

RP-HPLC Determination of vitamins B₁, B₃, B₆, folic acid and B₁₂ in multivitamin tablets

RADA AMIDŽIĆ, JASMINA BRBORIC*, OLIVERA ČUDINA and SOTE VLADIMIROV

Department of Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmacy, Vojvode Stepe 450,
P. O. Box 146, 11152 Belgrade, Serbia and Montenegro

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Abstract: A simple and sensitive reversed-phase, ion-pair HPLC method was developed and validated for the simultaneous determination of B-group vitamins, thiamine chloride hydrochloride (B₁), nicotinamide (B₃), pyridoxine hydrochloride (B₆) and folic acid in Pentovit[®] coated tablets. The cyanocobalamine (B₁₂) was determined separately, because of its low concentration in the investigated multivitamin preparation. RP-HPLC analysis was performed with a LKB 2150 HPLC system, equipped with a UV/VIS Waters M 484 detector. The procedures for the determination of B₁, B₂, B₆ and folic acid were carried out on a Supelcosil ABZ⁺ (15 cm × 4.6 mm; 5 μm) column with methanol-5mM heptanesulphonic acid sodium salt 0.1 % triethylamine TEA (25:75 V/V); pH 2.8 as the mobile phase. For the determination of B₁₂ a Suplex pKb-100 (15 cm × 4.6 mm; 5 μm) column and methanol-water (22:78 V/V) as the mobile phase were used. The column effluents were monitored at 290 nm for B₁, B₃, B₆ and folic acid, and at 550 nm for B₁₂. The obtained results and statistical parameters for all the investigated vitamins of the B-group in Pentovit[®] coated tablets were satisfactory and ranged from 90.4 % to 108.5 % (RSD. from 0.5 % to 4.1 %). The parameters for the validation of the methods are given.

Keywords: RP-HPLC, vitamin B₁, B₃, B₆, B₁₂, folic acid, multivitamin preparation.

INTRODUCTION

The use of therapeutic multivitamins are indicated in cases of deficiency in pathological conditions in which the nutritional requirements are greatly increased or in conditions in which absorption, utilization, or excretion of vitamins are abnormal. Multivitamin pharmaceutical preparations containing mixtures of these substances are very interesting for analysis, and most of them include the water-soluble B-group. The term B-group vitamins usually refers to thiamine, riboflavin, pyridoxine, nicotinic acid, pantothenic acid, biotin, cyanocobalamine and folic acid. As their chemical structures are not related, a considerable number of papers have been published in which the use of different physical, chemical and biological methods are described. The simultaneous determination of several wa-

* Author for correspondence.

ter-soluble vitamins is difficult and often many different analyses have to be performed. Different instrumental methods have been used for the determination of B-group vitamins, including electrochemical methods,¹ spectrophotometry,^{2,3} derivative UV spectrophotometry,^{4–7} spectrofluorimetry,^{8–10} normal phase and reversed phase TLC^{11–13} and HPLC procedures,^{14–19} as well as capillary electrophoresis.^{20,21} The determination of B-complex mainly in tablets using HPLC methods have been extensively described. The most widely used methods for the determination of B-group vitamins are reversed-phase HPLC, using a C18 column and aqueous–organic mobile phases, in acidic media. Other chromatographic systems separate B₁, B₆ and B₁₂ when they are present in the same concentration range¹⁴ but are hampered when the amount of B₁ and B₆ exceeds by a hundred or even a thousand fold the amount of B₁₂ present in the complex. The methods reported in the literature are unable to determinate simultaneously the five vitamins. Ivanovic *et al.*¹⁹ developed and validated a method to assay some water soluble vitamins (B₁, B₂, B₃, B₆, vitamin C and PABA) in solution dosage forms. The recent literature attaches importance to those dosage forms which contain folic acid and B₁₂, important for the treatment of anaemia, especially in pregnancy. Applying the proposed reversed-phase, ion-pair HPLC method, it is possible to identify and determinate simultaneously all vitamins, except vitamin B₁₂, in analyzed pharmaceutical preparation with only one injection.

The present paper describes a sensitive and simple RP-HPLC method with UV/VIS detection for determination of B-group vitamins: Thiamine chloride hydrochloride (vitamin B₁) (3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride hydrochloride); nicotinamide (vitamin B₃) (3-pyridine carboxamide); Pyridoxine hydrochloride (vitamin B₆) (5-hydroxy-6-methyl-3,4-pyridine dimethanol hydrochloride); folic acid, (*N*-[4-[[[(2-amino-1,4-dihydro-4-oxo-6-pteridiny)]methyl]amino]benzoyl]-L-glutamic acid); and Cyanocobalamine (vitamin B₁₂) (5,6-dimethyl benzimidazolyl cyanocobamide) in a multivitamin preparation.

EXPERIMENTAL

Reagents and solvents

All chemicals and reagents were of analytical grade and the water was distilled and filtered through a membrane filter. Thiamine chloride hydrochloride, pyridoxine hydrochloride, nicotinamide, folic acid and cyanocobalamine (ICN Biomedicals Inc.) were used as working standards. Methanol for HPLC (Merck, Darmstadt, Germany) heptanesulphonic acid sodium salt (Sigma), triethylamine, TEA (Aldrich Chemical Company, Inc.) were used to prepare the mobile phase and orthophosphoric acid (Merck) for adjusting the pH values.

Dosage form

Pentovit[®] tablets, manufactured by VORONJEZHIMFARM-VREMYA (Pentovit[®] coated tablet: thiamine chloride hydrochloride 10 mg or thiamine bromide hydrobromide 12.9 mg, pyridoxine hydrochloride 5 mg, nicotinamide 20 mg, folic acid 400 µg and cyanocobalamine 50 µg).

Standard solutions

Standard stock solution of vitamin B₁ was prepared by dissolving 25.0 mg of thiamine chloride hydrochloride in 50.0 ml of water.

Standard stock solution of vitamin B₃ was prepared by dissolving 25.0 mg of nicotinamide in 50.0 ml of water.

Standard stock solution of vitamin B₆ was prepared by dissolving 25.0 mg of pyridoxine hydrochloride in 50.0 ml of water.

Standard stock solution of folic acid was prepared by dissolving 25.0 mg of folic acid in 50.0 ml of water. 1 ml of the standard stock solution of folic acid was diluted to 50 ml with 15 % methanol solution.

The working standard solution of B₁, B₃, B₆ and folic acid was obtained by diluting 0.4 ml of standard stock solution of B₁, 0.8 ml of standard stock solution of B₃, 0.2 ml of standard stock solution of B₆ and 0.8 ml of standard stock solution of folic acid to 10 ml with 15 % methanol solution.

Standard stock solution of vitamin B₁₂ was prepared by dissolving 10.0 mg of cyanocobalamin in 100.0 ml of water.

The working standard solution of B₁₂ was obtained by diluting 1 ml of the standard stock solution of vitamin B₁₂ to 10 ml with water. 1 ml of this solution was diluted to 10.0 ml with the same solvent.

Sample preparations

Sample preparation of B₁, B₃, B₆ and folic acid. Twenty tablets were weighed and triturated to a fine powder. The average mass of one tablet was transferred into a 50 ml volumetric flask and 15 % of methanol solution was added. The mixture was sonicated (15 min) and diluted to the mark with the same solvent. 1 ml of this solution was transferred into a 10 ml volumetric flask, diluted to the mark with the same solvent and filtered through a 0.2 µm Millipore filter.

Sample preparation of B₁₂. Twenty tablets were weighed and triturated to a fine powder. The average mass of two tablets was transferred into a 100 ml volumetric flask and water was added. The mixture was sonicated (20 min), diluted to the mark with the same solvent and filtered through a 0.2 µm Millipore filter.

Apparatus, mobile phase and chromatographic conditions

A chromatographic LKB 2150 HPLC System, equipped with a Waters M 484 UV/VIS detector, was connected with a computed integrator Maxima 820 Work Station. The detection wavelength was adjusted to 290 nm with a sensitivity of 0.05 AUFS for the determination of B₁, B₃, B₆ vitamins and folic acid and to 550 nm with a sensitivity of 0.05 AUFS for the determination of vitamin B₁₂. A Supelcosil ABZ⁺ column (15 cm × 4.6 mm; particle size 5 µm) was used for the determination of B₁, B₃, B₆ vitamins and folic acid. A Suplex pKb-100 column (15 cm × 4.6 mm; particle size 5 µm) was used for the determination of vitamin B₁₂. The experiments were conducted at 35 °C for the determination of B₁, B₃, B₆ vitamins and folic acid and at 25 °C for the determination of vitamin B₁₂. The mobile phase for the determination of B₁, B₃, B₆ vitamins and folic acid was of methanol – 5 mM heptanesulphonic acid sodium salt / 0.1 % TEA (25:75 V/V). The pH 2.8 was adjusted with orthophosphoric acid. The flow rate was 1 ml/min and the injected volume 10 µl. The mobile phase for the determination of vitamin B₁₂ was methanol–water (22:78 V/V). The flow rate was 0.8 ml/min and the injected volume 100 µl. The prepared mobile phases were filtered through a 0.2 µm Anotop filter and degassed with an ultrasonic bath.

HPLC procedure

Prior to injection into the chromatographic system, all analytical solutions were degassed by sonication. All the prepared sample solutions were first chromatographed to ensure that interfering peaks were not present. 10 µl and 100 µl aliquots of the standard solutions and sample solutions were injected. Triplicate injections were made for each solution.

RESULTS AND DISCUSSION

The optimal conditions for the identification and quantification of B₁, B₃, B₆ vitamins and folic acid in Pentovit[®] tablets, using heptanesulphonic acid sodium salt as the ion pairing reagent were investigated and established. The best results for the simultaneous determination of B₁, B₃, B₆ vitamins and folic acid were obtained with the following mobile phase: methanol – 5 mM heptanesulphonic acid sodium salt / 0.1 % triethylamine TEA

(25:75 V/V). Cyanocobalamine (B₁₂) was determined separately because of its low concentration in the investigated multivitamin preparation. The optimization procedure included studies concerning the composition of the mobile phase, flow-rate and temperature. After establishing the optimal conditions for the separation, the selectivity, linearity, precision, limit of detection and limit of quantification were determined.

The chromatographic parameters, *i.e.*, capacity factor, selectivity factor, resolution factor and factor symmetry, were calculated on the basis of the experimentally obtained values of retention times and width peaks for all the investigated B-complex vitamins.

Under the described experimental conditions, the values of retention times were: 2.27 min for B₃, 3.30 min for B₆, 4.74 min for folic acid and 7.63 min for B₁. The retention time for B₁₂ was 6.53 min.

The values of selectivity factor were 1.5 for pyridoxine hydrochloride/nicotinamide; 1.4 for folic acid/pyridoxine hydrochloride and 1.6 for thiamine chloride hydrochloride/folic acid.

The resolution factors R_s between the chromatographic peaks were calculated from the equation $R_s = 2(t_2 - t_1)/(W_1 + W_2)$, where t_2, t_1 are the retention times of the two components and W_1, W_2 are the peak widths at the base of the two respective peaks: 4.1 for pyridoxine hydrochloride/nicotinamide; 4.8 for folic acid/pyridoxine hydrochloride; and 8.3 for thiamine chloride hydrochloride/folic acid.

The representative chromatograms of the working standard solution of B₁, B₃, B₆ vitamins and folic acid and of the sample solution are presented in Fig. 1. The assay was selective, no significant interfering peaks were observed at the retention times of the vitamins. All excipients eluted at a different times and did not interfere with the analyzed compounds. The representative chromatograms of the working standard solution of vitamin B₁₂ and a sample preparation are presented in Fig. 2.

The linearity of the method was determined by injecting five solutions of concentration between 50 % and 150 % of the expected concentration. Analysis were performed in triplicate to determinate the linearity of the assay. Good linearities were obtained with correlation coefficients above 0.99. The important parameters of calibration curves, *i.e.*, slope (a), intercept (b) and correlation coefficient (r) are presented in Table I.

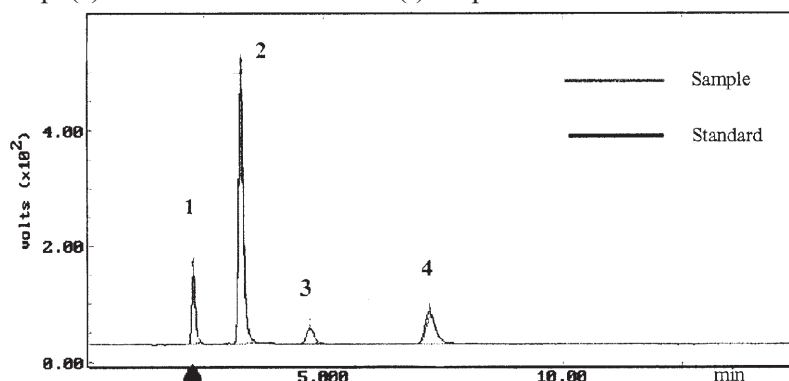


Fig. 1. Representative chromatograms of the standard solutions of vitamins B₃ (1), B₆ (2), folic acid (3), B₁ (4) and a sample solution.

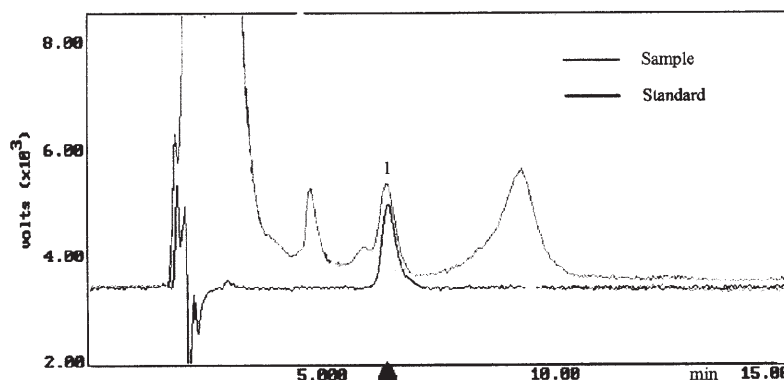


Fig. 2. Representative chromatograms of the standard solution of vitamin B₁₂ (I) and a sample solution.

TABLE I. The important parameters for the calibration curves

Vitamin	$y = ax + b$	r	Concentration range
B ₁	$y = 4153.206x - 1118$	0.9992	10–30 $\mu\text{g/ml}$
B ₃	$y = 1550.58x + 2484.68$	0.9995	20–60 $\mu\text{g/ml}$
B ₆	$y = 30332.36x - 2616.38$	0.9980	5–15 $\mu\text{g/ml}$
Folic acid	$y = 33452.2x + 679.08$	0.9995	0.4–1.2 $\mu\text{g/ml}$
B ₁₂	$y = 38045.88x - 399.28$	0.9991	0.5–1.5 $\mu\text{g/ml}$

a – Slope; b – intercept; r – correlation coefficient

The precision of the procedure was checked by analysis of ten working standard solutions (B₁ 20 $\mu\text{g/ml}$; B₃ 40 $\mu\text{g/ml}$; B₆ 10 $\mu\text{g/ml}$; folic acid 0.8 $\mu\text{g/ml}$ and B₁₂ 1 $\mu\text{g/ml}$). The *RSD* values 1.2 %; 0.7 %; 0.1 %; 1.5 % and 0.4 % for B₁, B₃, B₆, folic acid and B₁₂, respectively, were indicative of the satisfactory repeatability of the system. The precision of the method was checked for within-day and between-day variation.

The limit of detection (*LOD*) and limit of quantification (*LOQ*) for the investigated vitamins were experimentally determined and they are presented in Table II.

The results of the determination of B-group vitamins in Pentovit[®] coated tablets are given in Table III. The values obtained for the *RSD* (below 5 %) show the accuracy and reproducibility of the method.

TABLE II. Limits of detection (*LOD*) and limits of quantification

Vitamin	<i>LOD</i> /($\mu\text{g/ml}$)	<i>LOQ</i> /($\mu\text{g/ml}$)
Thiamine chloride hydrochloride (B ₁)	0.6250	1.250
Nicotinamide (B ₃)	0.6250	1.250
Pyridoxine hydrochloride (B ₆)	0.0195	0.039
Folic acid	0.0125	0.025
Cyanocobalamine (B ₁₂)	0.0625	0.125

LOD – Limit of detection; *LOQ* – limit of quantification

TABLE III. Results of the determination of B-group vitamins in Pentovit[®] coated tablets

Vitamin	Amount in Pentovit [®] tablet	Found	Recovery/%	RSD/%
B ₁	10.0 mg (8.5–11.5 mg)	9.04 mg	90.4	1.3
B ₃	20.0 mg (17.0–23.0 mg)	21.44 mg	107.2	1.9
B ₆	5.0 mg (4.25–5.75 mg)	5.26 mg	105.2	2.0
Folic acid	400 µg (320–480 µg)	433.9 µg	108.5	0.5
B ₁₂	50.0 µg (40–60 µg)	45.86 µg	91.7	4.1

**n* = 6

CONCLUSIONS

The results obtained confirm that the proposed method is simple, accurate, precise and can be successfully applied for the routine analysis of B₁, B₃, B₆, folic acid and B₁₂ in B-complex tablets. The investigated vitamins were completely separated and the excipients present in the dosage forms did not interfere with the peaks of interest.

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ИЗВОД

RP-HPLC ОДРЕЂИВАЊЕ ВИТАМИНА В₁, В₃, В₆, ФОЛНЕ КИСЕЛИНЕ И В₁₂ ИЗ МУЛТИВИТАМИНСКИХ ТАБЛЕТА

РАДА АМИЦИЋ, ЈАСМИНА БРБОРИЋ, ОЛИВЕРА ЧУДИНА И СОТЕ ВЛАДИМИРОВ

Институт за фармацеутичку хемију и аналитику лекова, Фармацеутички факултет, Војводе Степе 450, 11152 Београд

За истовремено одређивање витамина В-комплекса, тиамин-хлорида-хидрохлорида (В₁), никотинамида (В₃), пиридоксин-хидрохлорида (В₆) и фолне киселине у Pentovit[®] таблетама примењена је и валидирана једноставна и осетљива метода реверзно-фазне јон-пар течне хроматографије под високим притиском. Због ниске концентрације у испитиваном препарату цијанокобаламин (В₁₂) је одређен под другим условима. Одређивање витамина В₁, В₃, В₆ и фолне киселине изведено је на LKB 2150 HPLC систему, са UV/VIS Waters М 484 детектором, коришћењем Supelcosil ABZ⁺ колоне (15 cm × 4,6 mm; 5 µm) са мобилном фазом метанол – 5 mM натријум-хептансулфонат/0,1 % триетиламин (25:75 V/V) при рН 2,8. За одређивање витамина В₁₂ коришћена је колона Suplex рКb-100 (15 cm × 4,6 mm; 5 µm) и мобилна фаза метанол–вода (22:78 V/V). Витамини В₁, В₃, В₆ и фолна киселина детектовани су на 290 nm, а витамин В₁₂ на 550 nm. Добијене вредности садржаја и статистички параметри за све витамине В-комплекса у Pentovit[®] таблетама су задовољавајући и крећу се у опсегу од 90,4 % до 108,5 % (RSD од 0,5 % до 4,1 %). Приказани су и параметри за валидацију методе.

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