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# RP-HPLC Determination of vitamins $B_1$, $B_3$, $B_6$, folic acid and $B_{12}$ 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 ($B_1$), nicotinamide ($B_3$), pyridoxine hydrochloride ($B_6$) and folic acid in Pentovit® coated tablets. The cyanocobalamine ($B_{12}$) 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 $B_1$, $B_2$, $B_6$ 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 $B_{12}$ 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 $B_1$, $B_3$, $B_6$ and folic acid, and at 550 nm for $B_{12}$. 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 $B_1$, $B_3$, $B_6$, $B_{12}$, 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 chemicals 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.

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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 $B_1$, $B_6$ and $B_{12}$ when they are present in the same concentration range[14] but are hampered when the amount of $B_1$ and $B_6$ exceeds by a hundred or even a thousand fold the amount of $B_{12}$ 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 ($B_1$, $B_2$, $B_3$, $B_6$, vitamin C and PABA) in solution dosage forms. The recent literature attaches importance to those dosage forms which contain folic acid and $B_{12}$, 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 $B_{12}$, 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 $B_1$) (3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-5-(2-hydroxyethyl)-4-methyl thiazolium chloride hydrochloride); nicotinamide (vitamin $B_3$) (3-pyridine carboxamide); Pyridoxine hydrochloride (vitamin $B_6$) (5-hydroxy-6-methyl-3,4-pyridine dimethanol hydrochloride); folic acid, (*N*-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid); and Cyanocobalamine (vitamin $B_{12}$) (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 $B_1$* was prepared by dissolving 25.0 mg of thiamine chloride hydrochloride in 50.0 ml of water.

*Standard stock solution of vitamin $B_3$* was prepared by dissolving 25.0 mg of nicotinamide in 50.0 ml of water.

*Standard stock solution of vitamin $B_6$* 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 $B_1$, $B_3$, $B_6$ and folic acid* was obtained by diluting 0.4 ml of standard stock solution of $B_1$, 0.8 ml of standards stock solution of $B_3$, 0.2 ml of standard stock solution of $B_6$ and 0.8 ml of standard stock solution of folic acid to 10 ml with 15 % methanol solution.

*Standard stock solution of vitamin $B_{12}$* was prepared by dissolving 10.0 mg of cyanocobalamine in 100.0 ml of water.

*The working standard solution of $B_{12}$* was obtained by diluting 1 ml of the standard stock solution of vitamin $B_{12}$ 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 $B_1$, $B_3$, $B_6$ 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 $B_{12}$*. 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 $B_1$, $B_3$, $B_6$ vitamins and folic and to 550 nm with a sensitivity of 0.05 AUFS for the determination of vitamin $B_{12}$. A Supelcosil ABZ$^+$ column (15 cm × 4.6 mm; particle size 5 μm) was used for the determination of $B_1$, $B_3$, $B_6$ 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 $B_{12}$. The experiments were conducted at 35 ºC for the determination of $B_1$, $B_3$, $B_6$ vitamins and folic acid and at 25 ºC for the determination of vitamin $B_{12}$. The mobile phase for the determination of $B_1$, $B_3$, $B_6$ 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 $B_{12}$ 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 $B_1$, $B_3$, $B_6$ 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 $B_1$, $B_3$, $B_6$ 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 ($B_{12}$) 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 $B_3$, 3.30 min for $B_6$, 4.74 min for folic acid and 7.63 min for $B_1$. The retention time for $B_{12}$ 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 $B_1$, $B_3$, $B_6$ 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 $B_{12}$ 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.

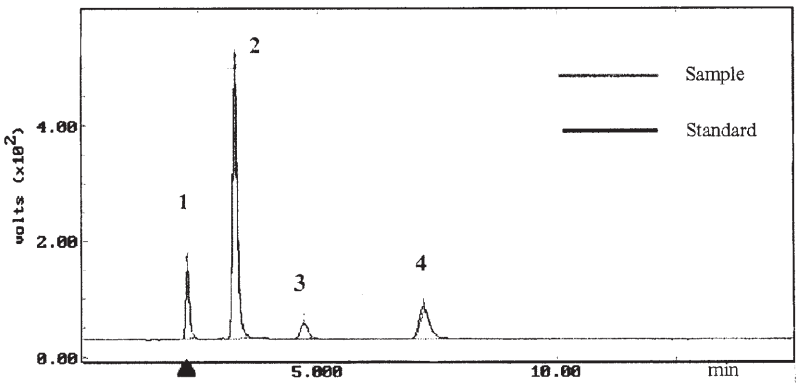


Fig. 1. Representative chromatograms of the standard solutions of vitamins $B_3$ (**1**), $B_6$ (**2**), folic acid (**3**), $B_1$ (**4**) and a sample solution.

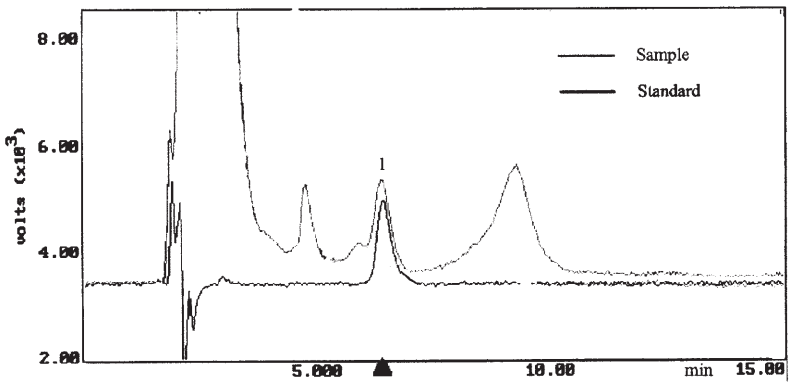


Fig. 2. Representative chromatograms of the standard solution of vitamin $B_{12}$ (**1**) and a sample solution.

TABLE I. The important parameters for the calibration curves

| Vitamin | $y = ax + b$ | $r$ | Concentration range |
|---|---|---|---|
| $B_1$ | $y = 4153.206\,x - 1118$ | 0.9992 | 10–30 μg/ml |
| $B_3$ | $y = 1550.58\,x + 2484.68$ | 0.9995 | 20–60 μg/ml |
| $B_6$ | $y = 30332.36\,x - 2616.38$ | 0.9980 | 5–15 μg/ml |
| Folic acid | $y = 33452.2\,x + 679.08$ | 0.9995 | 0.4–1.2 μg/ml |
| $B_{12}$ | $y = 38045.88\,x - 399.28$ | 0.9991 | 0.5–1.5 μg/ml |

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

The precision of the procedure was checked by analysis of ten working standard solutions ($B_1$ 20 μg/ml; $B_3$ 40 μg/ml; $B_6$ 10 μg/ml; folic acid 0.8 μg/ml and $B_{12}$ 1 μg/ml). The *RSD* values 1.2 %; 0.7 %; 0.1 %; 1.5 % and 0.4 % for $B_1$, $B_3$, $B_6$, folic acid and $B_{12}$, 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 | *LOD*/(μg/ml) | *LOQ*/(μg/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 $B_1$, $B_3$, $B_6$, folic acid and $B_{12}$ 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 $B_1$, $B_3$, $B_6$, ФОЛНЕ КИСЕЛИНЕ И $B_{12}$ ИЗ МУЛТИВИТАМИНСКИХ ТАБЛЕТА

РАДА АМИЦИЋ, ЈАСМИНА БРБОРИЋ, ОЛИВЕРА ЧУДИНА и СОТЕ ВЛАДИМИРОВ

*Институт за фармацеутску хемију и аналитику лекова, Фармацеутски факултет, Војводе Степе 450, 11152 Београд*

За истовремено одређивање витамина B-комплекса, тиамин-хлорида-хидрохлорида ($B_1$), никотинамида ($B_3$), пиридоксин-хидрохлорида ($B_6$) 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 ($B_{12}$) је одређен под другим условима. Одређивање витамина $B_1$, $B_3$, $B_6$ и фолне киселине изведено је на LKB 2150 HPLC систему, са UV/VIS Waters M 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 pH 2,8. За одређивање витамина $B_{12}$ коришћена је колона Suplex pKb-100 (15 cm × 4,6 mm; 5 μm) и мобилна фаза метанол–вода (22:78 V/V). Витамини $B_1$, $B_3$, $B_6$ и фолна киселина детектовани су на 290 nm, а витамин $B_{12}$ на 550 nm. Добијене вредности садржаја и статистички параметри за све витамине B-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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