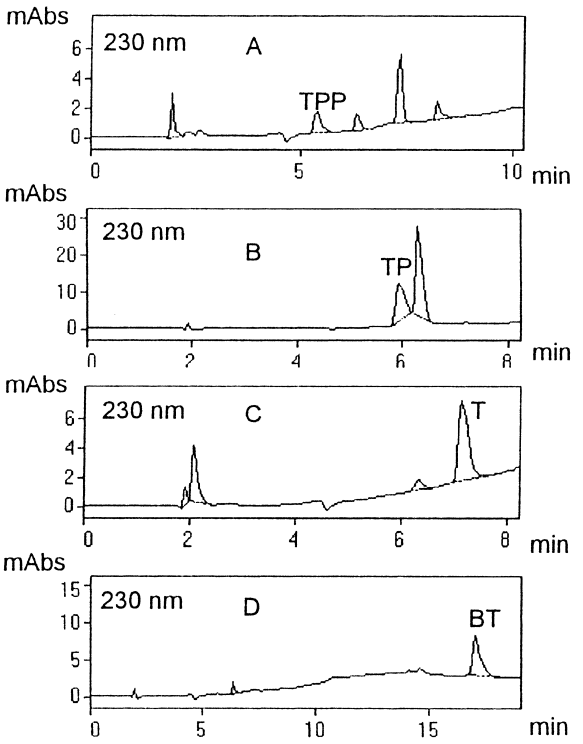

Fig. 4. Chromatograms for different pharmaceuticals. (A) Dynamin, (B) Menagil B_6, (C) Becozyme C-Forte and (D) Lacedermol complex using the AmideC_{16} column.

part where this curve deflects toward the negative side, impurities are considered to be included at that part. (b) The ratio between chromatograms obtained using two wavelengths, the purity of the target peak being displayed as a chromatogram ratio. The purity of a peak is considered greater as the shape of the chromatogram ratio becomes closer to that of a rectangle. Fig. 5 shows the peak purity curves obtained for the peaks corresponding to the thiamine-like compounds present in the pharmaceuticals. The results showed that no impurities coeluted with the analytical peaks. The values for the purity index were: thiamine pyrophosphate, 0.9985; thiamine phosphate, 0.9960; thiamine, 0.9996 and *S*-benzoylthiamine, 0.9874. These values indicate that the purity of the peaks was satisfactory.

After identification of the thiamine-related compounds and once the absence of a matrix effect had been confirmed, the compounds were quantified in the different pharmaceuticals. Table 3 shows the results obtained by the proposed procedure and the contents labelled by the laboratories. The Wilcoxon test was used to compare the results. The values obtained at 95% confidence interval [$T+=1.000$; $T-=-2.000$; P(est.)$=1.000$] indicated that there was no significant difference between the results obtained and the labelled levels. The variability day-to-day was calculated using the RSD for all the pharmaceuticals analysed in different days and the mean value was $\pm 3.5\%$ ($n=12$).

Because the chromatographic technique offers advantages for the determination of analytes in complex mixtures, the procedure was applied to the analysis of biological fluids such as urine. Absolute recoveries were evaluated by adding different amounts (1 and 2 μg/ml) of all the analytes to urine sample. These were submitted to the chromatographic procedure and the concentrations were obtained using the calibration graphs. When all spike and recovery data were combined, an average recovery±SD ($n=14$) of $98.1\pm 1.3\%$ was obtained. Then, several volunteers were administered oral therapeutic dose of the different pharmaceuticals analysed and the corresponding urine samples were collected. Fig. 6A shows the blank chromatogram for an urine sample, demonstrating the absence of compounds co-eluting with the thiamine-compounds. The analytes found in the urine were thiamine for all pharmaceuticals ingested and both thiamine and thiazole when the product administered contained benzoylthiamine. The chromatogram obtained for the last product is shown in Fig. 6B. Fig. 6C shows the chromatogram obtained when the thiamine-related compounds were added to that urine sample. The spectral scans of urine samples and standards showed good overlap. Thus, the peak purity curves and chromatogram ratios obtained for the peaks corresponding to the spiked urine sample showed that no impurities coeluted with the analytical peaks. The averaged values (mean±SD) for the purity index were: thiamine, 0.9254 ± 0.08 ($n=4$) and thyazole, 0.9973 ($n=1$). These values indicate that the purity of the peaks was satisfactory.

After administration of a pharmaceutical containing 15 mg of thiamine to a volunteer, a pharmacokinetic study was carried out. Urine samples were collected from 0 to 10 h after the ingestion of the drug and analysed using the proposed procedure. A plot of the urinary excretion shows that the thiamine concentration increased quickly within 4 to

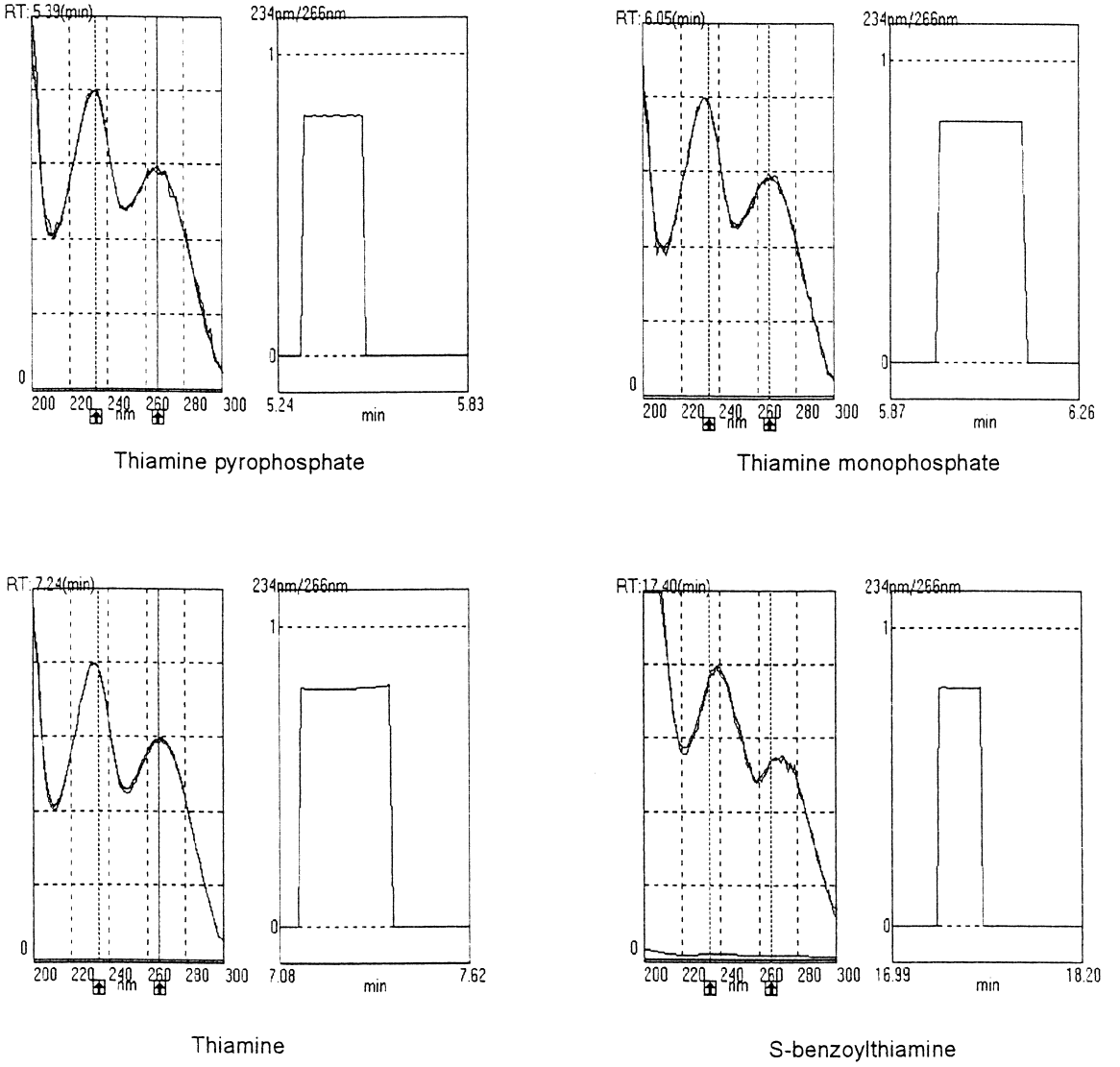

Fig. 5. Peak purity curves obtained for the peaks corresponding to the thiamine-like compounds present in pharmaceutical samples.

Table 3
Determination of thiamine-related compounds in pharmaceutical products

Sample (laboratory)	Compound	Content	
		Labelled (mg)	Found[a] (mg)
Dynamin (Menarini)	Thiamine pyrophosphate	10	10.0±0.3
Menagil B_6 (Lácer)	Thiamine phosphate	250	245±6
Becozyme C-Forte (Roche)	Thiamine	15	15.0±0.8
Lacedermol complex (Ern)	*S*-Benzoylthiamine	50	50.5±1.7

[a] Mean±standard deviation, $n=3$.

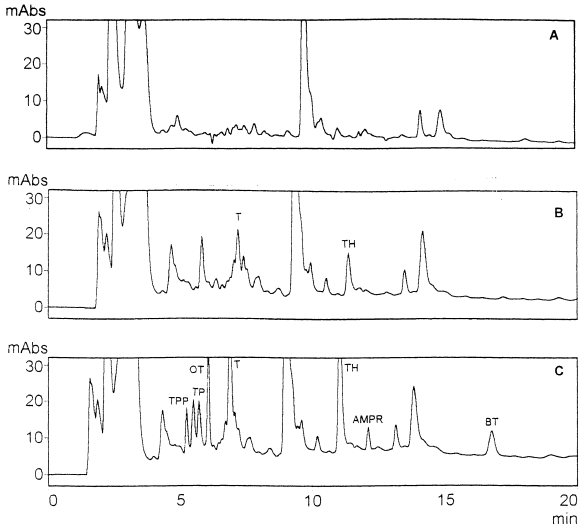

Fig. 6. (A) Chromatogram for a blank urine sample; (B) chromatogram for a urine sample after the ingestion of a dose of 50 mg benzoylthiamine (Lacedermol complex); (C) chromatogram for the same urine sample spiked with 1 μg/ml of the thiamine-related compounds.

6 h, then decreased. The percentage of thiamine excreted during the first 10 h was 65%.

4. Conclusion

The use of reversed-phase liquid chromatography using an AmideC_{16} column is well suited to determining the thiamine-related compounds of a basic character. A procedure for analysing seven compunds in a single chromatogram using gradient elution is proposed. Diode array detection was used for peak purity evaluation using the peak purity curve and the ratio of the chromatogram at two wavelengths. The procedures could be applied to the determination of the thiamine-like compounds in pharmaceutical products and biological fluids such as urine. Pharmacokinetic studies to evaluate the assimilation of the drug can also be performed.

Acknowledgements

The authors are grateful to the Spanish Ministery of Science and Technology (project BQU2000-0218) for financial support.

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