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Micellar liquid chromatography determination of B vitamins with direct injection and ultraviolet absorbance detection

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Abstract

A micellar reversed-phase liquid chromatographic procedure was developed for the control of five water-soluble vitamins, B (nicotinamide), B_1 (thiamine), B_2 (riboflavin), B_6 (pyridoxine and pyridoxamine), in multivitamin pharmaceutical formulations (capsules, pills and syrups). Optimization procedure includes studies about the composition of the mobile phase (sodium dodecyl sulphate and the modifiers propanol, butanol or pentanol), flow-rate and temperature. Chromatographic analysis of all vitamins was carried out using a single mobile phase of 0.1 *M* SDS–4% (v/v) pentanol at pH 3, in a C_{18} column in isocratic mode, and UV-detection at 270, 290 and 325 nm. The flow-rates selected were 1.0 ml/min in the interval 0 to 6 min, and 2.0 ml/min until the end of the chromatogram and temperature was 45 °C. In the micellar liquid chromatographic system, the samples were injected without pretreatment, and the analysis time was below 12 min. Repeatabilities and intermediate precision were achieved according to ICH, and were below 5%. When the method is applied to real samples, the amount found with respect to the declared compositions were within the 91–105% range. These results were similar to those obtained with a conventional 60:40 (v/v) methanol–water mixture for some of the vitamins, but with the advantage of use a single mobile phase for the analyses of the five vitamins, with direct injection of the samples and reduced toxicity, flammability, environmental impact and cost of the micellar–pentanol solutions.
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Keywords: Mobile phase composition; Vitamins; Pentanol; Sodium dodecyl sulfate

1. Introduction

Therapeutic multivitamins are advisable for use in cases of deficiency in pathological conditions in which nutritional requirements are greatly increased (e.g. alcoholism, hyperthyroidism, severe illness or injury and cachexia) or in conditions in which absorption, utilization, or excretion of vitamins is abnormal. Therapeutic multivitamins should not be used as dietary supplements, and medical supervision is important when these preparations are dispensed. Multivitamin pharmaceutical preparations containing complex mixtures of these substances are very interesting for analysis, and most of them include the water-soluble B group (Table 1) nicotinamide, pyridoxamine, pyridoxine, riboflavin and thiamine [1].

The most widely used methods in the determination of vitamins of the B-group are reversed-phase high-performance liquid chromatography (RP-

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Table 1
Structures, wavelength of maximum absorption (nm), acid–base constants and partition coefficients octanol–water

Compost	Structure	λ_{max}	pK_a	log $P_{o/w}$
Nicotinamide (B)		260	0.5 3.35	−0.37
Pyridoxamine dihydrochloride (B_6)	2 HCl · H_2O	325	–[a]	–[a]
Pyridoxine hydrochloride (B_6)	HCl	290	5.0 8.96	−0.77
Riboflavin (B_2)		268	1.9 9.69 10.2	–[a]
Thiamine hydrochloride (B_1)	HCl Cl^-	260	4.8 9.0	–[a]

[a] Values not found.

HPLC), using a C_{18} column and aqueous–organic mobile phases, in acidic media, containing acetonitrile [2], 15% (v/v) methanol [3], or in gradient elution with 7.5 to 30% (v/v) methanol [4], or 5 to 30% [5]. C_8 columns could also be used with a mobile phase of 15% (v/v) methanol [6]. The methods reported in the literature are unable to analyze the five vitamins and require analysis times in the 15–20 min range, including extraction procedures [3–6], column switching [2] or gradient elution [5].

Micellar liquid chromatography (MLC) has proved to be a useful technique in the control of diverse groups of drugs in pharmaceutical preparations, such as sulfonamides [7], antihistamines and phenethylamines [8], diuretics [9], β-blockers [10] and benzodiazepines [11]. In MLC, the retention behavior of compounds can be predicted to a high degree of accuracy [12]. This fact simplifies the optimization of mobile phase composition. The most common organic modifier is propanol, but the use of other alcohols such as butanol and pentanol can be more convenient to decrease the retention and increase the efficencies. Another advantage of the MLC is that it allows the direct injection of the samples.

This work describes the development of a rapid and simple MLC procedure that is selective for the simultaneous determination of five B group vitamins, using a mobile phase containing sodium dodecyl sulfate (SDS) and pentanol and with direct injection of the sample. The proposed method has been used

for the quantitative determination of commercialized pharmaceuticals which contain these substances.

2. Experimental

2.1. Reagents

The vitamins (nicotinamide, pyridoxamine dihydrochloride, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride) were supplied by Sigma–Aldrich (Steinheim, Germany). Stock solutions containing 100 μg/ml were prepared by dissolving the compounds in nanopure water from Barnstead (Sybron, Boston, MA, USA), with the aid of a Selecta Mixtasel (Barcelona) ultrasonic bath. All solutions were stored at 4 °C in light-resistant glass bottles. Stock solutions were always filtered directly into the autosampler vials through 0.45 μm Nylon membranes 13 mm in diameter.

The stability of the vitamin solutions was checked at 25 and 5 °C during 2 months, by recording the absorption spectra and the chromatogram. Results indicated that these solutions were stable throughout this time.

The mobile phases were prepared with the surfactant sodium dodecyl sulfate (99% purity, Merck, Darmstadt, Germany) and the modifiers 1-propanol, 1-butanol or 1-pentanol (Scharlab, Barcelona, Spain), buffered with phosphate system (Panreac, Barcelona). After preparation, the mobile phases were filtered through 0.45 μm Nylon membranes (Micron Separations, Westboro, MA, USA). Methanol (Scharlab) was used in the preparation of the aqueous–organic mobile phase and for conditioning the column.

2.2. Apparatus

The pH of the mobile phases was measured with a Crison GLP 22 (Barcelona) potentiometer, equipped with a combined Ag/AgCl/glass electrode. UV spectra and absorbance measurements were obtained with a Perkin-Elmer UV–Vis–NIR spectrophotometer Lambda 19 (Norwalk, CT, USA). Maximum absorption wavelengths of the vitamins are given in Table 1.

An Agilent Technologies 1100 chromatograph (Palo Alto, CA, USA), equipped with a quaternary pump, an autosampler and a UV–Visible detector was used. The column used for the analysis was a Kromasil C_{18} (Scharlab, 5 μm particle size, 120 mm×4.6 mm I.D.) thermostatized at 45 °±0.2 °C. The column was washed before any change of the mobile phase with 90 ml of water at a flow of 0.5 ml/min and then passed the new mobile phase, at the working flow-rate, until the stability of the base line, that normally is accomplished in 30 min. Weekly, or before stopping the chromatograph, we wash the column and system with 100 ml of water and 100 ml of methanol, pumped at 0.2 ml/min.

The composition of the optimum mobile phase was 0.1 *M* SDS–4% (v/v) pentanol/phosphate buffer 0.1 *M* at pH 3. The injection volume was set at 20 μl. In the MLC method changes in the flow-rate were 1.0 ml/min in the interval 0 to 6 min, and 2.0 ml/min until the end of the chromatogram. The wavelengths used for monitoring were 270 nm until 3.5 min, which was then changed to 290 nm until 8 min and again to 325 nm up to 10.5 min, to finally return to 270 nm until the end of the chromatogram.

The signal was acquired through a Chemstation (Hewlett–Packard) connected to the chromatograph and this was also used for the measurement of peak properties: capacity factor (k), efficiency (N) and asymmetry (T). The dead volume was determined as the mean value of the first significant deviation from the base line in the chromatograms of the analytes. The chromatographic data were then processed with Excel and Michrom [12].

For comparison purposes, the pharmaceuticals were also determined by an aqueous–organic mobile phase with a composition of methanol–water 60:40 (v/v) at pH 3.0. In the selection of this mobile phase, we started working with 15% of methanol [6] that gives great retention times for the determination of pyridoxine and riboflavine. After we increase the content of methanol (without changes in the selectivity) in order to obtain adequate analysis times for these two substances.

2.3. Sample preparation

The pharmaceutical preparations were presented in the form of capsules, pills and syrups. For the analyses, ten capsules or pills were weighed, ground

to fine powder, and homogenized; three portions were then taken and weighed separately. The syrups were well shaken and three aliquots of the homogenized samples were mixed separately with a small amount of methanol and diluted with 0.1 *M* SDS. Some excipients were not soluble and hence the sample solutions had to be filtered before injection into the chromatograph. The filtration was always performed directly into the autosampler vials through 0.45 μm Nylon membranes 12.5 mm in diameter (Micron Separations).

3. Results and discussion

3.1. Selection of the pH, modifier and surfactant concentration

The vitamins studied in this work have a high polarity, with an octanol/water coefficient (log $P_{o/w}$) in the −0.4 to −0.8 range (Table 1). This parameter therefore indicates that they must appear near or in the dead volume. The values of log *K* (Table 1) obtained for some of these vitamins [13] shows that all the vitamins are protonated within the working pH range of the C_{18} columns, this being the reason why they appear when chromatographed in micellar liquid chromatography.

Using a flow-rate of 1 ml/min, 25 °C and SDS pure micellar mobile phases at pH 7, thiamine does not elute and the other vitamins show low efficiencies (*N* below 500), and high asymmetry factors (above 2). Using propanol, thiamine appears in 15 min, but the other vitamins appear near the dead volume. After, the vitamins were chromatographed at pH 3 in mobile phases which present an intermediate eluent strength: SDS 0.1 *M*, SDS 0.1 M–7.5% (v/v) propanol, SDS 0.1 M–4% (v/v) butanol or pentanol. In SDS 0.1 *M*, neither thiamine nor the B6 group give a signal till 30 min. In SDS–propanol the B6 group shows retention times in the 20–25 min range, while thiamine remains undetected. The use of butanol or pentanol was therefore preferred. Butanol and pentanol give good efficiencies ($N=2000$–3000) with asymmetry factors below 1.5. The five vitamins could be resolved in butanol and pentanol with analysis times below 22 min, but with butanol, the efficiency and asymmetry factors for thiamine were unacceptable ($N=200$, $T=2.8$). For these reasons, mobile phases with SDS–pentanol at pH 3 were preferred for carrying out the optimization procedure.

The optimization criteria was to obtain the mobile phase that allows the complete separation (maximum resolution) in an appropriate analysis time. This will be useful for analysis of all the mixtures of these substances. The development of this strategy was facilitated by the capability of MLC to predict the retention of compounds using simple equations. The model (Eq. (1)) employed for these predictions was [12]:

$$k=\frac{K_{AS}\dfrac{1+K_{SD}\varphi}{1+K_{ADS}\varphi}}{1+K_{AM}\dfrac{1+K_{MD}\varphi}{1+K_{ADM}\varphi}[M]} \qquad (1)$$

where $[M]$ and φ are the concentrations of surfactant and modifier, K_{AS}, K_{AM}, K_{MD}, K_{SD}, K_{ADS}, and K_{ADM}, correspond to the equilibria between solute (A) in stationary phase (S), micelle (M), or bulk water (D). This equation was non-linearly fitted according to the Powell method [14] using the retention data obtained from injections of the vitamin solutions in seven mobile phases containing SDS–pentanol (0.05/2, 0.05/6, 0.1/4, 0.15/2, 0.15/6, 0.15/5, 0.1/2), in concentrations *M* and % (v/v), respectively. They all contained phosphate buffer at pH 3. In all cases, the retention factors (*k*), efficiencies (*N*) were measured according to Foley and Dorsey [15]. Tailing factors (*T*) were measured at 10% of peak. Table 2 shows the coefficients in Eq. (1) for each drug when pentanol was used. These allow the prediction of mobile phase composition containing pentanol for any desired retention time and provide a simple way of optimizing the separation of mixtures.

The accurate prediction of the retention according to Eq. (1) allowed the application of an interpretive procedure to predict the optimal resolution, following a criterion that utilizes the valley-to-peak ratios [16]. The global function of resolution, may vary from 0 to 1, and the proximity to one indicates the performance of the separation. The function was maximized to obtain the optimal mobile phase. Incorporation of the shape of the chromatographic peaks in the optimization procedure improves the

Table 2
Coefficients for Eq. (1)

Vitamin	K_{AS}	K_{AM}	K_{MD}	K_{ADM}	K_{SD}	K_{ADS}
Nicotinamide	39,422.5	1376.8	−16.7	49.0	−16.3	8604.5
Pyridoxine	93,235.5	143.6	−16.7	36.3	−14.0	91,593.4
Pyridoxamine	197.3	105.9	−16.7	−18.0	−16.7	−17.4
Riboflavine	860,263.9	61.7	−17.1	33.0	−9.4	8,098,101.0
Thiamine	209.2	98.2	−16.7	−16.7	−16.6	−15.9

results. The reliable simulation of peak shape for any mobile phase in the variable space was carried out with an asymmetrical Gaussian function where the standard deviation is a first-degree polynomial function [15,17]. The parameters obtained were interpolated from the data obtained in the three experimental mobile phases closest to the simulated mobile phase. Using Eq. (1) and the mathematical treatment here described, the relative global error in the prediction of capacity factors for five vitamins is below 5% for most of the substances studied.

The efficiencies in SDS–pentanol are somewhat greater than those achieved when SDS or SDS–propanol or SDS–butanol mixtures are used. The hydrophilic layer formed by the sulfate head groups of SDS above the surface of the silica influences the retention of the compounds. The hydroxyl groups on the silica surface play a less important role in the separation as a result of SDS adsorption. Since the hydrophilic layer exists above the silica surface, the association kinetics which is controlled primarily by the electrostatic interaction are more facile than ion-exchange processes involving the silanol groups on the silica surface. Furthermore, the interaction of the protonated vitamins at pH 3 with the hydrophilic layer formed by SDS reduces the penetration depth of the compounds into the bonded phase. The net effect is an improvement in efficiency when a micellar mobile phase is utilized since the role of the silanol groups on the silica surface have been diminished with respect to their participation in the retention mechanism. As expected, the elution strength of pentanol was greater. It can be observed that, in the studied concentration ranges, the changes in the retention produced by a change in SDS were higher than those produced by the modifier when butanol was used, but for pentanol the behaviour was opposite. Pentanol wets better the bonded phase than butanol, thereby reducing to a greater degree the amount of SDS adsorbed on the bonded phase. Anyway, the strength of SDS shown in the elution of the vitamins was large, which suggests the large affinity of the compounds for the micelles. The strong retention of the compounds in the surfactant-modified stationary phase is also indicative of the strong association of the vitamins with the surfactant molecules. For this reason the hydrophilic vitamins can only be eluted with adequate retention times only when pentanol is used.

The global resolution diagram, simulated and real chromatograms obtained are depicted in Fig. 1. Fig. 1a shows how resolution values near one (maximum value) can be obtained using mobile phases that contain pentanol in concentrations below 4% (v/v). The best resolution value was obtained in 0.1 *M* SDS–4% (v/v) pentanol ($R=0.999$) with an analysis time of 22 min, and thus this mobile phase was selected as optimum. Fig. 1b shows the simulated chromatogram for the mixture of the five vitamins in the optimum mobile. The agreement between the simulated and experimental chromatograms is optimum (Fig. 1c). The retention times were: riboflavin (1.9), nicotinamide (3.4), pyridoxine (5.2), pyridoxamine (17.6) and thiamine (19.8).

3.2. Temperature, flow and injection volume optimization

In order to reduce the analysis time of the procedure, the parameters temperature, flow and injection volume were studied in the 25 to 55 °C, 1 to 3 ml/min, and 5 to 50 μl ranges, respectively.

When temperature throughout the separation process is varied in the 25 to 55 °C range, the chromatographic parameters efficiency and asymmetry factor remain constant, but retention time of thiamine is reduced by 2.3 or 1 min for nicotinamide. At 45 °C the reduction in retention time for thiamine and the

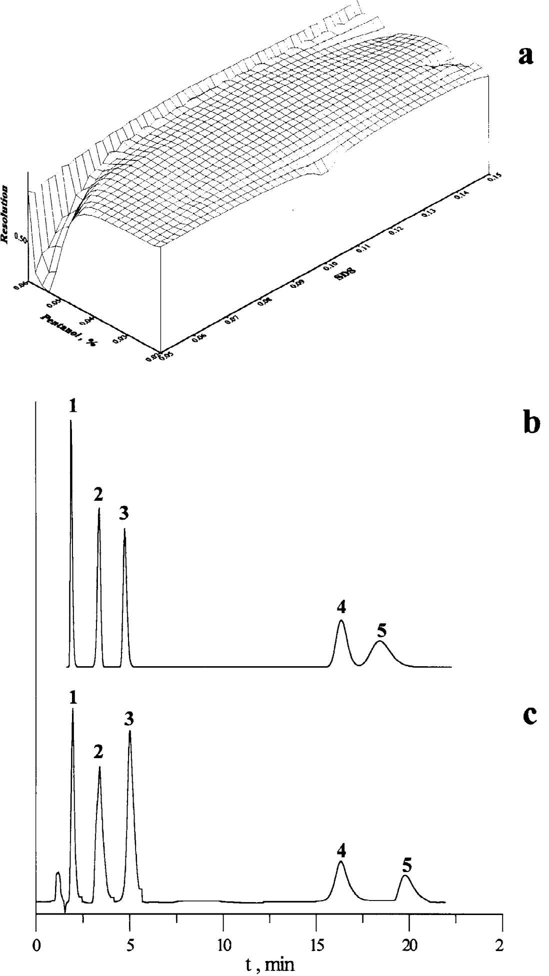

Fig. 1. (a) Global resolution diagram, (b) simulated and (c) real chromatogram for a mixture of riboflavin (1), nicotinamide (2), pyridoxine (3), pyridoxamine (4) and thiamine (5). Work conditions: mobile phase SDS 0.1 M–4% pentanol—pH=3, 20 μl simple, flow-rate 1 ml/min and room temperature). $\lambda=270$ nm (riboflavin, nicotinamide and thiamine), $\lambda=290$ nm (pyridoxine) and $\lambda=325$ nm (pyridoxamine).

most retained compounds was approximately 1 min, and thus this retention time could be reduced to 20 min, with good resolution and the following retention times (min): riboflavin (1.8), nicotinamide (2.9), pyridoxine (4.8), pyridoxamine (16.7) and thiamine (18.7).

To reduce the retention time of pyridoxamine and thiamine we have worked with a flow of 1 ml/min until 6 min, later increasing to values of 1.5, 2 and 3 ml/min. Using 1.5 ml/min the retention time of the thiamine was reduced by 4 min. When 3 ml/min was used the asymmetry factors were higher than 3 and the pyridoxamine–thiamine peaks are overlapped, and the peak of riboflavin appears in the dead volume. These problems are avoided by using a flow of 2 ml/min, and that is why it was selected. With a flow of 1 ml/min up to 6 min and 2 ml/min until the end of chromatogram, the retention times of the vitamins were riboflavin (1.8), nicotinamide (2.9), pyridoxine (4.8), pyridoxamine (10.1) and thiamine (11.2). The retention time of the last substance, and thus the analysis time of the procedure, has been reduced by 7 min.

Finally, the effect of increasing the volume of injection, in the 5 to 50 μl range, was investigated. The area of the peak increases linearly in the range 5 to 20 μl. For volumes above 20 μl two adverse effects are observed due to the sample overloading: a decrease in the resolution and efficiency, and an increase in the asymmetry factors. Thus, an injection volume of 20 μl was selected.

3.3. Figures of merit

Calibration graphs were constructed by triplicate injection of five solutions of the vitamins at increasing concentrations in the 0.5–25 μg/ml range. Table 3 summarizes the parameters of the calibration curves obtained by measuring peak areas for each vitamin eluted with the micellar–organic and aqueous–organic mobile phases. Linear regression coefficients were always $r>0.999$. The limits of detection (LODs) was calculated using the 3s criterion that correspond to a signal equal to three times the standard deviation of the background noise (i.e. the signal-to-noise ratio is equal to 3), and these values appear also in Table 3, and were well below then those required for the analysis of the pharmaceuticals.

Repeatabilities or intra-assay precision (average of ten measurements made the same day), and intermediate precision (average of ten measurements of repeatabilities taken on 10 days over a 3-month period and made by a different analyst, equipment, etc.) are indicated in Table 4 at three different drug concentrations: 0.5, 5 and 25 μg/ml. The relative standard deviations (RSD) were below 3.3%.

Table 3
Slope, intercept, correlation coefficient (r) and LOD (ng/ml) in the calibration of the vitamins eluted with 0.1 *M* SDS–4% (v/v) pentanol—pH 3.0 for the MLC method and 60:40 (v/v) methanol–water/pH 3.0 in the RPLC method

Compound	MLC				RPLC			
	Slope	Intercept	r	LOD	Slope	Intercept	r	LOD
Nicotinamide	57.3±0.1	−46.5±0.5	0.99995	10	100.5±1.8	12.4±11.0	0.9992	12
Pyridoxamine	35.6±0.2	−12.8±0.2	0.99997	5	45.1±0.8	15.9±3.6	0.9991	20
Pyridoxine	60.5±0.1	−11.6±0.4	0.99993	12	15.9±0.6	14.2±1.5	0.9995	12
Riboflavin	264.7±1.4	−42.9±4.0	0.99991	3	134.5±2.6	42.8±3.2	0.9996	4
Thiamine	27.2±0.2	−13.5±1.4	0.99994	20	38.7±0.1	23.0±0.5	0.9991	25

Table 4
Repeatability and intermediate precision (C.V. %, $n=10$) using the optimum mobile phase 0.1 *M* SDS–4% (v/v) pentanol—pH 3.0

Compound	Repeatability			Intermediate precision		
	c_1	c_2	c_3	c_1	c_2	c_3
Nicotinamide	2.1	1.9	1.7	2.3	2.1	1.8
Pyridoxamine	2.6	1.5	1.6	2.5	2.1	1.9
Pyridoxine	1.8	1.8	1.2	2.2	1.9	1.4
Riboflavine	3.0	2.4	1.8	3.3	2.7	2.0
Thiamine	2.3	1.9	1.4	2.5	2.3	1.8

Concentrations were $c_1=0.5$, $c_2=5$ and $c_3=25$ μg/ml.

3.4. Analysis of pharmaceutical preparations

The results obtained in the analysis of six pharmaceutical preparations, which contained the studied vitamins, using 0.1 *M* SDS–4% pentanol and 60:40 (v/v) methanol–water are given in Table 5. Both methods shows the same selectivity. Fig. 2 illustrates the chromatograms of the pharmaceuticals: Becozyme Forte (nicotinamide, pyridoxine, riboflavin and thiamine), Benexol (pyridoxine and thiamine) and Glaan (vitamin B_6, vitamin B_2 and vitamin B_1).

No interference was found from other drugs accompanying the assayed drugs in the pharmaceutical preparations, such as ascorbic acid, biotin, calcium pantothenate, β-caroten, cholecalciferol, cyanocobalamine, L-cystine, folic acid, hesperedin, hydroxicobalamine, menadione, orotic acid, pan-

Table 5
Analysis of multivitamin pharmaceutical preparations

Pharmaceutical (Laboratory)	Composition (mg per capsule, pill or 5 ml syrup)	Amount found (%)±RSD (%) ($n=5$)	
		MLC	RPLC
Albintil	Nicotinamide (12.5),	98.4±0.6	–
(Solvay Pharma)	Pyridoxine hydrochloride (2),	96.5±1.6	92.3±3.6
	Riboflavin sodium phosphate (2),	97.5±2.9	–
	Thiamine hydrochloride (1.5).	99.6±0.5	92.3±0.7
Becozyme C Forte	Nicotinamide (50),	95.4±0.6	–
(Roche)	Pyridoxine hydrochloride (10),	97.6±0.3	97.4±0.2
	Riboflavin (15),	95.3±0.9	–
	Thiamine (15).	91.5±0.6	92.9±3.0
Hidropolivit Mineral	Nicotinamide (DCl) (15),	93.7±3.4	–
(Menari)	Pyridoxine hydrochloride (1),	98.5±0.2	96.4±0.9
	Riboflavin (1),	100.2±2.0	–
	Thiamine (2).	101.5±5.6	99.5±1.2
Glaan	Vitamin B_6 (1.2),	91.3±3.0	90.5±1.9
(Madaus)	Vitamin B_2 (1.6),	94.7±1.6	–
	Vitamin B_1 (1.2).	97.5±0.5	95.6±1.9
Benexol	Pyridoxine hydrochloride (250),	97.9±0.5	97.1±0.4
(Roche)	Thiamine hydrochloride (250).	92.5±0.2	94.8±0.5
Bester	Pyridoxine hydrochloride (150),	105.4±0.7	101.9±1.8
(Salvat)	Thiamine hydrochloride (100).	93.9±1.5	95.3±2.2

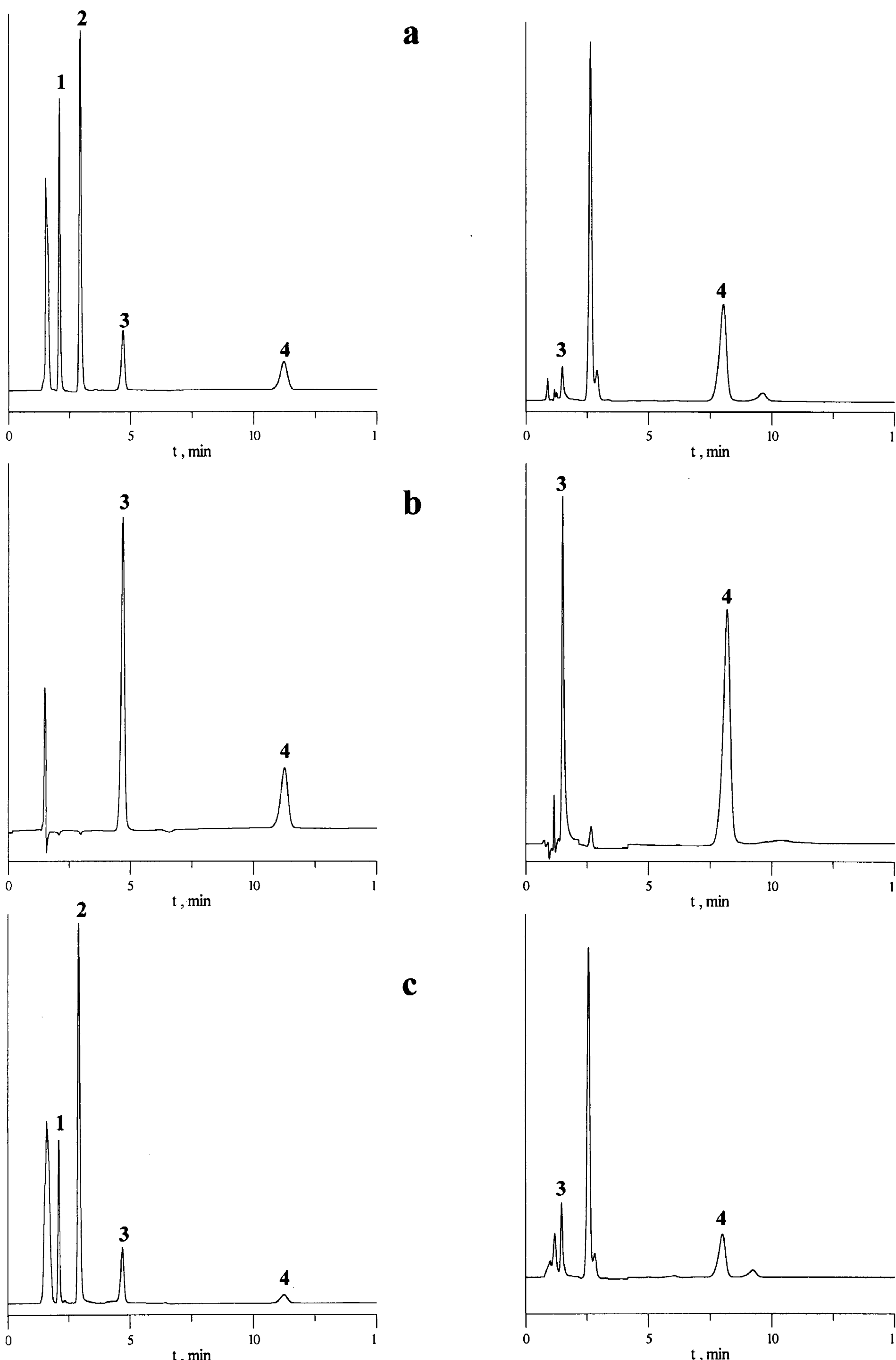

Fig. 2. Chromatograms of the pharmaceuticals in micellar mobile phase (left) and aqueous–organic (right): (a) Becozyme Forte (nicotinamide, pyridoxine, riboflavin and thiamine), (b) Benexol (pyridoxine and thiamine), and (c) Glaan (vitamin B_6, vitamin B_2 and vitamin B_1). Compounds: (1) riboflavin, (2) nicotinamide, (3) pyridoxine and (4) thiamine.

tenol, retinol and α-tocopherol. For most of these compounds the high elution strength of pentanol cause the elution at the beginning of the chromatogram.

The assayed drugs show retention times below 12 min for both micellar and aqueous–organic mobile phases, and the amounts found in the analyses of the pharmaceuticals are similar and in the 91–105% and 90–102% ranges, respectively. The procedure with micellar mobile phases we have proposed has the advantage of using a single mobile phase for the analyses of the five vitamins, allows the direct injection of the samples, avoiding sample pretreatment, extraction procedures, column switching techniques or gradient elution of mobile phases, is simple, rapid, uses a small amount of pentanol (highly retained in the micellar solution, reduces the risk of evaporation, and made the micellar mobile phase stable for a long time), instead of a large amount of methanol, reducing thus the toxicity, flammability, environmental impact and cost of RPLC.

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