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Simultaneous determination of thiamine and its phosphate esters by a liquid chromatographic method based on post-column photolysis and chemiluminescence detection

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ABSTRACT

A sensitive method for the post-column detection of thiamine (T) and its phosphate esters is described. The procedure is based on the on-line photolysis of the analytes into photoproducts which have a strong enhancing effect on the CL permanganate–luminol reaction. The complete separation of the thiamines was obtained on a RP-amide C_{16} column in isocratic elution with an analysis time of less than 7 min. Under the optimum conditions, analytical curves, based on standard solutions, were linear over the range 10–1000 nM for thiamine and 100–2000 nM for its mono- and di-phosphate esters. Intra- and inter-day precision values of less than 1.14% relative standard deviation (RSD) ($n = 10$) and 1.86% RSD ($n = 15$), respectively, were obtained. The method was successfully applied to the determination of the thiamines in pharmaceutical preparations and baby foods.

1. Introduction

Thiamine (vitamin B_1), the antineuritic or antiberiberi factor, is distributed widely in natural materials in the forms of free thiamine (T) and its mono- (TMP), di- (TDP) and triphosphate (TTP) esters. There is now ample documental evidence supporting two independent roles of T: the well-established role of TDP as a coenzyme in carbohydrate metabolism and the role of a phosphorylated form of T in the process of nerve conduction [1–3].

Several methods for assessing the thiamine status by direct measurement of T and its esters using liquid chromatography (LC) have been described. The most common procedure is the alkaline oxidation of T and its phosphate esters to highly fluorescent thiochromes by potassium hexacyaneferrate(III). This can be done either by post-column or pre-column derivatization techniques [4–6].

The above reported oxidation of T also provides a chemiluminescence (CL) emission in batch [7] and in a flow injection (FI) [8] systems, and resulted in poor sensitivity. The CL response has also been obtained electrochemically at a platinum electrode and enhanced by Rhodamine B [9]. CL sensors based on the inhibition effect of T on the luminol–periodate [10] or luminol–hexacyaneferrate(III) [11] systems have been developed. The enhancing effect of T on the luminol–hydrogen peroxide CL reaction has been used for the determination of T in a flow system [12].

Because of their inherent advantages over ordinary chemical reactions, photochemical reactions have been widely used for post-column derivatization coupled to fluorimetric and CL detection in LC [13]. Photochemical derivatization has been also used for the determination of T. Thus, FI photochemical approaches have been proposed based on the photometric [14] or fluorimetric detection of the compound generated from UV irradiation of thiamine [15,16]. The combination of photolytic reaction and CL detection has been employed for the FI determination of this vitamin. In this method photo-fragments of T emit light by oxidation with permanganate in acid medium, and the resultant CL is measured [17].

This paper describes for the first time the implementation of a photochemically induced CL post-column detection system for thiamines. It is based on the strongly enhancing effect of the photoproducts, generated on-line from the photolysis of thiamines, on the CL permanganate–luminol reaction. The proposed LC method was used for assessment of thiamine status by measurement of T, TMP and TDP. TTP measurements could not be performed since a suitable TTP pure standard was not available to our group. The separation was carried out with a stationary phase involving a ligand with amide groups (RP-amide C_{16}) and phosphate buffer of pH 7 as mobile phase. The proposed LC method provides a high degree of resolution, sensitivity, reproducibility and quantitative determination, advantages much in demand in food and pharmaceutical laboratories.

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2. Experimental

2.1. Reagents and solutions

All reagents were of analytical reagent grade and were purchased from Sigma–Aldrich (Madrid, Spain) unless otherwise stated. Solvents were of chromatographic grade and provided by Romil (Loughborough, UK). Water was deionised and further purified by means a Milli-Q Plus Water Purification system (Millipore; Madrid, Spain). Taka-diastase was obtained from Fluka (Buchs, Switzerland).

Stock solution of cetyltrimethylammonium bromide (CTAB) (5 mM) was prepared by dissolving 0.455 g of the compound in ultrapure water.

Stock solution of 0.025 M phosphate buffer of pH 7 was prepared by dissolving 3 g of sodium dihydrogen phosphate in ultrapure water, adjusting to pH 7 with 2 M sodium hydroxide and diluting to 1 L with ultrapure water.

Stock solution of luminol (0.01 M) was prepared by dissolving 0.177 g of luminol in 5 ml of 1 M sodium hydroxide and diluting to 100 ml with ultrapure water.

Stock solution of potassium permanganate (0.02 M) was prepared by dissolving 0.316 g of $KMnO_4$ in 100 ml of boiled ultrapure water, filtering through glass wool and protecting from light.

Stock standard solutions of 1 mM T, TMP and TDP were prepared in ultrapure water. These solutions were kept in dark bottles at 4 °C. Working standard solutions were prepared by appropriate dilution with ultrapure water just before use.

2.2. Apparatus and procedure

The instrumental setup used in this study (Fig. 1) consisted of an LC Beckman Coulter instrument (Fullerton, CA, USA), composed of a System Gold 125 NM Solvent Module, a System Gold 186 diode array detector and a Rheodyne model 7725 injection valve fitted with a 100 μl loop. The analytical column was a RP-amide C_{16} (Supelco, 15 cm × 4 mm, particle size 5 μm). A guard column packed with the same stationary phase was also used. The mobile phase consisted of 0.025 M phosphate buffer of pH 7 at a flow rate of 1.1 ml min^{-1}.

The CL detector consisted of a Photomultiplier Detection System Model 814 from Photon Technology International (Monmouth Junction, NJ, USA) placed in a box. A Hellma 170.700 QS (two windows; inner volume, 31 μl; path length, 0.5 mm) was placed adjacent to the photomultiplier tube (PMT). The flow cell exposed a large surface area to the PMT and was backed by a mirror for maximum light collection. Extreme precautions were taken to ensure that the sample compartment and PMT were light-tight. The CL signal produced in the flow cell was detected without wavelength discrimination. The CL detector was connected to the LC equipment by means of a SS420x interface (Beckman). An IBM personal computer using a 32 Karat software (Beckman) was used for data acquisition and treatment.

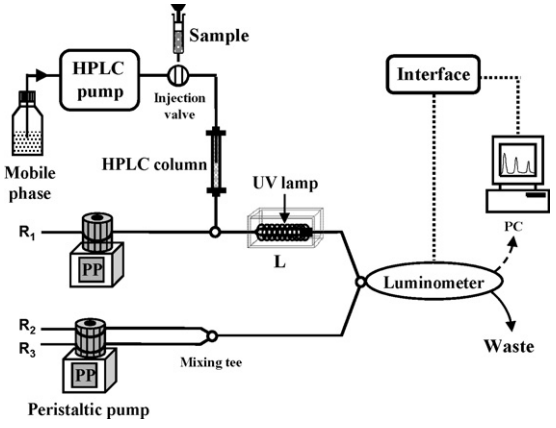

Fig. 1. Schematic diagram of the LC post-column CL detection used in the determination of T, TMP and TDP. Mobile phase = 25 mM phosphate buffer pH 7; R_1 = CTAB, 5 mM; R_2 = potassium permanganate, 5 μM; R_3 = luminol, 8 μM and phosphate buffer of pH 11.2, 0.2 M.

The post-column detection system is depicted schematically in Fig. 1. A Gilson (Middleton, WI, USA) Minipuls-3 peristaltic pump was used for the addition of reagents. Except for the pump tubing (Tygon), PTFE tubing (0.5 mm i.d.) was used throughout the manifold. The photoreactor was constructed from PTFE tubing (0.5 mm i.d., 100 cm length) coiled around a Spectronic (Westbury, NY, USA) rod-shape low pressure mercury lamp (50 mm × 5 mm diameter) (power 6 W; main spectral line, 254 nm). The photoreactor–lamp assembly was housed in a fan-ventilated metal box covered with a mirror to increase the photon flux. The eluate from the LC column merged with the 5 mM CTAB stream (flow rate 1.8 ml min^{-1}) and was irradiated with UV light as it passed through the photoreactor where thiamines were photoderivatized. The effluent of the photoreactor merged with the premixed streams of luminol (8 μM, 0.2 M phosphate buffer pH 11,2; flow rate 1.8 ml min^{-1}) and permanganate (5 μM; flow rate 1.8 ml min^{-1}) in a mixing point as close as possible to the inlet of the flow cell. The tubing of the premixed permanganate and luminol streams and that leading from the photoreactor to the flow cell was covered with black insulating tape in order to prevent a fibre optic effect from introducing stray light into the detector.

2.3. Sample preparation

2.3.1. Pharmaceutical formulations

Seven different multivitamin formulations were used, i.e., tablets, capsules and ampoules. After finely powdering a tablet in a porcelain mortar, a portion of average tablet weight was quantitatively transferred into a 100 ml volumetric flask and diluted to volume with ultrapure water. In the case of ampoules an aliquot of 500 μl was quantitatively transferred into a 100 ml volumetric flask and diluted to volume with ultrapure water. Aliquots of these solutions were further diluted with ultrapure water, so that the concentration of the analytes was in the range 0.2–0.4 μM.

2.3.2. Baby foods

The procedure used for the preparation of the samples was similar to that described by the Analytical Methods Committee [18]. Briefly: 10 g of the sample was weighed into a 100 ml amber glass bottle and 25 ml of 0.1 M hydrochloric acid added. The mixture was homogenized by vortex-stirring for 1 min, sonicated for 10 min, heated in a water bath at 90 °C for 30 min, and allowed to cool. Then, the pH was adjusted to 4–4.5 with 2 M sodium acetate, and 0.1 g of taka-diastase was added. The suspension was maintained at 50 °C on a shaking water bath for 2 h, followed by the addition of 1 ml of 50% (w/v) trichloroacetic acid and heated again at 90 °C for 10 min. After cooling and adjusting the pH to 7 with 5 M sodium hydroxide, the sample was quantitatively transferred to a 100 ml volumetric flask and diluted to volume with ultrapure water. Aliquots were successively filtered through a No. 4 Whatman paper and a 0.45 μm nylon chromatographic filter and injected into the chromatograph.

3. Results and discussion

3.1. Post-column detection system

The principle behind the post-column detection system is that the products from the photolysis of T, TMP and TDP strongly

increase the weak radiation emitted during the CL oxidation of luminol by permanganate in basic medium. The two reactants are continuously mixed and introduced into the flow cell and the weak CL emission is continuously recorded as the baseline. When the on-line photolysed thiamines are introduced in the flow system, the CL intensity is enhanced in proportion to their concentration.

In order to establish optimal conditions for the lowest possible detection limits, the effects of various parameters on the CL system were investigated using a flow manifold with the chromatographic column removed from the system represented in Fig. 1.

The concentrations of luminol and potassium permanganate were important parameters in the experiments. The effect of different concentrations combination of luminol (2–20 μM) and $KMnO_4$ (1–50 μM) was studied. The experimental results showed that the maximum CL signal and signal-to-blank ratio were obtained when 8 μM of luminol and 5 μM of $KMnO_4$ were used.

The CL signal was greatly affected by pH of the solution in the photoreactor and in the flow cell. The influence of pH on the photolysis of thiamines was first studied. 0.02 M phosphate buffer solutions of different pH values were pumped through the R_1 channel while maintaining a pH of 11 in the flow cell. It was found that the CL intensity increased up to pH 7; a quasi plateau was observed between pH 7 and 9 while a higher pH the intensity decreased. A pH of 7 was selected for further experiments.

After selecting the optimum pH for the photolysis of thiamines, the effect of pH in the flow cell was tested by delivering luminol solutions (8 μM) prepared in 0.2 M phosphate buffers of different pH values. The CL signal of the reaction in the presence and absence of the photolysed thiamines increased with the increase in pH. A pH of 11.2 was chosen as a compromise between sensitivity and blank value.

Micelles aggregates have been used to manipulate the microenvironment of analytes and reagents and to control the reactivity, equilibrium and pathway of chemical and photochemical process [19,20]. Thus, the effects of anionic (sodium dodecyl sulfate), cationic (tetradecyltrimethylammonium bromide and CTAB) and non-ionic (Brij 35, polyvinyl alcohol and Triton X-100) surfactants were examined. Experiments showed that the presence of CTAB produced the highest CL, which increased as the CTAB concentration was increased up to 0.01 M. Higher concentrations of CTAB lowered the CL intensity. The greatest difference between the CL signal in the presence and absence of the photolysed thiamines was observed at a CTAB concentration of 5 mM.

The residence time of the sample in the photoreactor had a great influence of the photochemical reaction and hence on the sensitivity attained. The irradiation time was controlled by the length of the tubing around the lamp and/or the flow rates of the sample and the CTAB streams. The photoderivatization of T, TMP and TDP was rapid. The analytical signal decreased by only 15% when the flow rate through the photoreactor was varied over the range 2.1–4.2 ml min^{-1}. The highest CL with the lowest blank was achieved using flow rates of 1.1 ml min^{-1} for the sample and 1.8 ml min^{-1} for the CTAB streams.

The total flow rate is a very important parameter for CL emission in the flow cell. By maintaining the analyte residence time in the photoreactor constant, the CL intensity increased with increasing flow rate. An overall flow rate of 6.5 ml min^{-1} was selected, because higher flow rates caused too much pressure in the flow tubes and connections and excessive consumption of reagents. In order to ensure efficiency in the photolysis of thiamines, the flow rates of the sample (the effluent when the detection system is connected to LC column) and the CTAB streams were fixed at 1.1 and 1.8 ml min^{-1}, respectively; therefore the flow rates of the CL reagents must be 3.6 ml min^{-1} (1.8 ml min^{-1} for each channel).

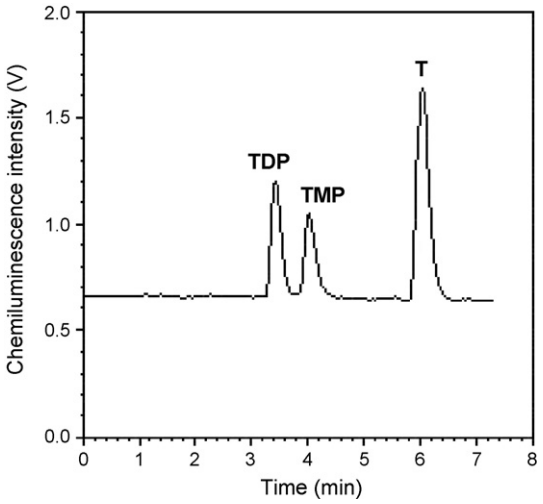

Fig. 2. Typical chromatogram of a standard solution containing T (0.25 μM), TMP (0.50 μM) and TDP (0.50 μM) under the optimum conditions as indicated in Fig. 1.

3.2. Chromatography

In most cases T, TMP and TDP are separated on C_{18} columns. Problems with the reverse phase condition on C_{18} columns are: (i) making sure that the peaks of the polar phosphorylated thiamines are clearly separated from the injection peak, (ii) that there is sufficient resolution between the TDP and TMP peaks and (iii) that the retention time for T be reasonably short. Ion-pairing or normal modes have been proposed, but with inherent problems in terms of mobile phase preparation (gradient elution), stabilisation and lifetime of the column. Recently, the RP-amide C_{16} column has been proposed for the separation of thiamines using pre- and post-column derivatization to thiochromes [21–23]. This technique was used with UV [21] and fluorescence [22,23] detection. It was decided to evaluate the feasibility of analysing T, TMP and TDP using post-column photochemical derivatization and CL detection on a RP-amide C_{16} column, after adapting the chromatographic conditions.

The mobile phase should not only be suitable for the separation of the thiamines but also compatible with the post-column detection system. The mobile phases reported for the separation of these vitamins on an amide C_{16} column use mixtures of phosphate buffer with methanol or acetonitrile. When these binary mixtures were tested, it was found that the CL signal strongly decreased with increasing amounts of acetonitrile. Methanol did not affect the CL signal but retention decreased for the three vitamins and the resolution of the TDP/TMP pair was very poor. Therefore, 100% phosphate buffers of different concentrations and with pH values between five and eight were tested. The best results were obtained with a 0.025 M phosphate buffer of pH 7. The flow rate of the mobile phase was fixed at 1.1 ml min^{-1}, because it corresponded to the optimum value found for the sample channel in the detection system and, in addition, TDP, TMP and T were well separated. Fig. 2 shows a typical chromatogram obtained from a standard solution; as can be seen the background was rather low, the total separation took less than 7 min, and resolution values for the TDP/TMP and TMP/T were 1.6 and 2.7, respectively.

3.3. Method validation

The proposed LC method for the simultaneous determination of T, TMP and TDP was validated by determining its performance characteristics regarding linearity, detection limit, repeatability, precision and selectivity. To test the linearity, a series of standard

Table 1
Parameters of calibration graphs.

Parameter	T	TMP	TDP
Intercept (10^6 area units)	−0.87	−1.08	−2.25
Slope (10^4 area units/nM)	8.79	1.04	1.63
Correlation coefficient	0.9998	0.9994	0.9995
Linearity (nM)	10–1000	100–2000	100–2000
Detection limit (nM)	0.61	4.76	2.97

solutions of T, TMP and TDP in the concentration range 10–2000 nM was analysed (at least 18 samples covering the whole range were used). Each point of the calibration graphs corresponds to the mean value from three independent peak measurements. The linearity between peak area and concentration was good for the three analytes, as shown by the fact that the regression coefficients (r) were greater than 0.999 for all the curves. The detection limits, estimated according to IUPAC recommendations, were lower than 4.8 nM [24]. The regression equations and other parameters are shown in Table 1.

The intra-day precision was tested with 10 repeated injections of two samples solutions containing the three analytes at two concentrations. The relative standard deviations (RSD) were always less than 1.14%. The inter-day precision was studied by analysing the above samples on five consecutive days. Each sample was injected three times every day; the RSD for peak area were less than 1.86%. Detailed results of precision and accuracy are shown in Table 2.

Table 2
Precision and accuracy data.

Concentration (μM)	Intra-day		Inter-day	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
T				
0.25	0.89	99.1	1.60	97.2
1.0	0.81	98.8	1.52	95.1
TMP				
0.50	1.09	100.6	1.86	98.4
2.0	1.13	99.1	1.84	97.9
TDP				
0.50	1.14	97.7	1.65	99.0
2.0	1.06	96.4	1.59	100.6

The method can be considered very selective because very few compounds will show a response in this post-column detection sys-

Table 3
Determination of thiamines in pharmaceutical formulations and baby food samples.

Sample (supplier)[a]	Vitamin	Labelled content	Amount added	Amount found	Recovery (%)
Pharmaceuticals					
Benerva (Bayer)	T	100 mg/ampoule	–	100.3	100 ± 1
			50	144.7	96 ± 3
			100	202.6	101 ± 1
Perfus multivitamínico (Rius Garriga)	T	15 mg/ampoule	–	15.1	101 ± 1
			5	19.8	99 ± 2
			10	25.3	101 ± 1
Hidroxil B_{12}–B_6–B_1 (Almirall Prodesfarma)	T	250 mg/tablet	–	250.6	100 ± 1
			50	298.2	99 ± 2
			100	343.8	98 ± 2
Dynamín (ERN)	T	2 mg/tablet	–	2.04	102 ± 3
			1	3.04	101 ± 2
			2	3.87	97 ± 2
Menalgil B_6 (Menarini)	T	250 mg/tablet	–	249.8	100 ± 1
			100	344.3	98 ± 1
			200	462.5	103 ± 2
Nervobión 5000 (Merck)	T	100 mg/ampoule	–	100.2	100 ± 1
			50	152.1	101 ± 1
			100	196.6	98 ± 3
Pluribios (Madariaga)	TDP	10 mg/capsule	–	9.96	100 ± 1
			5	14.5	97 ± 2
			10	20.0	100 ± 4
Baby foods					
Nidina H.A. (Nestlé)	T	3.60 μg/g	–	3.59	100 ± 2
			0.5	4.23	103 ± 4
			1	4.47	97 ± 5
Hero baby 1 (Hero)	T	5.23 μg/g	–	5.25	100 ± 1
			0.5	5.52	96 ± 2
			1	6.09	98 ± 3

[a] Composition: Benerva (per 1 ml): thiamine hydrochloride 100 mg; excipients: glycerol, sodium dihydrogen phosphate, sodium bicarbonate, phenol, water. Perfus multivitamínico (per 3 ml): thiamine hydrochloride 15 mg, ascorbic acid 150 mg, retinol palmitate 3000 UI, cholecalciferol 300 UI, rivoflavin-5-phosphate 4.11 mg, nicotinamide 30 mg, pyridoxine hydrochloride 4.5 mg, D-panthenol 7.5 mg, α-tocopheryl acetate 1.5 mg; excipients: polysorbate 80, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, sodium bicarbonate and water. Hidroxil B_{12}–B_6–B_1 (per tablet): thiamine hydrochloride 250 mg, pyridoxine hydrochloride 250 mg, hydroxycobalamin 500 mg; excipients unspecified. Dynamín (per tablet): thiamine hydrochloride 2 mg, caffeine 30 mg, pyridoxine hydrochloride 2.5 mg, cyanocobalamin 2 mg, folic acid 400 mg, L-glutamine 50 mg; excipients: sucrose, mannitol, starch, microcrystalline cellulose, magnesium stearate, copolymer of methyl butyl, 2-methyl dimethylaminoethyl (1:2:1), gum arabic, calcium carbonate, E-122, white and carnauba wax. Menalgil B_6 (per tablet): thiamine hydrochloride 250 mg, pyridoxine hydrochloride 250 mg, cyanocobalamin 1000 mg; excipients: microcrystalline cellulose, glyceryl palmitostearate, calcium phosphate, magnesium stearate, polyvinyl alcohol, TiO_2, talc, lecithin, E-124, xanthan gum, carnoisine. Nervobión 5000 (per 3 ml): thiamine hydrochloride 100 mg, pyridoxine hydrochloride 100 mg, cyanocobalamin 5 mg; excipients: benzyl alcohol, NaOH, potassium cyanide. Pluribios (per capsule): thiamine di-phosphate 10 mg; retinol acetate 5000 IU; riboflavin-5-phosphate 10 mg; nicotinamide 25 mg, ascorbic acid 200 mg, tocopherol acetate 30 mg; excipients: microcrystalline cellulose, TiO_2, E-102. Nidina H.A.: hydrolysed serum proteins, vegetable oils, lactose, dextri-maltose, salts of potassium, chloride, calcium, phosphorus, sodium, magnesium, iron, zinc, copper and iodine, vitamins (C, E, niacin, pantothenic acid, A, riboflavin, B_6, thiamine, folic acid, K, biotin, B_{12} and D), taurine, choline, L-arginine, inositol, L-carnitine and L-histidine. Hero baby 1: demineralised whey, vegetable oils, skim milk, lactose, minerals, lecithin, vitamins (C, E, niacin, pantothenic acid, B_1, B_6, A, B_2, folic acid, K, biotin, D_3 and B_{12}), taurine, inositol, choline and carnitine.

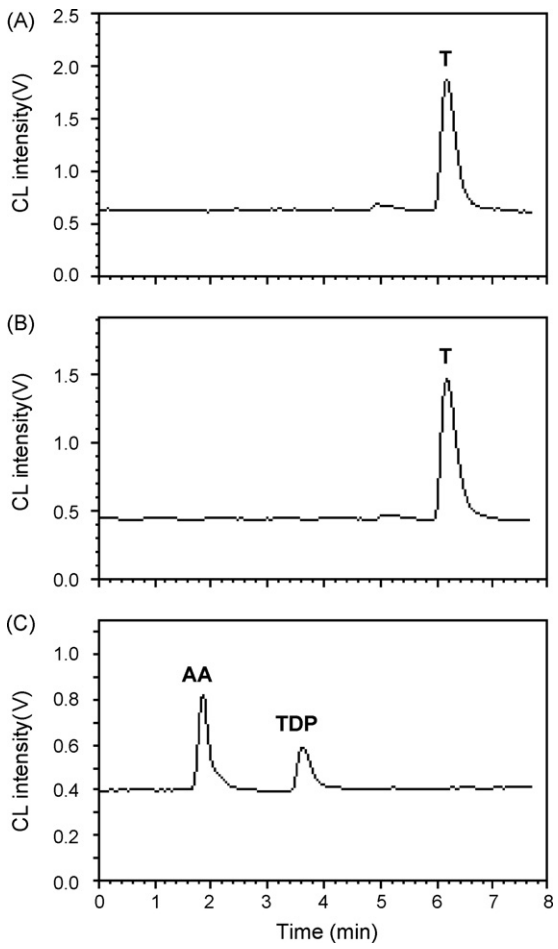

Fig. 3. Chromatograms for different pharmaceuticals. (A) Hidroxil B_{12}–B_6–B_1; (B) Menalgil B_6; (C) Pluribios. Peaks: T, thiamine; TDP, thiamine di-phosphate; AA, ascorbic acid.

tem. It is worth noting that biotin, cyanocobalamin, nicotinamide, riboflavin, retinol, cholecalciferol, pantothenic acid, tocopherol and folic acid do not give CL signal. Ascorbic acid and pyridoxine give only a very weak CL; in addition, these vitamins did not elute at the times corresponding to the thiamines.

3.4. Applications

The characteristics of this detection system provided very simple chromatograms for the determination of thiamine and its phosphate esters in real samples. To evaluate the applicability of the present method for the determination of these compounds, pharmaceutical formulations and vitamin-enriched baby nutrition products were analysed.

The chromatographic peaks in the samples analysed were identified by comparing spectra and retention data obtained for the samples, the standards and spiked samples under optimal method conditions. After identification of the thiamines and once the absence of a matrix effect had been confirmed, the analytes were quantified in the different samples.

The results obtained in the analysis of seven multivitamin formulations and the contents labelled by the laboratories are shown in Table 3. The Wilcoxon test was used to compare the results. The values obtained at 95% confidence interval ($W = 16$; $T+ = 22$; $T- = -6$; $P = 0.219$) indicated that there was no significant difference between the results obtained and the labelled levels. The inter-day precision was calculated using the RSD for all the pharmaceuticals analysed in five consecutive days and the mean value was ±1.54% ($n = 100$). Fig. 3 shows the chromatograms obtained when injecting a sample of three of the pharmaceuticals analysed.

The proposed method was also used in the analysis of two baby nutrition products. Table 3 shows that the results obtained were in excellent agreement with the declared values. The inter-day precision, corresponding to five consecutive days, gave a mean value of RSD of ±1.88%.

4. Conclusions

The introduction of a new CL detection system based on the strongly enhancing effect of on-line photolysed T, TMP and TDP on the CL permanganate–luminol reaction permitted us to develop an LC method for the determination of these analytes. The separation was obtained on a RP-amide C_{16} column in isocratic elution and with an analysis time of less than 7 min.

The proposed method has satisfactory precision (inter-day precision, RSD over the range 1.52–1.86%) and sensitivity (LOD between 0.6 nM and 4.8 nM), which is comparable with other sensitive methods. As most of the vitamins do not provide a CL signal or are very weak, the proposed procedure is recommended for the determination of thiamine and its phosphate esters in the fields of pharmaceutical and food industries.

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