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Development and Analytical Validation of the Methodology for Vitamins in Tablets by Ultra-Performance Liquid Chromatography

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Abstract

In the present study, we developed a reliable and robust chromatographic method for the quantification of multivitamins in tablet samples by ultra-performance liquid chromatography (UPLC) with photodiode array detection. The vitamins nicotinamide, pyridoxine, riboflavin, and thiamin were analyzed and quantified in a total analysis time of 2.5 minutes, using hydrophilic interaction liquid chromatography stationary phase. Tocopherol acetate and cyanocobalamin were analyzed and quantified in a total analysis time of 2.5 minutes, using reversed-phase (RP)-UPLC. The analysis time reported here is lower than that of similar methods reported in the literature for single vitamin determination. The method linearity exhibits a good correlation coefficient (R^2 = 0.998) with the relative residual standard deviation in the acceptable limit of 2.0%. The developed methods were validated, and the results demonstrated that the proposed analytical method showed to be selective, sensitive, accurate, and robust for the quantification of evaluated vitamins in multivitamin tablets. The work was fully developed in the quality control laboratory of a pharmaceutical industry in the Agroindustrial District of Anápolis (DAIA, Goiás, Brazil), where the product is manufactured.

Key words: UPLC, Multivitamin Tablets, Hydrophilic Interaction Liquid Chromatography, Analytical Validation

Introduction

Vitamins are important organic compounds synthesized by the body; their existence was reported in the great voyages period and was related to crews' diseases due to poor nutrition related to diets without fruits or vegetables. Advances in basic science in the 19th century made possible the discovery of vitamins, their main sources, and many diseases related to their deficiency (1), as well as the establishment of artificial supplements that can complement the intake of such compounds (2).

Currently, supplements are used to treat vitamin deficiency and marketed in several dosage forms, alone or in combination in the formulation, with different application requirements (3). In accordance with pertinent legal provisions, such as the Resolution number 17 of the Collegiate Board of the National Health Surveillance Agency of Brazil, which deals with pharmaceutical good manufacturing practices, to confirm the safety and effectiveness of pharmaceuticals, manufacturers must evaluate their physical and chemical characteristics in vitro, to ensure the identity, quality, and safety of the product purchased by the consumer (4).

According to the United States Pharmacopeia (3,5) the official analytical methods are tedious and involve sample pretreatment to eliminate the interferences. Besides this, it is related to an individual analytical technique for each different vitamin. The usual methods include photometric (5–7), polarographic (8), and fluorometric (9–11), besides the chromatographic (12) and electrophoretic, methods (13). The chromatographic separations of water-soluble vitamins are described in the literature using neither normal phase or reversed-phase modes with ultraviolet (UV) (14–17), photodiode array (DAD) (18,19), or mass spectrometry (20,21) detection.

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Ultra-performance liquid chromatography (UPLC) is a separation technique that uses reduced chromatographic columns usually packed with sub 2 μm resulting in shorter analysis time, higher resolution, and less solvent consumption and decreases the time and cost of analysis when compared to traditional high-performance liquid chromatography, which has been considered an ambient friendly technique (22). Another chromatographic method to determine water-soluble vitamins in tropical fruits is UPC^2 (ultra-performance convergence chromatography), which was reported in (23). The literature presents a few methods for determining the content of vitamins in tablet dosage specially using UPLC technologies. Beside this, almost UPLC-published methods often use a mass spectrometry detector (20,24). Published vitamin determination methodologies usually cover only one vitamin, most of them in food matrixes and do not adequately evaluate multivitamin formulation. Also, these published methodologies showed long running times, a lack of selectivity or reproducibility, and sometimes use of expensive techniques such as mass spectrometry (15,18,21,24–28). The separation of water-soluble vitamins in different matrixes is frequently done using reversed-phase high-performance liquid chromatography with the C-18 column as a stationary phase, but there are a few papers that use HILIC to quantify the mix of B vitamins and tocopherol acetate together, and in most of them the retention times for this compounds are between 3.5 and 40 minutes (21,28–30).

Here, we present a study of UPLC separation and quantification of nicotinamide, pyridoxine, riboflavin, and thiamine, in multivitamin tablets, using the hydrophilic interaction liquid chromatography (HILIC) stationary phase, with diode array detection (DAD). The separation of tocopherol acetate and cyanocobalamin was performed by RP-UPLC with the DAD detector. To the best of our knowledge, HILIC is usually used for single-vitamin determination in pure standard or food samples (21,28–30). In quality control laboratories of raw materials and products in pharmaceutical industries, the development of fast, safe, reproducible, and economical methods has been desirable. To the best of our knowledge, this is the first time that a validated methodology for water-soluble vitamins in multivitamin tablets with short-time methodology (2.5 minutes) using the HILIC column was proposed. This total analysis time is lower than that in those reported in the literature for the analysis of water-soluble vitamins (11,16,17,21,28–31). The study present here was fully developed in the quality control laboratory of a pharmaceutical industry in the Agroindustrial district of Anápolis (DAIA, Goiás, Brazil), where the product is manufactured. The UPLC/DAD method was developed and validated and is currently being used in the industry to determine the content of water-soluble vitamins in multivitamin tablets in the quality control laboratory.

Experimental

Test sample and placebo

A single pilot batch of multivitamin tablets was produced and used as a test material for the development and validation of the analytical method. The constituents of the tablet and their respective roles in the formulation are as follows: microcrystalline cellulose PH 200—suspending agent, adsorbent; copovidone—binder; sorbitol powder—diluent and sweetening agent; colloidal silicon dioxide—sliding; magnesium stearate—lubricant; polyvinyl alcohol (PVA), talc—polymer; dyes Red 40 aluminum lacquer and bright aluminum lacquer blue—colors. The vitamins and their respective dosage per tablet are as follows: thiamine, vitamin B_1—10.5 mg; riboflavin, vitamin B_2–10.5 mg; nicotinamide, vitamin PP—10.5 mg; pyridoxine, vitamin B_6–10.5 mg; cyanocobalamin, vitamin B_{12}–10.5 μg; tocopherol acetate, vitamin E—10.5 mg. The average weight of the tablets obtained by weighing of 20 units was 980.0 mg. This amount was used in the analytical validation tests.

Reagents

The reagents used for development and validation of the methods were acetonitrile and methanol, HPLC grade, from Merck (Darmstadt, Germany); ammonium acetate and ammonium hydroxide, analytical grade, from JT Baker (NJ); and formic acid, analytical grade, from Merck (Darmstadt, Germany). The water used in analytical determinations was of HPLC grade, prepared by a purification system with two modules: Elix® 35 and Milli-Q Gradient, both from Millipore, France. Vitamin standards were obtained from USP (United States Pharmacopeia—Rockville, MD).

Instruments

The weight measurements were performed using an analytical balance of Toledo brand, model OHAUS Adventurer AR2140, accurate to 0.1 mg, which was checked daily; the ultrasound machine was of brand Unique, USC-5050A model; the bath was of brand NovaEtica, model 314/10D; and the pH meter was of Micronal brand, B474 model.

Sample preparation

Tocopherol acetate content analysis. Standard preparation. Tocopherol acetate (52.5 mg) was weighed and transferred to a 50 ml beaker, to which 5.0 ml of water was added. The mixture was placed in a water bath for 10 minutes at 40 °C and after cooling (room temperature) was transferred to a 50 ml volumetric flask. The residue in the beaker was rinsed twice with 10.0 ml of methanol and transferred to the flask. The flask was made up to the final volume with methanol. After 15 minutes in the ultrasound bath, 5.0 ml of the solution was pipetted and transferred to a 50 ml flask. The flask volume was completed with methanol. An aliquot of this solution was filtered through a 0.45 μm membrane directly in the vial (concentration: 0.105 mg/ml).

Preparation of samples. Finely powdered tablets (980.0 mg, equivalent to 10.5 mg of tocopherol acetate) was weighed and transferred to a 100 ml beaker. To this, 5.0 ml of water was added, and it was then placed in the water bath at 40 °C for 10 minutes. The content of the beaker was transferred to a 100 ml volumetric flask. The beaker was rinsed twice with 10.0 ml of methanol and this was transferred to the flask. The flask volume was completed with methanol. The mixture was then placed in the ultrasonic bath for 15 minutes and filtered using a membrane of 0.45 μm directly in the vial (concentration: 0.105 mg/ml).

Analysis of cyanocobalamin content. Standard preparation. Cyanocobalamin (21.0 mg) was weighed and transferred to a 100 ml amber volumetric flask. The volume was completed with water and stirred until completely dissolved. Then, 2.0 ml of the solution was pipetted and transferred to a 100 ml amber volumetric flask and completed to volume with water. An aliquot of the solution was filtered through a 0.45 μm membrane directly into an amber vial (concentration: 0.0042 mg/ml).

Sample preparation. Finely powdered tablets (980.0 mg, equivalent to 0.105 mg cyanocobalamin) was weighed and transferred to a 30 ml amber bottle. To this, 25.0 ml of water was added by a volumetric pipette before being stirred vigorously for 2 minutes while

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observing whether complete disintegration of lumps occurred. The mixture was placed in the ultrasonic bath for 15 minutes. An aliquot of the solution was filtered using a 0.45 μm membrane directly into an amber vial (concentration: 0.0042 mg/ml).

Content analysis of vitamins: nicotinamide, pyridoxine, riboflavin, and thiamine. For the extraction of vitamins and solubilization of the standards, we used a diluent with the following composition: 37.5% (vol/vol) of acetonitrile, 12.5% (vol/vol) methanol, 49.9% (vol/vol) of water, and 0.1% (vol/vol) formic acid.

Standard preparation. Each of the vitamin (26.25 mg) was weighed and transferred to a 50 ml beaker. Then, 25.0 ml of diluent was added, and the mixture was placed in a water bath maintained at 60 °C for 10 minutes until the solution remained translucent, indicating complete solubilization of the vitamins. After cooling, the solution was transferred to a 50 ml volumetric flask, and the beaker was rinsed twice with 10.0 ml of diluent, transferring the rinses to the flask and making the solution up to a final volume with the same diluent. The solution was homogenized, and 5.0 ml was pipetted and transferred to another 50 ml volumetric flask, which was then completed with mobile phase A (described in the "Chromatographic conditions" section). An aliquot of this homogenized solution was filtered through a membrane of 0.45 μm directly to the vial (concentration 0.0525 mg/ml of each of the vitamins).

Sample preparation. Finely powdered tablets (980.0 mg, equivalent to 10.5 mg of each of vitamins) was weighed and transferred to a 50 ml beaker. To this, 20.0 ml of diluent was added, and the solution was placed in a water bath maintained at 60 °C for 10 minutes. After cooling, the solution was transferred to a 200 ml volumetric flask. The beaker was rinsed twice with 10.0 ml of mobile phase A, and the rinses were transferred to the flask. The flask volume was completed with the same solvent. The solution was homogenized, and an aliquot was filtered through a membrane of 0.45 μm directly to the vial (concentration 0.0525 mg/ml of each of the vitamins).

Placebo solution preparation. Tablets for placebo tests were prepared by the pharmaceutical development sector of the manufacturing industry, with the same design and process used in the preparation of the product, excluding the vitamins. For each analysis, the placebo solution was prepared in the same manner as that in sample preparation, except that instead of the sample, an equivalent mass of the placebo was weighed, and the corresponding masses of vitamins not analyzed by the method concerned were analyzed. Thus, in the preparation of tocopherol acetate placebo, 937.9 mg of placebo; 10.5 mg of nicotinamide, pyridoxine, riboflavin, and thiamine; and 0.1 mg cyanocobalamin were weighed. In the preparation of cyanocobalamin placebo, 937.9 mg of placebo and 10.5 mg of nicotinamide, pyridoxine, riboflavin, thiamine, and tocopherol acetate were weighed. In the analysis of nicotinamide, pyridoxine, riboflavin, and thiamine, 937.9 mg of placebo, 10.5 mg of tocopherol acetate, and 0.1 mg cyanocobalamin were weighed.

Chromatographic conditions. The chromatography analyses were performed on a UPLC Acquity model chromatograph, manufactured by Waters Corporation, composed of a quaternary solvent pump, Acquity Class H model, an autosampler, Sample Manager FTN model, and a photodiode array detector, PDA eλ model. The collected data were then processed using the Software Empower 2, developed by Waters Corporation (32).

For tocopherol acetate analysis, an Acquity UPLC BEH C18 column, 2.1 mm inner in diameter and 50 mm in length and packed with particles 1.7 μm in diameter, was used. As a mobile phase, acetonitrile was used at a flow rate of 0.5 ml/min. The injection volume of the samples was 1.0 μl, and the chromatographic analysis time was 2.5 minutes. The wavelength of analyses was 285.5 nm. The column temperature was maintained at 30 °C, and the sample compartment was maintained at 10 °C.

The column used in the determination of cyanocobalamin analysis was the same as that employed in the analysis of tocopherol acetate. The mobile phase used was a mixture of water and methanol at a ratio of 80 to 20 at a flow rate of 0.5 ml/min. The injection volume was 4.0 μl, and analysis time was 2.5 minutes. The wavelength of analyses was 550.0 nm. The column temperature was maintained at 30 °C, and the compartment containing samples was cooled at 10 °C.

For nicotinamide, pyridoxine, riboflavin, and thiamine analysis, the Acquity UPLC BEH amide column, 2.1 mm in internal diameter and 50 mm in length and packed with particles of 1.7 μm in diameter, was used. Separation was achieved by adopting the mobile phase gradient as follows: from 0.0 to 0.2 minutes, a ratio of 90:10 (phases A: B) was maintained; from 0.2 to 2.0 minutes, a linear gradient to 70:30 ratio (phase A: B) was used; and from 2.0 to 2.5 minutes, the column returned to the initial condition of a linear gradient. The injection volume of the samples was 1.0 μl. The column temperature was maintained at 30 °C, and the sample compartment was cooled to 10 °C. The most suitable wavelength for the detection and quantification of the four active components was 265 nm at a data capture rate of 20 points per second. The mobile phase flow rate was maintained at 0.5 ml/min. The preparation of mobile phases A and B is described as follows.

1. Mobile phase A: ammonium acetate was added to a mixture of 900 ml of acetonitrile and 100 ml of water to give a concentration of 0.01 mol/L. The pH of this solution was adjusted to 9.0 with ammonium hydroxide.
2. Mobile phase B: ammonium acetate was added to the mixture of 500 ml of acetonitrile and 500 ml of water to have a concentration of 0.01 mol/L. The pH of this solution was adjusted to 9.0 with ammonium hydroxide.

Tests to validate the analytical methodologies

System suitability. To carry out this evaluation, standard solutions were injected five times.

Specificity. The solutions (standard, sample, placebo, diluent, and mobile phase) were injected in triplicate for standard solution and duplicate for the other solutions, and their results were compared. The spectral scan was performed on these injections to determine the spectral purity of the peaks by using a photodiode array detector collecting the absorbance in the wavelength range 200 to 400 nm (200 to 700 nm in cyanocobalamin analysis).

Stability of solution. To study the stability of the analytical solutions, standard, and sample, they were analyzed in duplicate injections immediately after its preparation and reexamined at 6, 12, and 18 hours elapsed from the preparation, being maintained at ambient temperature at light shelter.

Linearity. The linearity test was designed to cover both the range required for the evaluation of the content as to the uniformity test.

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Table 1. Variation of Conditions Used to Evaluate the Robustness

Method/Vitamin	Type of Variation	Variation Level
Nicotinamide, pyridoxine, riboflavin, and thiamine	Mobile phase flow rate	± 2.0%
	Mobile phase composition	± 1.0%
	Chromatograph column batch	3 different batches
	Column temperature	± 3 °C
	Mobile phase pH	± 0.20 pH units
Tocopherol acetate	Mobile phase flow rate	± 2.0%
	Chromatograph column batch	3 different batches
	Column temperature	± 3 °C
Cyanocobalamin	Mobile phase flow rate	± 2.0%
	Mobile phase composition	± 1.0%
	Chromatograph column batch	3 different batches
	Column temperature	± 3 °C

Vitamin standard solutions were prepared at concentrations corresponding to 70, 80, 90, 100, 110, 120, and 130% of the concentration of the vitamins in the sample solution, starting from the standard stock solution and making up the required dilutions. Each solution, corresponding to each level, was injected in triplicate.

Quantification limit and detection limit. The quantification and detection limits were determined just for cyanocobalamin due to its presence in the formulation in a critical concentration. They were determined by means of three curves of each of the vitamins. Because the method works near the range of the quantification limit, standard solutions were prepared at concentrations corresponding to 70, 80, 90, 100, 110, 120, and 130% of the concentration of the vitamin in the sample solution, starting from the standard stock solution and making up the required dilutions. The value of limits was calculated as follows:

$$DL = {}^{3.3 \times \sigma}\big/_{S} \quad (1)$$

$$QL = {}^{10 \times \sigma}\big/_{S} \quad (2)$$

where

σ = the standard deviation of y-intercepts of regression lines

S = the slope of the calibration curve estimated from the calibration curve of the analyte

DL = the detection limit

QL = the quantification limit

Precision. For each method, two analysts, on different days, prepared six sample solutions starting from the same material, and each solution was injected in duplicate. The values of peak areas were used to calculate the relative standard deviation (%) between preparations. The repeatability was evaluated by the deviation of areas of multiple preparations of the same analyst whereas the intermediate precision was evaluated by the deviation of areas of the preparations of the two analysts.

Accuracy. The preparation of the samples for evaluation of accuracy was like the description in the "Sample preparation" section. Instead of weighing the sample, the mass of the placebo and active substance was separately weighed and transferred into the flask, except on cyanocobalamin analysis, as described below. To evaluate the accuracy of each method of analysis, nine samples were prepared, three for each of the levels 80, 100, and 120% of the theoretical concentration. The amount of analyte was determined based on the response of the standard solutions at the level of 100%.

To evaluate cyanocobalamin measurement accuracy, a cyanocobalamin stock solution was prepared and the corresponding amount of vitamin that should be weighed was transferred from this solution with a volumetric pipette to achieve the active mass (0.1050 mg cyanocobalamin). Each prepared solution was injected in triplicate.

Robustness. To evaluate method robustness, the standard and sample solutions were prepared and injected, five and twice each, respectively. Table 1 summarizes the conditions that were evaluated and a variation level for each variable.

Results

Content analysis of vitamins: nicotinamide, pyridoxine, riboflavin, and thiamine

The chromatograms showing the retention times of each of the vitamins are shown in Figure 1. There was no significant interference in vitamin retention times with diluent and placebo solutions. Figure 1 also shows the chromatogram of the standard, sample, diluent, and placebo solutions under the given conditions. The chromatographic analysis time was 2.5 minutes, and the column was reequilibrated for 1 minute under the initial conditions. The chromatographic conditions were established prior to the start of the validation tests.

Tocopherol acetate content analysis

Placebo and sample solutions were injected, and there was no interference in the tocopherol acetate retention time, 1.9 minutes. Figure 2 shows the chromatogram of the set condition. Other vitamins were eluted next to the void volume in the sample and placebo solutions, forming a much higher peak than that of tocopherol acetate. In standard solution, the addition of tocopherol acetate was observed, as a peak close to the dead volume corresponding to the diluent.

Analysis of cyanocobalamin content

The critical point for the determination of cyanocobalamin is that it is present at a lower concentration than the other vitamins in tablets. Good sensitivity was achieved under conditions described in the "Experimental" section, with a higher signal-to-noise ratio of 60

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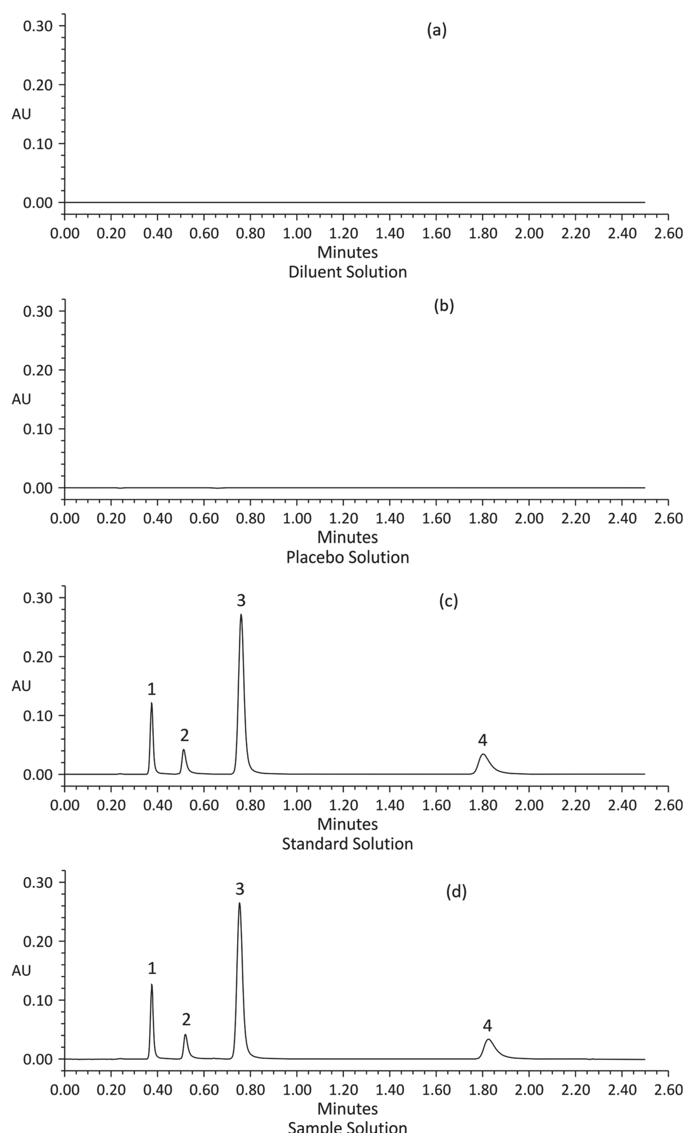

Figure 1. Typical chromatogram of the analysis of nicotinamide (1), pyridoxine (2), riboflavin (3), and thiamine (4) under the proposed conditions. (a) Diluent, (b) placebo, (c) standard solution, and (d) tablet sample analysis.

and a standard deviation of multiple injections of less than 2.0%. The chromatogram of this analysis can be seen in Figure 3.

Validation of analytical methods

System suitability. The suitability of the system for the validation test has been verified using five consecutive injections of each standard solution. The relative standard deviation of the chromatographic peak area of each vitamin was less than 1.50% in all cases; the resolution of the vitamins and any interfering compound was greater than 4.9 in all cases, whereas the signal-to-noise ratio of cyanocobalamin, a critical parameter for its quantification, was greater than 67. Other chromatographic parameters are presented in Supporting Information.

Specificity. There was no evaluated substance at the same retention time as the vitamins on the chromatogram of placebo solutions, diluents, or mobile phase. The evaluation of the purity of the peak, using Empower 2 Software, related the parameters Purity Angle (purity angle—PA) and Threshold (threshold—TH) with its spectral homogeneity (25).

Stability of solution. The stability of the prepared solutions was 18 hours to the vitamins nicotinamide, pyridoxine, riboflavin, thiamine, and tocopherol acetate and 24 hours to cyanocobalamin. The results have shown that, except for the standard vitamin thiamine solution, all others are stable for up to 18 hours. The maximum time that must elapse was 6 hours, the period in which the result of the thiamine content in standard solution is in a range of ±2.0% of the initial value.

Linearity. The linearity of the analytical method was evaluated for each vitamin by calculating the linear correlation coefficient, straight-line equation, and their residues by the method of least squares. The linear correlation coefficients (R) found for all vitamins were greater than 0.998. The curve parameters of the tests of linearity are present in Supporting Information. The relative residual standard deviation

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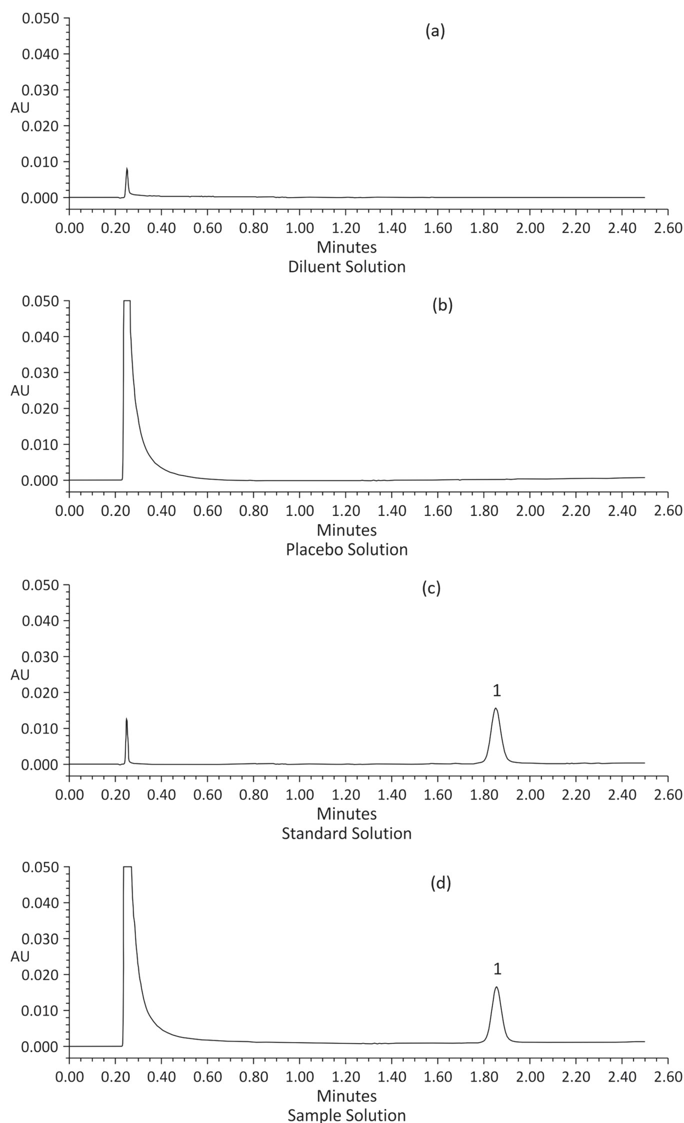

Figure 2. Typical chromatogram of the analysis of tocopherol acetate (1) under the proposed conditions. (a) Diluent, (b) placebo, (c) standard solution, and (d) tablet sample analysis.

was in the acceptable limit of 2.0%. Table 1 shows the percentage difference of the residues of each chromatographic analysis for each vitamin and the relative residual standard deviation.

Quantification and detection limits of cyanocobalamin. The value of quantification limit and the detection limit of cyanocobalamin were 0.000585 ansd 0.000175 mg/ml, respectively. The curve parameters of each of three curves used for their determination, calculated as recommended by regulators (33), are present in Supporting Information.

Precision. The results for the precision and accuracy test are presented in summary form in Table 2. The results show that the coefficient of variation for repeatability and intermediate precision were adequate in all cases, less than 2.0%.

Accuracy. To evaluate the accuracy, the efficiency of extraction of the vitamins in the sample preparation procedure was determined. The standard deviation of each level was below 2.0%, and the average results at each level are summarized in Table 3.

Robustness tests. The study of the analytical methodology of robustness is designed to compare the values of the main chromatographic parameters against the variations of the original conditions of the method that may compromise the results. Variation in the flow of the mobile phase, the mobile phase composition, the batch of the chromatographic column, the column temperature, and the pH of the mobile phase, when applicable, have been chosen for this evaluation. The parameters evaluated were the relative standard deviation of the standard solution injections, the separation efficiency (number of theoretical plates), the symmetry of the peak, the capacity factor, and the variation in the content of the samples compared to the standard (see Tables S4–S30 in Supporting Information).

Discussion

Content analysis of vitamins: nicotinamide, pyridoxine, riboflavin, and thiamine

To establish the extraction procedure of the vitamin, present in the formulation, it was diluted in a solvent so that all the

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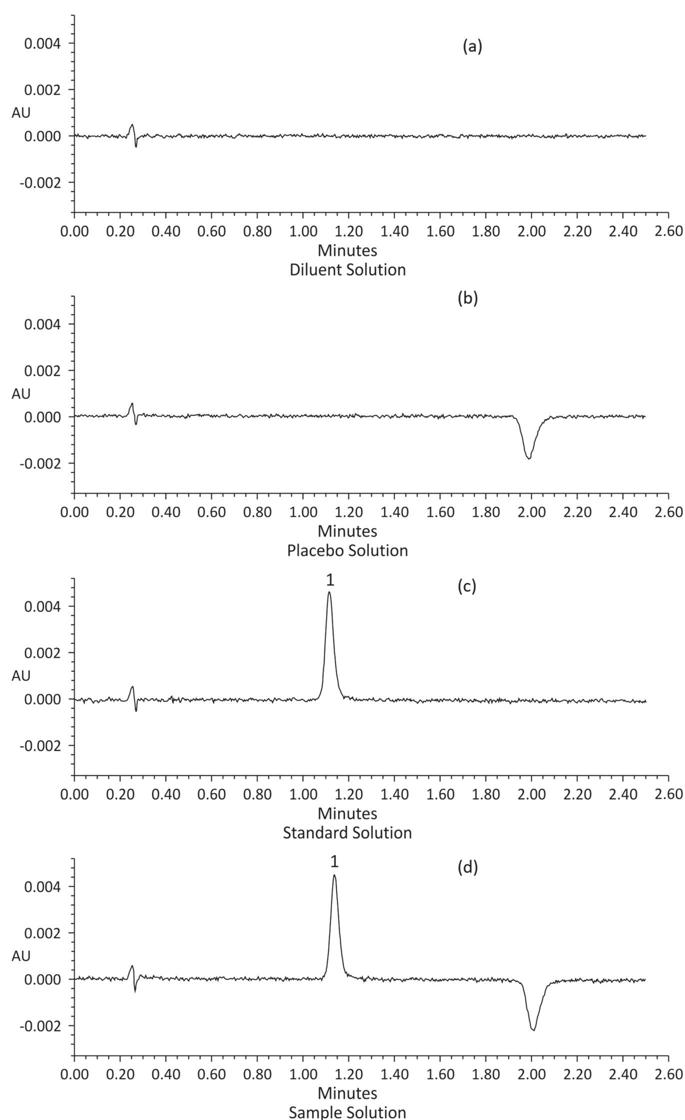

Figure 3. Typical chromatogram of the analysis of cyanocobalamin (1) under the proposed conditions. (a) Diluent, (b) placebo, (c) standard solution, and (d) tablet sample analysis.

vitamins would be considered soluble. The solubility was best achieved by using the diluent already described in the "Sample preparation" section. The extraction efficiency was evaluated on accuracy procedure. The wavelength of 265.0 nm was employed because the absorption spectrum of all interest compounds is close to this value (nicotinamide—261.7 nm, riboflavin—267.7 nm, and thiamine—266.5 nm). Pyridoxine absorbs at this wavelength well, despite having an absorption peak above 20 nm (at 283.1 nm).

The chromatographic conditions for the vitamins nicotinamide, pyridoxine, riboflavin, and thiamine were selected after a series of gradient tests, starting from the condition set on Waters' (34) work. The analysis time of the proposed gradient was, however, two times lower than that proposed by Waters. The column manufacturer guarantees that due to the technology used in the synthesis of the silica particles (Ethylene Bridged Hybrid (BEH)—particulate hybrid ethylene bridge), it is possible to work at any pH value within the range 1–12. The amide group of the stationary phase retains the analytes mainly by polar interactions, with hydrogen bonds being the more expressive of them. At the pH of the mobile phase, the molecules can be considered to be in the nonionized state based on its pKa values (35,36). A direct relationship between the number of hydroxyls, amides, and amines in vitamin molecules and the retention time of nicotinamide, pyridoxine, and riboflavin was observed. Nicotinamide had the shorter retention time as it is a small molecule and has only two hydrogen-bonding hydrogen atoms and one amide group (which form hydrogen bonds less strong than the hydrogen atoms of the hydroxyl groups). Pyridoxine has three hydroxyls, whereas riboflavin has four hydroxyl groups and one hydrogen from an amide group, which justifies riboflavin having been retained more than pyridoxine.

Thiamine, in addition to the hydrogen bonds that can be formed, is positively charged and may have strong interactions with the negative electric potential of oxygen and nitrogen of the amide groups of the stationary phase. As a result, thiamine was the vitamin that was more retained. Thus, the elution order of vitamins is

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Table 2. Relative Residual Standard Deviation Analysis

Concentration	In %					
%	Nicotinamide	Pyridoxine	Riboflavin	Thiamine	Tocopherol acetate	Cyanocobalamin
70	98.78	99.73	98.40	99.61	100.32	101.39
	99.08	100.09	98.67	99.72	100.31	101.83
	98.99	100.16	98.66	99.79	100.33	98.87
80	101.62	100.37	102.93	100.68	99.93	99.12
	99.48	101.63	99.47	99.93	100.35	101.86
	98.93	100.88	98.81	99.07	100.29	99.45
90	99.98	99.36	98.86	100.14	99.59	98.56
	98.84	100.63	98.99	99.00	99.34	100.33
	100.52	102.34	100.63	100.58	100.32	100.10
100	99.03	99.97	99.08	99.09	99.76	99.78
	99.61	100.79	99.72	99.94	99.58	99.62
	99.15	99.96	99.23	99.26	99.73	101.99
110	101.12	101.24	101.26	101.07	100.70	101.06
	101.03	101.68	100.98	101.27	99.75	101.74
	100.69	101.35	100.63	100.67	99.85	100.19
120	99.28	99.37	99.22	98.98	99.66	100.99
	98.62	98.38	98.52	97.87	99.89	100.67
	98.75	98.19	98.62	97.87	99.66	99.42
130	100.13	99.91	100.20	100.42	100.06	99.15
	101.46	101.15	101.55	101.65	99.76	99.84
	101.71	100.63	102.69	101.70	99.99	99.50
DPR residual	1.05	1.04	1.37	1.08	0.35	1.07

Table 3. Precision Results – Relative Standard Deviation of Multiple Preparations and Recovery of Vitamins on Accuracy Test

Vitamin	Analyst 1[a]	Analyst 2[a]	Intermediate precision*	Level %		
				80	100	120
Nicotinamide	1.19	0.61	0.90	110.3	100.0	100.5
Pyridoxine	1.13	0.63	0.88	99.1	99.1	99.4
Riboflavin	1.15	0.81	0.98	100.0	100.3	100.9
Thiamine	1.10	0.72	0.91	100.4	100.8	101.3
Tocopherol acetate	0.42	1.05	0.74	100.4	99.4	99.5
Cyanocobalamin	1.10	0.95	1.03	100.2	100.3	99.9

[a]Relative standard deviation (%) of multiple preparations

roughly consistent with their molecular structures and with the polar characteristic of the stationary phase (amide). The gradient used in chromatographic analysis secured a minimum resolution of 4.9 among all the chromatographic peaks of analyzed vitamins. Figure 1 shows the retention times of each of the vitamins, with values in the range of 5 to 10 times smaller than those found for the methods using conventional HPLC conditions (8,14,15,18,26,27,37,38). There was no significant interference in vitamin retention times in the chromatographic analysis of diluent and placebo solutions. The chromatographic analysis time was 2.5 minutes, and the column was reequilibrated for 1 minute under the initial conditions of the gradient between injections so that there were no changes in the retention times of vitamins between multiple chromatographic analyses. The chromatographic conditions were established prior to the start of validation tests.

Tocopherol acetate content analysis

Methanol is a good solvent for tocopherol acetate and was therefore used as a solvent for the standard solution. When preparing the samples, however, 5.0 ml of water at 40 °C was required to disperse the excipient before the addition of methanol. The accuracy, discussed below, demonstrated that this procedure was enough to extract the vitamin. The wavelength of detection adopted for the method was determined by evaluating the spectrum extracted at the apex of the peak. The value 285.5 nm was chosen because

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it provided a clear baseline with a good signal-to-noise ratio of about 400. The elution with acetonitrile, based on the work of Wielinski and Olszanowski, (39), was satisfactory and unnecessary to try another condition. Placebo and sample solutions were injected, and it was found that there was no interference in the tocopherol acetate retention time (1.9 minutes, clear improvement compared with the method developed by Wielinski and Olszanowski, whose duration was 20 minutes and the eluting time of tocopherol acetate was 6 minutes) (40). Figure 2 shows the chromatogram; it can be observed that the other vitamins were eluted next to the void volume in the sample and placebo solutions, forming a much higher peak than that of tocopherol acetate. In the standard solution with tocopherol acetate, a peak close to the dead volume corresponding to the diluent was observed.

Analysis of cyanocobalamin content

Water, in which cyanocobalamin is soluble, was used as the solvent for preparing the standard solution and for extracting cyanocobalamin from the pharmaceutical preparation. The precaution of using only amber glassware was taken due to cyanocobalamin solution, which is prone to photodegradation (40). The critical point for the determination of cyanocobalamin is that it is present at a much lower concentration than the other vitamins, whose presence may interfere with the quantification. The wavelength used for detection in chromatographic analysis was 550.0 nm because, although this was not the most absorbing wavelength, it is more specific in the presence of interferences, including vitamins and other related compounds that may be present in the formulation. The mobile phase used initially was composed of water and methanol at a ratio of 70:30 vol/vol, based on the work of Hua-Bin Li and coworkers (41). Some adjustments were made, and the use of water and methanol at a ratio of 80 to 20 vol/vol was shown to result in better peak shape and intensity. To improve the signal-to-noise ratio of the cyanocobalamin peak, the injection volume was increased to 4.0 µl. Good sensitivity was achieved under these conditions, with a higher signal-to-noise ratio of 60 and a standard deviation of multiple standard solution injections of less than 2.0%. The chromatogram of this analysis can be seen in Figure 3.

Validation of analytical methods

System suitability. The suitability of the system for the validation test using five consecutive injections of each standard solution was considered adequate (5). The values of these parameters showed no significant change during the validation assay.

Specificity. As PA was lower than TH, the spectrum is homogeneous throughout extension of the chromatographic band. This purity was achieved for all peaks; therefore, it is possible to assign a single component to a chromatographic peak.

Stability of solution. Evaluation of the stability of analytical solutions was performed prior to other tests for the preparation of solutions, and the chromatographic analysis was programmed within a time in which the solutions were stable. The stability of the freshly prepared solutions was assessed over a period of 18 hours to the vitamins nicotinamide, pyridoxine, riboflavin, thiamine, and tocopherol acetate, and 24 hours to cyanocobalamin. The results have shown that, except for the standard vitamin thiamine solution, all others are stable for up to 18 hours. However, as nicotinamide, pyridoxine, riboflavin, and thiamine analyses were performed simultaneously, the maximum time that must elapse from the preparation of the solutions to the end of the analysis in this method was 6 hours, which corresponds to the period in which the result of the thiamine content in standard solution is within a range of ±2.0% of the initial value. It is suggested that the degradation observed for thiamine is caused by the rapid decay of the thiazolium ring in alkaline solutions, because the pH of the standard solutions and the sample is 9.0 (42). Washabaugh and coworkers (42) studied the kinetics of the thiazolium ring degradation of thiamine in a pH range from 3 to 11 and proposed that degradation in basic pH occurs by the nucleophilic addition of hydroxide ions to the carbon existing between positive nitrogen and sulfur heteroatoms. The formed intermediate structure is unstable, and the ring breaks up, forming an acyclic structure (42). The scheme of the degradation mechanism based on Washabaugh and coworkers' (42) study is present in Supporting Information. The analytical solutions prepared for the determination of tocopherol acetate and those prepared for the determination of cyanocobalamin were shown to be stable for a period of 18 and 24 hours, respectively; these are the maximum times that can elapse from the preparation of the solutions to the end of the chromatographic analyses.

Linearity. The linearity of the method was evaluated for each vitamin by calculating the linear correlation coefficient, straight-line equation, and their residues by the method of least squares. The linear correlation coefficients (R) found for the responses of all vitamins were greater than 0.998. According to the literature, a value above 0.990 is sufficient to demonstrate linearity of a method for determining the pharmaceutical product content (32,43). The results present in Table 1 shows that for each vitamin the relative residual standard deviation is in the acceptable limit of 2.0%.

Quantification and detection limits of cyanocobalamin. The amount of cyanocobalamin in the formulation is much lower than the concentration of other vitamins. The value of quantification limit and the detection limit of cyanocobalamin were 0.000585 and 0.000175 mg/ml, respectively. Although the detection limit is not as good as those reported by Li and coworkers (41) using fluorescence detection, the limit obtained by our method (UV/VIS) is perfectly adequate for analysis of the analyte in the formulation in question.

Precision. As the coefficient of variation for repeatability and intermediate precision were adequate in all cases, less than 2.0%. The method was considered precise.

Accuracy. The accuracy and efficiency of extraction of the vitamins in the sample preparation procedure were determined. The standard deviation of each level was below 2.0%. The average results at each level are summarized in Table 3. The degree of recovery accepted for analytical methods in the pharmaceutical industry is between 98.0 and 102.0% (43), so it can be concluded that this methodology can be considered adequate.

Robustness tests. The parameters evaluated in the robustness test showed that there were no significant deviations in these parameters, which means that the method is robust in the face of proposal variations.

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Conclusions

The six vitamins present in multivitamin tablets—nicotinamide (10.5 mg), pyridoxine (10.5 mg), riboflavin (10.5 mg), thiamine (10.5 mg), tocopherol acetate (10.5 mg), and cyanocobalamin (10.5 μg)—were determined by a UPLC-DAD method. In this method, the use of a RP-UPLC BEH C18 and other BEH amide column allowed the analysis of multivitamin tablets 5 to 10 times faster than that commonly found in the literature, 2.5 minutes for total analysis of tocopherol acetate and cyanocobalamin and for the other four vitamins present in the formulation. The short times of chromatographic runs of developed methods represent a reagent and solvent economy, as well as others. The methodologies were individually validated, and the results demonstrate that the methods are reliable for the intended applications. The mobile phases used in the proposed methods are composed of solvents and buffers compatible with detection by mass spectrometry, by making the chromatographic conditions compatible with this technology. In addition, the simplicity of the process makes it desirable in quality control laboratories in pharmaceutical companies that manipulate this kind of sample. The problem of low levels of vitamin B_{12} in the formulation has been solved using a particular method that has been optimized especially for this analysis.

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