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Determination of eight water- and fat-soluble vitamins in multi-vitamin pharmaceutical formulations by high-performance liquid chromatography

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Abstract

In the present work, a reversed-phase high-performance liquid chromatographic procedure has been developed for the determination of water-soluble vitamins (thiamine hydrochloride, pyridoxine hydrochloride, nicotinamide, riboflavin phosphoric ester and cyanocobalamine) and fat-soluble vitamins (retinol palmitate, cholecalciferol, α-tocopherol acetate) in multi-vitamin pharmaceutical formulations. The sample treatment proposed consists of a solid-phase extraction with C_{18} AR cartridges that allow the separation of fat-soluble vitamins, which were retained on the sorbent, from water-soluble vitamins. Afterwards, the water-soluble vitamins were analysed by HPLC on a Nova-Pack C_{18} (150×3.9 mm, 4 μm) analytical column, using CH_3OH–0.05 *M* CH_3COONH_4 as mobile phase The chromatographic analysis of the fat-soluble vitamins was carried out after their sequential elution with methanol and chloroform from C_{18} sorbent, on the above column. The mobile phase employed was MeOH–CH_3CN (95:5, v/v) working at a flow-rate of 2 ml min^{-1} in isocratic mode. The solid-phase extraction for these vitamins had been previously optimised. The experimental variables studied were: application volume, elution solvents and cleaning solutions. The UV–Vis detection of vitamins was made at 270 nm for all the water-soluble vitamins (362 nm for B_{12}) and 285 nm for the water-soluble and fat-soluble vitamins present in real samples at different concentration levels. The accuracy of the method was tested obtaining an average recovery ranging between 78 and 116%.

Keywords: Pharmaceutical analysis; Vitamins

1. Introduction

As its well known, vitamins are a broad group of organic compounds that are minor, but essential, constituents of food required for the normal growth, self-maintenance and functioning of human and animal bodies. These compounds can be classified in two main groups: water-soluble and fat-soluble vitamins. Among water-soluble vitamins, the B group including B_1, B_2, B_6 and B_{12} are the most important. They play different specific and vital functions in metabolism, and their lack or excess produces specific diseases.

Increasing interest in good eating habits in human and also animals, has meant greater awareness of the vital role that vitamins play in growth and health. In addition, the presence of fruits and vegetables in the daily diet and the consume of vitamin-supplemented and preserved foods, has substantially increased. Together with a possible loss through chemical reactions, vitamins might be leached during storage and processing of food. In this sense, it is extremely

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important to have available preparations to replace the possible lack of the vitamins in daily diet which is why multi-vitamin pharmaceuticals are becoming widely employed. These facts, together with the introduction of food labelling regulations, lead to a need for very powerful analytical separation techniques for the quality control of these complex preparations. This has stimulated research on accurate and efficient analytical methods for the determination of vitamins which is problematic because of their instability and the complexity of the matrices in which they are usually analysed.

The standard [1] and official analytical methods [2], which are tedious, sometimes non-specific and time-consuming, involve pre-treatment of the sample through complex chemical, physical or biological reactions to eliminate the interferences commonly found, followed by individual methods for each different vitamin. These methods include spectrophotometric, polarographic, fluorimetric, enzymatic and microbiological procedures.

In contrast, several papers have been published concerning the separation and quantification of vitamins by more simple methodologies. Among them the techniques most widely used are chromatographic, capillary electrophoresis [3–5], and, in particular, HPLC [6], which provides rapid, sensitive and accurate methods for vitamin determination and have the advantages of solvent economy, easy coupling with other techniques, and that small amounts of sample are required.

Individual vitamins can be chromatographed isocratically as well as certain combinations of two or three vitamins; the simultaneous chromatography of more complicated mixtures may require a gradient elution program. Determinations can be carried out, among others by ion-exchange [7], normal-phase [6] or ion-pairing [8–10] chromatography, reversed-phase [11–16] being the most common method. Most of the published methods involve the use of complex buffered mobile phases. Several bonded and stationary phases or column packing materials are developed [17], and several detection methods can be applied, UV–Vis absorbance with a single or variable wavelength or photodiode array [18,19], fluorimetric [20,21], or electrochemical [22,23].

In the present work, a simultaneous determination of water-soluble vitamins [nicotinamide (PP), cyanocobalamine (B_{12}), riboflavin phosphate (B_2), pyridoxine hydrochloride (B_6), thiamine hydrochloride (B_1)], and fat-soluble vitamins [retinyl palmitate (A), α-tocopherol acetate (E), cholecalciferol (D_3)] is proposed. Solid-phase extraction is applied as an isolation method of the two fractions of the vitamins (water-soluble and fat-soluble), in case both of them exist in the same sample [24]. The appropriate conditions of sorbent, adsorption, washing and elution are investigated. Afterwards, high-performance liquid chromatography with solvent programming has been the appropriate tool in overcoming the overlapping of certain pairs of vitamins and also in adjusting the elution volumes of early and late elution peaks. One of the challenges we had was that the vitamins are not found in real samples within a uniform concentration range. Their biopotencies and subsequent 'recommended daily allowances' are widely scattered. Some of the vitamins must therefore exist in the samples in small quantities [25] (B_{12} and D_3) while others are at macroscopic levels. Vitamin B_{12} also has the lack of an adequate chromophore for UV detection, and has to be analysed at 362 nm.

2. Experimental

2.1. Equipment

A liquid chromatograph (Shimadzu, Kyoto, Japan), equipped with two pumps (LC-9A) an UV–Vis spectrophotometer (SPD 6A) and a C-R6A Chromatopac integrator was used to record chromatograms and to calculate peak areas. A Waters liquid chromatograph with two pumps (600E) and autosampler plus (717) and a dual wavelength detector (2487) was also used, acquisition of chromatographic data was made with a Millennium 32 PC programme. A Rheodyne 7725i injection valve with a 100 or 20-μl loop was used.

The HPLC column used was a reversed-phase Nova-Pack C_{18} (150×3.9 mm, 4 μm) from Waters (Milford, MA, USA).

2.2. Extraction columns

Solid-phase extraction (SPE) columns C_{18} AR (30

mg) consisting of glass fiber disks embedded with bonded silica were obtained from SPEC (ANSYS Diagnostics, USA) and used without sample processing station.

2.3. Chemicals and reagents

All solvents were HPLC grade and were employed as supplied by manufacturers. High purity water obtained through a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used in all procedures.

HPLC grade methanol was obtained from Promochem (Wesel, Germany) and from Carlo Erba (Milan, Italy). HPLC grade acetonitrile, chloroform and isopropanol were obtained from Carlo Erba along to ammonium acetate for analysis.

Vitamins B_6, PP, B_1, B_2, and D_3 (cryst.), B_{12}, E and A were of analytical-reagent grade. Vitamin B_2 had a purity of 78%. All the vitamins were supplied by Sigma (St. Louis, MO, USA) and were not further purified.

Fort Dodge kindly supplied multi-vitamin formulation, 'Duphafral Multi', as a commercial preparation and as synthetic preparations too. The composition of this pharmaceutical formulation is shown in Table 1.

2.4. Chromatographic conditions

2.4.1. Water-soluble vitamins

A reversed-phase Nova-Pack C_{18} (150×3.9 mm, 4 μm) column was used for the separation of water-soluble vitamins at room temperature. The mobile phase of the HPLC system consisted of a 0.05 *M* ammonium acetate (solvent A)–methanol (solvent B) gradient that follows: in the range 0–1.5 min, 92.5:7.5; at 1.6 min, 84:16; at 15 min, 70:30. The mobile phase flow-rate was 1 ml min^{-1} and the volume injected 40 μl. Column effluents were monitored at λ=270 nm for all the water-soluble vitamins except for cyanocobalamine where λ=362 nm was used. A liquid chromatograph with a dual wavelength detector was used in some of the experiments.

These conditions were chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution.

Table 1
Components of 'Duphafral Multi'[a]

Components	Concentrations
Vitamin B_1	10 mg ml^{-1}
Vitamin B_2 phosphate	5 mg ml^{-1}
Vitamin B_6	3 mg ml^{-1}
Vitamin PP	35 mg ml^{-1}
Dexpanthenol	25 mg ml^{-1}
Vitamin B_{12}	20 μg ml^{-1}
Vitamin A palmitate	9 mg ml^{-1}
Vitamin E acetate	20 mg ml^{-1}
Vitamin D_3	0.2 mg ml^{-1}

[a] Excipients and antioxidants: citric acid, tioglycolic acid, phenol, benzylic alcohol, EDTA, 2[3]-*tert*.-butyl-4-hyroxyanisole (BHA), 2,6-di-*tert*.-butyl-*p*-cresol (BHT).

2.4.2. Fat-soluble vitamins

A reversed-phase Nova-Pack C_{18} (150×3.9 mm, 4 μm) column was used for the separation of the fat-soluble vitamins at room temperature. A variety of mobile phases were tested in order to find out the optimum chromatographic conditions for the analysis of the vitamins. The final mobile phase selected was MeOH–CH_3CN (95:5, v/v) using a flow-rate of 2 ml min^{-1} and an injection volume of 20 μl. Column effluents were monitored at 285 nm.

The mobile phases were filtered through a 0.45 μm nylon membrane to remove the impurities that might be present and degassed by sonication prior to use.

3. Procedure

3.1. Preparation of standard solutions

Using the same proportions of all the vitamins as those in the pharmaceutical formulation investigated, stock and standard solutions of water-soluble vitamins and fat-soluble vitamins were prepared every third day in water and methanol, respectively, by accurately weighting and dissolving all the vitamins. A separated stock solution of vitamin B_{12} was needed because of the difference between the level of this vitamin and the others in the pharmaceutical formulation (see Table 1).

The working standards were prepared by appropriate dilution of the concentrated stock standard solutions. These solutions were sonicated and stored

in dark glass flasks, in order to protect them from light, and kept under refrigeration.

After optimisation, the chromatographic procedure was finally applied to a synthetic and a commercial formulation.

3.2. Solid-phase extraction

The SPE strategy generally comprises the isolation (and concentration) of the analytes from a complex matrix by adsorption onto an appropriate sorbent, the removal of interfering impurities by washing with a suitable solvent system and then the selective recovery of the retained analytes with a modified solvent system of suitable elution strength. If necessary, this process can be modified by selection of sorbent and solvent systems, so that the interfering components are retained by a sorbent and the analytes are then recovered in the eluate.

The sample is dissolved in a polar solvent and then the solution is passed through the reversed-phase cartridge; polar organic compounds will pass through the sorbent, while less polar constituents will be retained. A solvent of intermediate polarity is used to wash off the material that is more polar than the one retained. Then, this is eluted with a less polar solvent, leaving the hydrophobic material retained on the sorbent

The volume of sample applied to the cartridge and its degree of dilution were optimised in order to avoid saturation of the sorbent. The suitability of different washing and elution solvents was also assessed, as well as the volumes required. The best recoveries where achieved with the following conditions.

Solid-phase extraction cartridges (SPEC C_{18} AR 3 ml) were conditioned immediately prior to use with 500 μl of methanol followed by 500 μl of deionized water.

In order to optimise the separation between the two groups of vitamins, several injection volumes as well as elution and washing solvents were tested. Analysis of a synthetic sample showed that a washing step prior to the elution is necessary for a quantitative recovery of water-soluble vitamins. The composition of this washing solution was investigated in order to obtain the best recoveries for the water-soluble vitamins. Elution solutions of different solvent strengths compatible with the mobile phase were tested to remove fat-soluble vitamins from the cartridge (Table 2). We found that $CHCl_3$ is the most efficient although the low polarity of vitamin D_3 only allows for 78% recovery.

Table 2
Percentages of recovery of fat-soluble vitamins from 0.200 ml of sample with 3 ml of different elution solvents[a]

Elution solvent	Vitamin (%)		
	A	E	D_3
DMSO	41	30	20
Dioxane	60	35	22
THF	118	95	38
CH_3CN	113	98	44
MeOH	100	104	40
THF–CH_3CN(1:1)	115	103	45
THF–$CHCl_3$ (2:3)	100	102	69
$CHCl_3$	100	100	78

[a] DMSO, dimethyl sulfoxide; THF, tetrahydrofuran.

The sample (injection solution) was diluted one part in ten with water and spiked with 1 mg l^{-1} of B_{12}. It was necessary because the amount of B_{12} remaining after dilution and SPE was under the detection limit of the chromatographic method (the amount of B_{12} is much lower as the amounts of the other vitamins). Then, a 200-μl aliquot of sample were applied to the column. The fat-soluble vitamins were retained on the sorbent, while the water-soluble vitamins passed unretained. The water-soluble vitamins were collected into 10-ml volumetric flasks and diluted to volume with water. Then, the column was washed with one column volume of deionized water and one column volume of MeOH–water (6:4, v/v) in order to remove all the water-soluble vitamins from the column. The column was dried and the fat-soluble vitamins were eluted by applying one column volume of methanol followed by one column volume of chloroform collecting them into 10-ml volumetric flasks and diluted to volume with methanol. All solutions were allowed to gravity flow.

Aliquots of 40 μl for the water-soluble vitamins and of 20 μl for the fat-soluble vitamins were injected into the HPLC column.

4. Results and discussion

A solution of each single vitamin was injected

separately to determine the individual retention times and a synthetic multi-vitamin standard mixture solution was similarly treated to determine the degree of resolution of its components.

4.1. Water-soluble vitamins

Standards of the vitamins were chromatographed separately in order to determine the retention time for each of them. A standard mixture of vitamins was then analysed as a mixture and chromatographic conditions were optimised to maximise peak resolution. Elution of the vitamins from the column was in order of decreasing polarity of the mobile phase used: as the proportion of methanol increased the retention times for the studied vitamins decreased and some of them eluted with the solvent front. The greater the proportion of ammonium acetate the better peak shapes and resolution of the studied vitamins. Satisfactory separation was achieved using the gradient 0.05 *M* ammonium acetate–methanol changing from (92.5:7.5, v/v) to (70:30, v/v). Since vitamins B_2 and B_{12} are retained on the column quite firmly, they elute only with the use of 30% aqueous methanol, while the others elute with ammonium acetate.

The separation pattern of the water-soluble vitamins – B_6 showed at t_R~2.5 min, B_1 at ~2.85 min, PP at ~3.2 min, B_2 at 10 min and B_{12} at ~13 min – well separated in that order, is shown in Fig. 1a. Although day-to-day minor variations appeared in retention times, which are subject to temperature fluctuations, the sequence of elution remained unaltered. As can be seen, the peaks of the majority of vitamins present in the mixture can be distinguished from the baseline and they elute as sharp peaks.

Cyanocobalamine exists in small concentrations compared to the other vitamins and absorbs poorly above 230 nm; it is not visualised. This particular problem was overcome by working at 362 nm where only B_2, B_6 and B_{12} absorb (Fig. 2a). As was explained above, it was necessary to fortify the samples prior to their analysis, because the final concentration of B_{12} obtained after the solid-phase extraction is lower than the detection limit of the chromatographic method.

In order to test the applicability of this procedure to a commercial vitamin formulation, 'Duphafral Multi' was chromatographed as above (Figs. 1b and 2b). Integration of the separated peak areas was done with the integrator or the computer programme and each vitamin was determined using the peak area–concentration relationship obtained in the standardisation step. The sample peaks were identified by comparing both the relative retention times and the UV–Vis spectrum of each one with those of the standard reference vitamins; and their quantification is carried out by the external standard method. These results are all summarised in Table 3. The protocol affords reproducible quantification of the different vitamins with error less than 10% [26], which is the normal level in any pharmaceutical quality control.

4.2. Fat-soluble vitamins

The same procedure described for the calibration of the method for the water-soluble vitamins was followed for fat-soluble vitamins as well. Elution of the fat-soluble vitamins from the column was tested with several mobile phases, upon all, those containing methanol and acetonitrile gave the best results. An increase in the proportion of acetonitrile means increase of the retention times. Methanol–acetonitrile (95:5, v/v) at 285 nm was chosen because it gave good resolution of the vitamins A (t_R~7.3 min), E (t_R~2.7 min) and D_3 (t_R~2.1 min) in less than 10 min as can be seen in Fig. 3. A good agreement between the concentration values of the reference samples and the amounts of vitamins found after analysis has been obtained with the proposed method (Table 3).

5. Validation

This method has been validated with respect to reproducibility, linearity and accuracy by using a reference sample supplied by Fort Dodge.

5.1. Reproducibility

Complete triplicate analysis was performed on all samples to allow the calculation of average deviations as a measurement of chromatographic reproducibility.

The relative standard deviations obtained in the analysis of the vitamins can be found in Table 3.

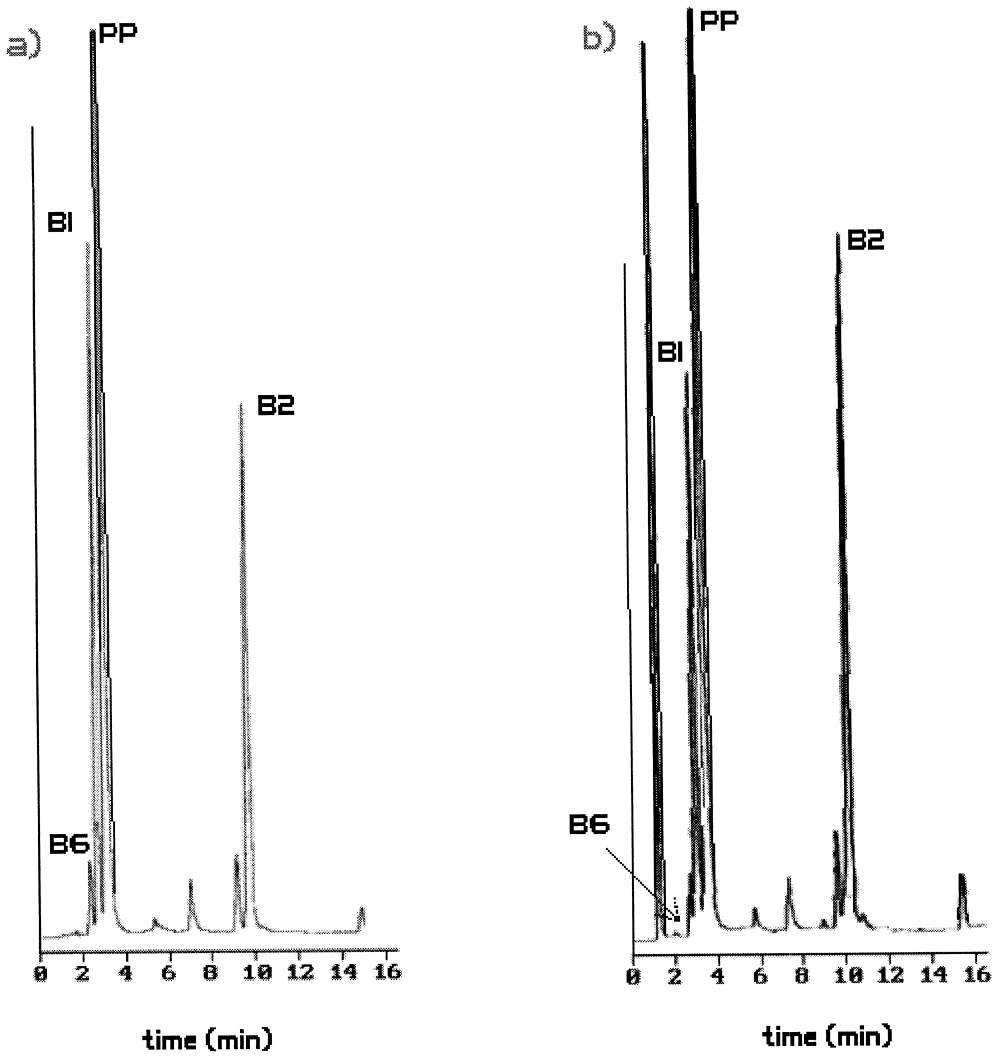

Fig. 1. High-performance liquid chromatograms (At.=7) of (a) standard solution containing 7.10 mg l^{-1} (2.56 min) of B_6, 19.72 mg l^{-1} (2.91 min) of B_1, 70.12 mg l^{-1} (3.36 min) of PP, 8.71 mg l^{-1} (10.52 min) of B_2, and 0.04 mg l^{-1} of B_{12}; and (b) sample solution containing 3.45 mg l^{-1} (2.51 min) of B_6, 37.90 mg l^{-1} (2.81 min) of B_1, 129.60 mg l^{-1} (3.23 min) of PP, 23.08 mg l^{-1} (10.16 min) of B_2 and 0.04 mg l^{-1} of B_{12} (spiked with 1 mg l^{-1}). Both chromatograms obtained by 40-μl loop injection at λ=270 nm.

5.2. Linear range

Linearity was obtained in the range of the standard concentration for each vitamin (Table 4). A series of five solutions at low and high concentration levels (from 50 to 150% of the nominal concentration levels) were prepared, each solution was injected three times and the regression was calculated by the method of least-squares. Peak areas were calculated and the results interpolated on the calibration graph for each vitamin.

5.3. Limits of detection

The detection limits were assessed using external

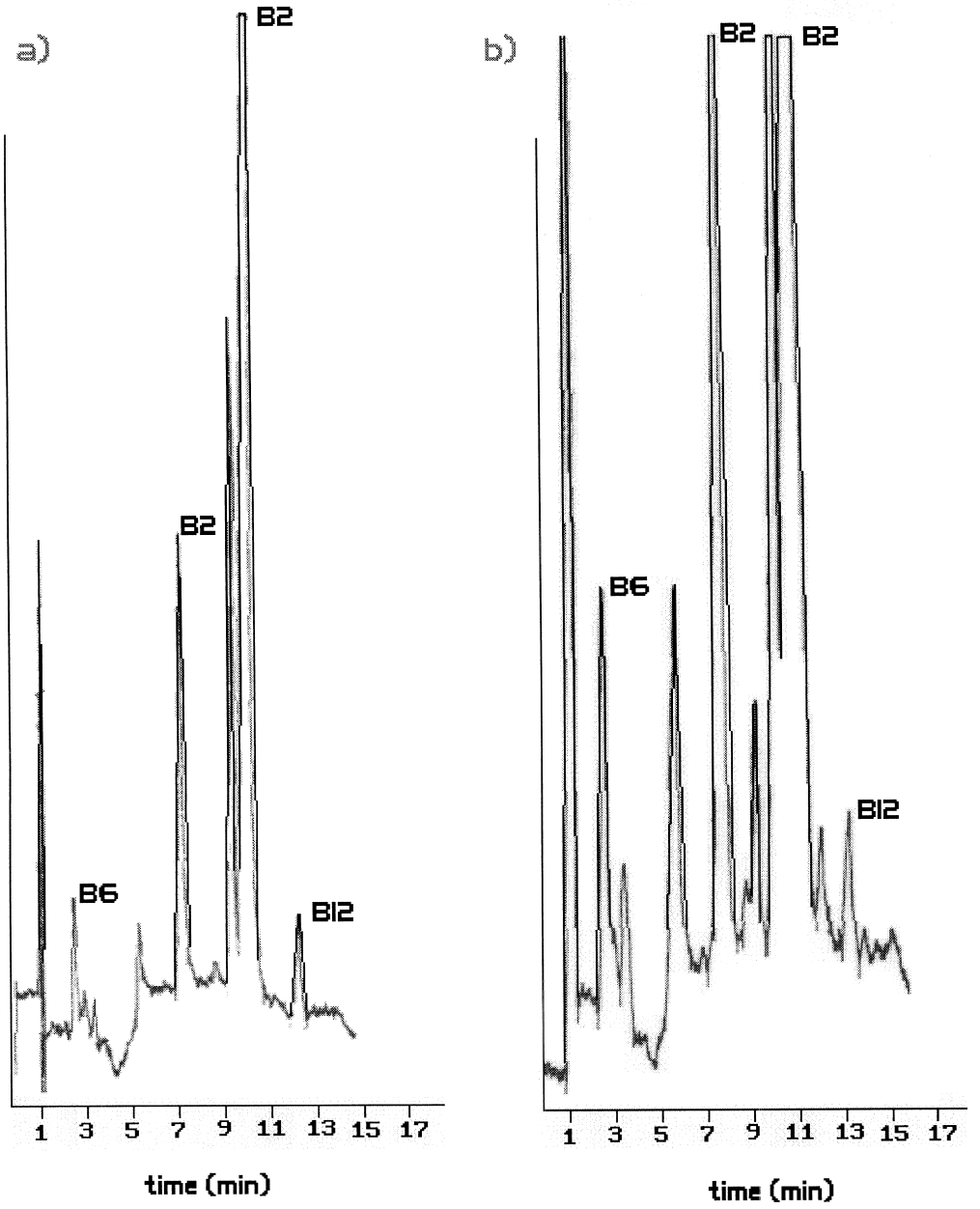

Fig. 2. High-performance liquid chromatograms (At.=0) of (a) standard solution containing 7.10 mg l^{-1} (2.56 min) of B_6, 19.72 mg l^{-1} of B_1, 70.12 mg l^{-1} of PP, 8.71 mg l^{-1} (10.52 min) of B_2 and 0.04 mg l^{-1} (13.39 min) of B_{12}; and (b) sample solution containing 3.45 mg l^{-1} (2.51 min) of B_6, 37.90 mg l^{-1} of B_1, 129.60 mg l^{-1} of PP, 23.08 mg l^{-1} (10.16 min) of B_2 and 0.04 mg l^{-1} (13.41 min) of B_{12} (spiked with 1 mg l^{-1}). Both chromatograms obtained by 40-μl loop injection at λ=362 nm.

Table 3
Quantification of water-soluble and fat-soluble vitamins

Vitamin	Vitamin content (mg l^{-1})	Mean[a] (mg l^{-1})	SD (mg l^{-1})	RSD (%)	Recovery (%)
B_6	6	5.90	0.37	6.23	98
B_1	20	20.03	0.63	3.16	100
PP	70	76.96	1.82	2.37	110
B_2	10	11.60	0.19	1.68	116
D_3	0.4	0.31	0.01	2.31	78
E	40	40.17	0.66	1.64	100
A	18	17.90	0.57	1.72	100
B_{12}	0.04+ 0.02 (spiked)	0.057	0.003	6.02	96

[a] Complete analysis was performed in triplicate.

Table 4
Linearity and detection limits for the water-soluble and fat-soluble vitamins

Vitamin	Linear range (mg l^{-1})	R^2	LOD[a] (mg l^{-1})
B_6	1.78–14.20	0.9996	1.37
B_1	4.93–39.44	0.9998	3.18
PP	17.53–140.24	0.9999	9.92
B_2	2.18–17.43	0.9998	1.84
B_{12}	0.04–0.12	0.9998	0.04[b]
D_3	0.16–1.30	0.9998	0.05
E	10.15–81.20	0.9998	3.09
A	4.94–39.49	0.9998	5.00

[a] LOD, limit of detection.
[b] Quantification limit.

standards. Those are considered to be the quantities that are producing a signal of peak height three times the size of background noise. The obtained values are collected in Table 4.

5.4. Precision

Method precision was determined by measuring repeatability and intermediate precision (between-

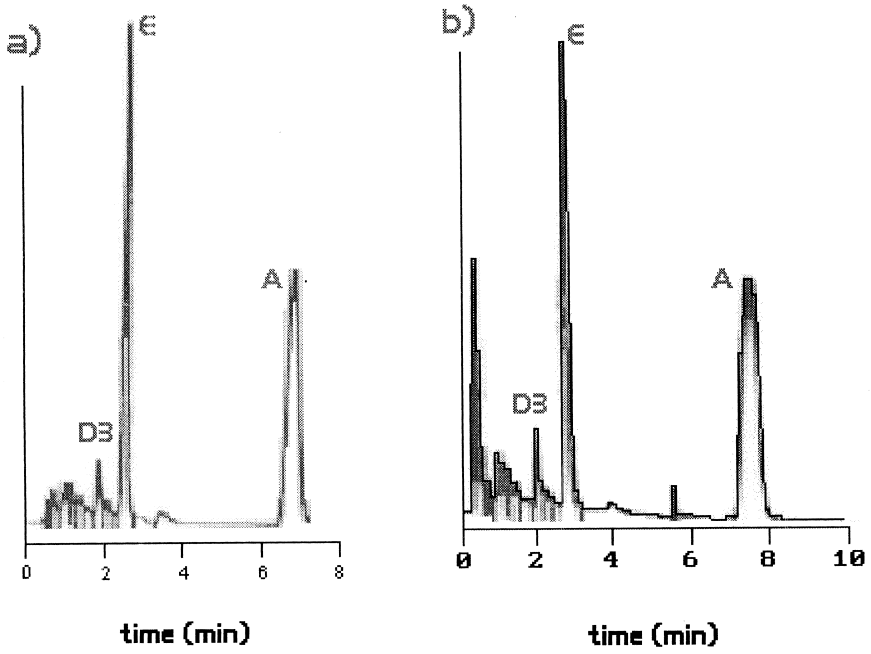

Fig. 3. High-performance liquid chromatograms (At.=3) of (a) a standard solution containing 0.65 mg l^{-1} (2.10 min) of D_3, 40.60 mg l^{-1} (2.67 min) of E, 19.74 mg l^{-1} (7.27 min) of A; and (b) a sample containing 0.97 mg l^{-1} (2.10 min) of D_3, 45.31 mg l^{-1} (2.69 min) of E and 22.52 mg l^{-1} (7.37 min) of A obtained by 20-μl loop injection at λ=285 nm.

day precision or time-different intermediate precision) for each vitamin during 3 days at the same concentration levels. The variation coefficients varied from 1.5 to 5.7%.

6. Conclusions

This work proposes a new method for the separation and quantification of five water-soluble (B_6, B_1, PP, B_2, B_{12}) and three fat-soluble vitamins (A, E, D_3). Solid-phase extraction proved to be an effective tool for performing adequate separation of the two groups of vitamins while HPLC provided a fast, accurate, and reliable method for their determination, with recoveries ranging from 78 to 116%. The binary eluent system used for water-soluble vitamins and the isocratic eluent system used for fat-soluble vitamins yielded low detection limits, good sensitivity and resolution within a minimum analysis time of 15 min for the fraction of water-soluble vitamins and 8 min for the fat-soluble vitamins.

The problems due to the different levels of concentration especially between B_{12} and the rest of the vitamins are solved spiking the samples with a standard solution of this vitamin, prior to the analysis.

The simplicity of the procedure should make it highly desirable for quality control of multi-vitamin products in the pharmaceutical and health food industries.

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