

**COMPARATIVE STUDY OF VITAMINS B CONTENT OF
SOME PHARMACEUTICAL AND DIETARY
SUPPLEMENTS***

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SUMMARY

A simple reversed-phase, HPLC method was developed and validated for the simultaneous determination and comparative assay of the B-group vitamins, thiamine hydrochloride (B1), nicotinic acid (B3), pyridoxine hydrochloride (B6) in vitamin tablets and dietary supplements using a isocratic elution with methanol:water, 10:90 as mobile phase. Riboflavin (B2) was determined separately on a methanol;water, 35:65 mobile phase, because of its high retention time on the previous mobile phase. The method was employed for the analysis of vitamin from dietary supplements. The method of acid hydrolysis with 0.1N sulphuric acid was used as the extraction procedure for the analysis of vitamin preparations obtained from various pharmaceutical outlets. The amounts obtained from the analysis were compared with the declared values. For most of these preparations, the amounts obtained were below the declared values, and in some of the preparations, the vitamins were found to be missing entirely.

Keywords: RP-HPLC, vitamin B₁, B₃, B₆, B₂, vitamin preparation., dietary supplements

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INTRODUCTION

Vitamins are organic molecules essential for normal metabolism and consequently required for normal health and physiological functions such as growth, development, maintenance, and reproduction. The human organism is unable to synthesize them in sufficient amounts to meet physiological demands and therefore must be obtained from the diet [1]

Vitamin B complex is a set of 12 water-soluble substances, out of which eight are considered vitamins, as the body cannot synthesize them: B1 (thiamin), B2 (riboflavin), B3 (niacin), B5 (pantothenic acid), B6 (pyridoxine), B7 (biotin), B9 (folic acid), and B12 (cobalamin). Since they are water-soluble, most are not stored to any great extent and must be replenished on a daily basis [2] .

As a group, the B vitamins have a broad range of functions. These include maintenance of myelin, which is the covering of nerve cells. A breakdown of myelin can cause a large and devastating variety of neurologic symptoms. B vitamins are also key to producing energy from the nutrients that are consumed. Three members of this group, folic acid, pyridoxine, and cobalamin work together to keep homocysteine levels low. This is quite important, since high homocysteine levels are associated with heart disease. Some B vitamins prevent certain birth defects (like neural tube defects), maintain healthy red blood cells, support immune function, regulate cell growth, aid in production of hormones, and may have a role in preventing some types of cancer. They also function in maintenance of healthy skin, hair, and nails [3] .

Therefore, a B vitamin supplement is considered a cheap, safe, and reliable way to improve overall wellbeing and mental abilities. Throughout the modern community, reports of the advantages of B vitamin therapy abound. Supplementary B vitamins have gained a widespread reputation for their ability to counteract stress and depression and promote vitality. Because of these benefits, B vitamins are widely prescribed by alternative practitioners, and yet, in spite of these facts, the number of people to benefit from extra B vitamins remains uncertain. In recent years, recommendations to take various vitamins (and other supplements) are so commonplace that often people are taking six, eight or even more vitamins each day [4] .

Unlike prescription and over-the-counter drugs, there is barely any government oversight of the safety and efficacy of vitamins, minerals and herbal and nonherbal

supplements. Compliance to the standards is voluntary, and only the manufacturer knows if the label information accurately reflects the actual contents and their strength.

According to the directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 [5], as well as to the corresponding regulations within our country [6], the labeling, presentation and advertising must not attribute to food supplements the property of preventing, treating or curing a human disease, or refer to such properties. Also, the nutrient advertised on the label must be present inside the vitamin formulation and must not exceed the stated recommended daily dose [7].

As some people may need supplements to correct deficiencies of particular vitamins caused either by an inability to convert the vitamins to their physiologically active forms or, on the other hand, by abnormalities of intestinal absorption, plasma transport, tissue storage, binding to proteins, or excretion [8], an incorrect dosage, or additional compounds, might prove harmful. In addition, as even large doses of B vitamins are considered generally non-toxic, it is not hard to ingest significant amounts of vitamins, several times the recommended daily allowance.

Analytical test methods for vitamins in food have been around for decades. In the last few years, new methods have been developed to replace many of the older tests. The methods of the AOAC (American Organization of Analytical Chemists) widely used for the estimation of water soluble vitamins B₁, B₂, B₆, B₁₂, C, niacin, niacinamide and folic acid involved extraction procedures and chemical reactions, followed by fluorimetry (B₁ and B₂), spectrophotometry (niacin and folic acid), titration (pyridoxine) and microbiological (B₁₂) [9]. These are time-consuming, tedious methods.

In recent years, high-pressure liquid chromatography (HPLC) has been shown to be a powerful tool for the determination of various compounds including water-soluble vitamins. HPLC methods have the advantage of being more specific, non-destructive and are capable of differentiating between different forms of a vitamin with varying biological activity [10].

There have been reported several HPLC analysis of the B vitamin group, either from food matrices [10-12], or tablet preparations [13-15]. To this date, no validation study of products commercialized as dietary supplements of the B vitamin complex on the Romanian market has been published.

The purpose of this study is to compare pharmaceutical vitamin preparation to

dietary supplements with the same declared vitamin amount by using suitable extraction methods and chromatographic techniques that would allow a fast, sensitive and non-destructive analysis of a large number of samples.

MATERIALS AND METHODS

REAGENTS AND SOLVENTS

All reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Methanol and water, doubly distilled on glass, were used to prepare the mobile phase.

Thiamine hydrochloride (injectable solution Sicovit, 50 mg/mL, Sicomed), pyridoxine hydrochloride (injectable solution Sicovit, 25 mg/mL, Sicomed), riboflavin (Fluka, Buchs, Switzerland) and nicotinic acid (Fluka, Buchs, Switzerland) were used as working standards.

APPARATUS, MOBILE PHASE AND CHROMATOGRAPHICAL CONDITIONS

The liquid chromatography system used consisted of a Merck Hitachi L-6200A Intelligent Pump, connected to a 20 μ L Rheodyne 7125 injection valve, a Merck LiCroSpher C₁₈ (5 μ) in LiChroCART (125 x 46 mm) column and a Waters Photodiode Array Detector PDA 996, data being processed by Millennium 32 software (Waters). The experiments were conducted at room temperature.

The three mobile phases were prepared by modifying the protocols used by Ekinci & Kadakal [12] and by Khor & Tee [14]:

- (1) MeOH : water; 10:90, pH 7, adjusted with phosphate buffer
- (2) MeOH : water; 10:90, pH 3, adjusted with H₃PO₄ 0.007M and KH₂PO₄ 0.043M
- (3) MeOH : water, 35:65, pH 7, adjusted with phosphate buffer

The flow rate was 1 mL/min and the injected volume 20 μ L. The prepared mobile phases were filtered on 0.2 μ m Millipore filter and degassed in a microwave oven prior using.

STANDARD SOLUTIONS

Standard stock solutions of 1 mg/mL water were prepared for each of the 4 vitamins. Working standard solutions were obtained by diluting 1 mL of each of the standard stock solutions to 10 mL with the mobile phase

SAMPLES

Pharmaceutical preparations: 4 different tablets containing amounts of several B vitamins (designated PhP 1, 2, 3, and 4)

Dietary supplements: 5 different 4Life vitamin preparations (referred to as DS 1, 2, 3, 4 and 5) a prenatal vitamin formula (designated DS 6), and an energy drink mix (DS 7).

Dietary supplements from natural extracts: 5 formulas with ginseng, spirullina and plant extracts (NDS 1 to 5)

SAMPLE PREPARATION

Vitamin content of the samples was extracted by acid hydrolysis following a protocol modified after Khor & Tee [15].

The tablets were finely ground with pestle and mortar and weighed accurately into flasks before adding 2 mL of 0.1N sulfuric acid. The sample was heated for 30 minutes in a boiling water-bath and stirred frequently. After that the sample was cooled in a basin of water and the pH adjusted to 7 with sodium acetate 3.75M using pH paper. The samples were filtered under pressure. 200 μ L pure methanol were added to 1.8 mL extract. The samples were filtered through Millipore 0.2 μ m filters.

All the experiments were performed in duplicate, and the mean error was 15.7%.

RESULTS AND DISCUSSION

CHROMATOGRAPHY OF THE VITAMIN MIXTURE

Figure 1 shows the HPLC chromatogram of a mixture of three vitamin standards, separated using the mobile phase of methanol:water; 10:90, pH 7. All vitamins gave quite good separation at the retention time (in minutes) of 1.6 for nicotinic acid, 2.5 for pyridoxine and 7.8 for thiamine. The chromatograms were integrated with MaxPlot subroutine and at 260 nm and 324 nm (for pyridoxine) respectively. Absorption spectra of the vitamins have been compared with those obtained from literature [16].

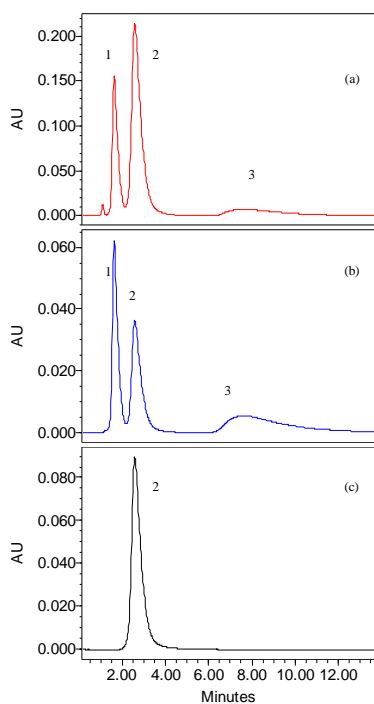


Figure 1. Chromatogram showing the separation of vitamin mixture of B₁, B₃ and B₆ in MeOH:water 10:90, pH 7. (a) MaxPlot; (b) 260nm; (c) 324nm. 1.nicotinic acid, 2.pyridoxine, 3. thiamine

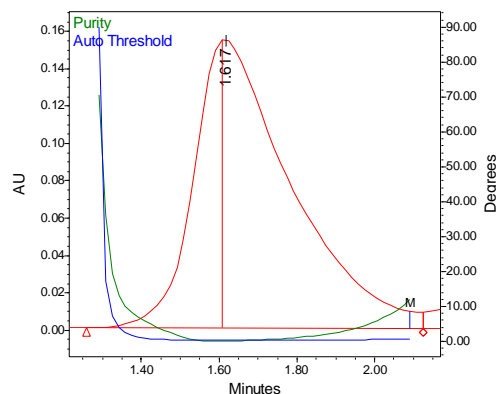


Figure 2 Purity assay of the pyridoxine standard

STANDARD PURITY

The evaluation of standard purity was performed using the built-in function Millennium 32 software. The M point of maximum impurity was outside peak area and 95 percent of peak area was above purity threshold level (Figure 2).

Thus, the purity of the standards prepared from injectable solutions of thiamine and pyridoxine hydrochloride was found satisfactory.

METHOD VALIDATION

The isocratic separation method in MeOH 10%, published by Ekinici & Kadakal [12] was modified by using a phosphate buffer to adjust the pH to 7, instead of only monopotassic phosphate, and increasing the flow rate from 0.7 mL/min to 1 mL/min.

The pH of the mobile phase was a critical factor for separation of the vitamins. We tested the separation resolution of the vitamin mixture on a 10% MeOH mobile phase at pH 3, as all three vitamins (thiamine, pyridoxine and nicotinic acid) have one of the pKs at

around 4.5-5. Therefore, at a pH below that value, the amount of protonated form of the vitamins would be higher, thus modifying the retention time and elution order of the compounds (Figure 3).

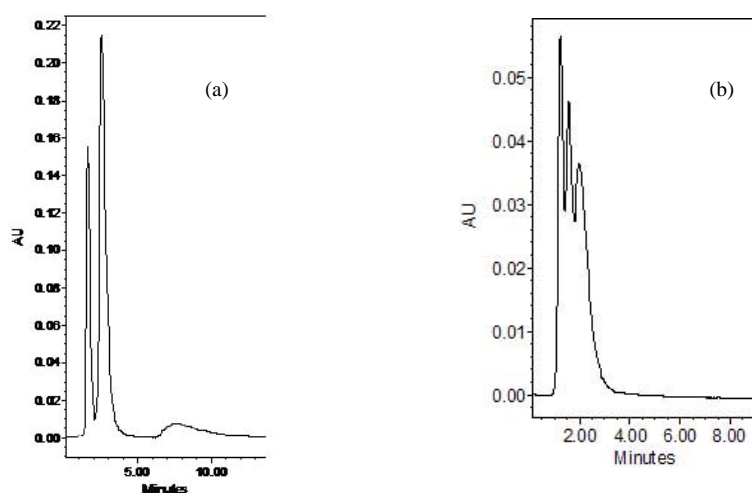


Figure 3 Separation of vitamin mixture at pH 7 (a) and at pH 3 (b), mobile phase MeOH : water, 10:90.

The vitamins eluted as follows: thiamine at 1.2 min, pyridoxine 1.6 min, nicotinic acid 2 min. The resolution of the separation was poor as all the vitamins eluted under 4 minutes and the vitamin absorption spectra were less characteristic at this pH, making the identification of the compounds less certain.

When we increased the percentage of methanol in the mobile phase to more than 10%, the vitamins nicotinic acid, pyridoxine and thiamine eluted in less than 3 min and resolution was very poor. In contrast, riboflavin eluted at around 5.5 min and resolution of riboflavin was good only when the proportion of methanol was increased to 35 %.

In view of the results obtained on the three mobile phases tested, we chose the mobile phase containing 10% methanol, at pH 7 for the analysis of vitamins B1, B3 and B6, as the separation was better than for the other mobile phases, while the retention time

remained under 10 minutes for the vitamins analyzed, allowing a rapid analysis of a large number of samples. For riboflavin, we used the mobile phase containing 35% MeOH in order to decrease the retention time for this vitamin, making thus possible a faster analysis of several samples within a given time limit.

At pH 7, the riboflavin peak was split into two peaks, as this pH is close to the isoelectric point (pH 6) of the vitamin and almost half of the compound is still protonated. During further trials, we propose to test a mobile phase of a lesser pH for riboflavin, while maintaining a high methanol content.

DETERMINATION OF PYRIDOXINE IN VITAMIN PREPARATIONS

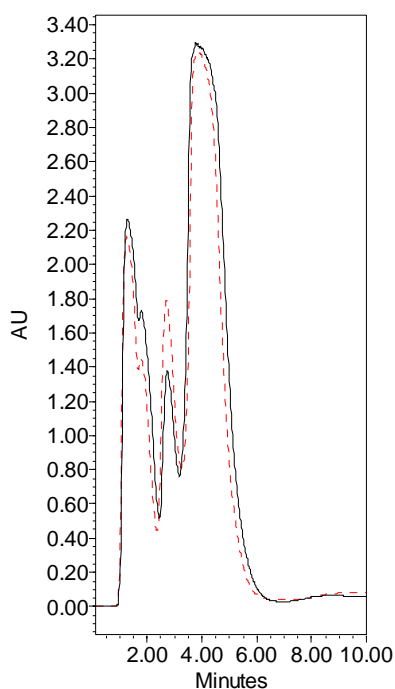
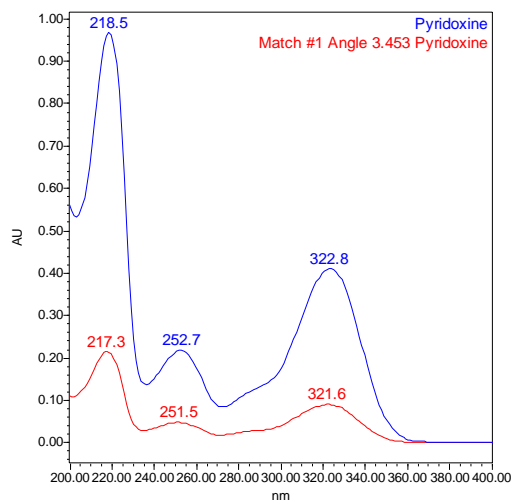


Figure 4. Chromatogram of samples PhP 2
(a)- cont. line, unspiked and (b)-dotted line, spiked

Figure 5. Absorption spectrum match for pyridoxine



In order to assay the loss of vitamin during extraction procedures a sample of a pharmaceutical multivitamin (PhP 2) was spiked with 2 mL of standard stock solution of pyridoxine (Figure 4).

The pyridoxine in the vitamin preparation was identified both from comparison with the retention time of the standard solution and by matching the absorption spectrum with the one recorded for the pure vitamin (Figure 5). The amount of vitamin in samples was calculated from standard calibration and external standard calibration (sample spiking)

The results are presented in Table I.

Table I. Determination of pyridoxine in vitamin preparation

Sample	Amount declared/tablet (mg)	Amount found (mg)	% found of the expected vitamin content
PhP 1	2	0.8275	41.37
PhP 2	5	2.2670	45.34
PhP 3	3	5.1973	173.24
PhP 4	5	4.3892	87.78
DS 1	1	0.4456	44.56
DS 2	0.7	0.3754	53.63
DS 3	1	0.5449	54.49
DS 4	2	1.3811	69.05
DS 5	1.2	0.4720	39.33
DS 6	2.6	0.6820	26.23
DS 7	0.37	nf	-
NDS 1	1	0.4001	40.01
NDS 2	unspecified several B vitamins	nf	-
NDS 3	unspecified several B vitamins	nf	-
NDS 4	unspecified several B vitamins	nf	-
NDS 5	unspecified several B vitamins	nf	-

nf – not found at the sensitivity of the method (1 µg/mL).

In calculating the amount of vitamin in the samples tested, loss during grinding and during extraction was taken into consideration. Loss during mechanical preparation of samples was assessed at around 20%. The method of determination of the loss during extraction needs further improving in order to allow for averaging solvent loss by

evaporation. Literature data place recovery of vitamin content after extraction by acid hydrolysis at 98% for thiamine, 110% for riboflavin and pyridoxine and around 90 % for nicotinic acid [15].

The amounts of pyridoxine recorded for each of the samples were in less than the amount declared, in almost all cases. In one case the amount was more than the amount declared (PhP 3). As for dietary supplements from natural extracts, only in one case pyridoxine was identified in the formula, though the prospects advertised the presence of B vitamins in all 5 dietary supplements on test. No pyridoxine was identified in the energy drink mixture (DS 7), even though the amount declared was high enough for analysis.

Figure 6 shows the chromatograms for some of the samples. As can be seen, there is no peak for pyridoxine in sample NDS 2.

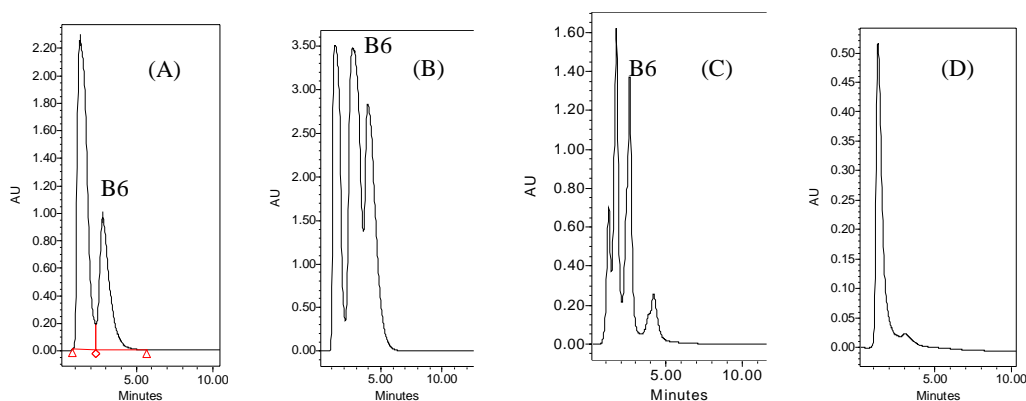


Figure 6. Chromatograms of vitamin samples, MeOH 10%, pH7.
(A) PhP 4. (B) PhP 3. (C) DS 4. (D) NDS 2

DETERMINATION OF NICOTINIC ACID IN VITAMIN PREPARATIONS

Although the presence of nicotinic acid was not proved by comparing the spectrum of the eluted peaks with the spectra from the library, we can assume that this vitamin can be present in the analyzed samples in form of nicotinamide or other forms. In order to identify these forms an appropriate standard has to be used.

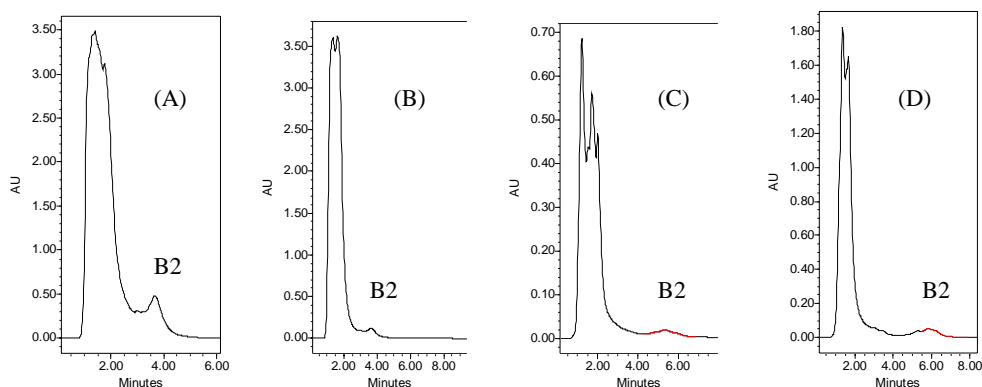
DETERMINATION OF RIBOFLAVIN IN VITAMIN PREPARATIONS

Figure 7. Chromatograms of vitamin samples, MeOH 35%, pH 7

(A) PhP 1; (B) PhP 3; (C) DS 3; (D) DS 4

Concerning the presence of riboflavin in the analyzed samples (see Figure 7) the results confirm its presence in all the samples. Although the quantities of B2 in the samples was not always as it was mentioned in the prospects, tacking into account the errors of determination, we can consider that the differences are on acceptable limits. An accurate quantitative estimation was difficult due to the split of the peak of B2 in the conditions of the separation.

CONCLUSION

The simultaneous determination of the water soluble vitamins B1, B3, B6, can be satisfactorily performed on a C18 column using mobile phase of 10% methanol, at pH 7. Riboflavin (B2) was determined separately on a methanol:water, 35:65 mobile phase, because of its high retention time on the previous mobile phase. At pH 7, riboflavin showed a split peak, making determinations difficult.

From complex samples the vitamins can be easily extracted by acid hydrolysis, filtered and brought to pH 7 in order to be injected in HPLC system.

For most of the pharmaceutical preparations tested, the amounts of vitamin assessed by HPLC (pyridoxine and riboflavin) were below the declared values (considering the limits of experimental errors), and in some of the preparations, the vitamins were found to be missing entirely.

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