

# Determination of Cyanocobalamin in Multivitamin Tablets by Multimode High-Performance Liquid Chromatography

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## Abstract

Quantitative determination of cyanocobalamin in B-complex tablets was performed by using multimode high-performance liquid chromatography. The multivitamin tablets (B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub>) were sonicated for 30 min in methanol-water (50:50, v/v) and diluted to appropriated volume with the same solvent. The resulting solution was filtered and the filtrate was analyzed on a (R)-phenylephrine-bonded silica column (150 x 4.6 mm i.d., 5 μm). The optimized mobile phase was 30 mM phosphate buffer (pH 3.0) containing 5% (v/v) acetonitrile at a flow rate of 1 ml min<sup>-1</sup> and the detection was measured at 360 nm. The calibration graph obtained was linear from 2.5 to 12.5 μg ml<sup>-1</sup>, with a coefficient of determination of 0.9999. The detection and quantitation limits were 0.25 and 1.25 μg ml<sup>-1</sup>, respectively. The accuracy was expressed as the percent of recovery and closed to 100%. An analysis was completed in 6 min. The new method is simple, rapid and precise.

**Key words:** Cyanocobalamin, Vitamins, HPLC, (R)-Phenylephrine

## INTRODUCTION

The problem of determination of cyanocobalamin (vitamin B<sub>12</sub>) is of importance in the analysis of this vitamin in multivitamin formulations. Separations of vitamin B<sub>12</sub> from others in the pharmaceutical preparations by HPLC have been reported by various workers (1-6). The most common method is reversed-phase high-performance liquid chromatography (RP-HPLC) using isocratic elution. This method is successful when the amounts of cyanocobalamin and others, such as vitamin B<sub>1</sub> and B<sub>6</sub>, is similar. For vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> formulations, the ratios between B<sub>12</sub> and the others are in the range 1:100 to 1:1000, and RP-HPLC in the isocratic mode could not be used because the chromatographic peaks are not well resolved. In order to solve this problem, gradient elution was applied (3-6). However, the method is time consuming and has low reproducibility. The alternative is to change the selectivity of the stationary phases by using multimode chromatography. A multimodal support was designed by coupling (R)-phenylephrine to silica and the chromatographic behavior of various compounds was investigated (7). The retention of all compounds on this stationary phase depends on a mixed mechanism, including ionic and hydrophobic interactions. It is of interest to apply this stationary phase to the analysis of vitamin B<sub>12</sub> in multivitamin formulations.

In this report, the retention of vitamins B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub> on (R)-phenylephrine bonded silica was investigated by modifying the pH of the mobile phase in the isocratic mode. An analytical application to the determination of vitamin B<sub>12</sub> in the vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets is given.

## MATERIALS AND METHODS

### *Instrumentation*

The chromatographic apparatus consisted of HPLC system (Spectra-Physics, San Jose, CA, USA) with Spectra system P1000 pump, a Spectra system UV1000 detector and a Rheodyne 7125 injector with 20 $\mu$ l loop. (Rheodyne, USA). Chromatograms

were recorded and processed with Barspec data system (Barspec, Rehovot, Israel).

### *Reagents and chemicals*

The (R)-phenylephrine bonded silica was prepared as previously described (7). Secondary standard of vitamin B<sub>12</sub> was from Division of Drug Analysis (Department of Medical Science, Ministry of Public Health, Nonthaburi, Thailand). Secondary standards of vitamin B<sub>1</sub> (thiamine mononitrate) and vitamin B<sub>6</sub> (pyridoxine hydrochloride) were from the Research and Development Institute (The Government Pharmaceutical Organization, Bangkok, Thailand). HPLC-grade acetonitrile was from J.T. Baker (USA). The water was deionized and distilled. All reagents were of analytical grade.

### *Sample of the vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets*

The sample used was vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets (The Government pharmaceutical Organization, Bangkok, Thailand). Each tablet contains: vitamin B<sub>1</sub> (thiamine mononitrate, 100 mg), vitamin B<sub>6</sub> (pyridoxine hydrochloride, 7.5 mg), vitamin B<sub>12</sub> (cyanocobalamin, 75  $\mu$ g).

### *Chromatographic conditions*

The (R)-phenylephrine-bonded support was packed into a 150 x 4.6 mm i.d. stainless steel column by conventional high-pressure slurry-packing procedures (8). The retention of vitamin B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub> was studied using 30 mM phosphate buffer (pH 2.5-6.5), 5% (v/v) acetonitrile, as eluent and detection was at 360 nm. A sample of the Vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets was analyzed by using 30 mM phosphate buffer (pH 3.0) containing 5% (v/v) acetonitrile, as eluent and detection was at 360 nm. In all cases, the flow rate was 1 ml min<sup>-1</sup>.

### *Chromatographic behavior of vitamins B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub>*

Stock solutions of each vitamin were separately prepared using methanol-water (50:50, v/v) as solvent at concentrations of 20  $\mu$ g ml<sup>-1</sup>. A 5- $\mu$ l volume was injected onto the column. Effect of mobile phase pH were studied using 5% (v/v) acetonitrile in 30 mM phosphoric acid previously adjusted to the desired pH (2.5-6.5) with 1N and/or 0.1N NaOH.

### Preparation of calibration curves

A calibration graph for vitamin B<sub>12</sub> was measured in the range 2.5–12.5 µg ml<sup>-1</sup> using methanol-water (50:50, v/v) as the diluting solvent.

### Sample preparation

Weighed and finely powdered 20 tablets. Transfer an accurately weighed portion of the powder equivalent to 375 µg of vitamin B<sub>12</sub>, to a 50-ml volumetric flask and 35 ml of methanol-water (50:50, v/v) was added. After sonication in an ultrasonic bath for 30 min the solution was diluted to volume with the same solvent and mixed well. The solution was then filtered through Whatman No. 1 filter paper (Whatman, Maidstone, UK) and the first 10-ml filtrate discarded. A 20-µl portion of each of final solutions was injected onto the column after filtration through a 0.45-µm membrane filter (Whatman).

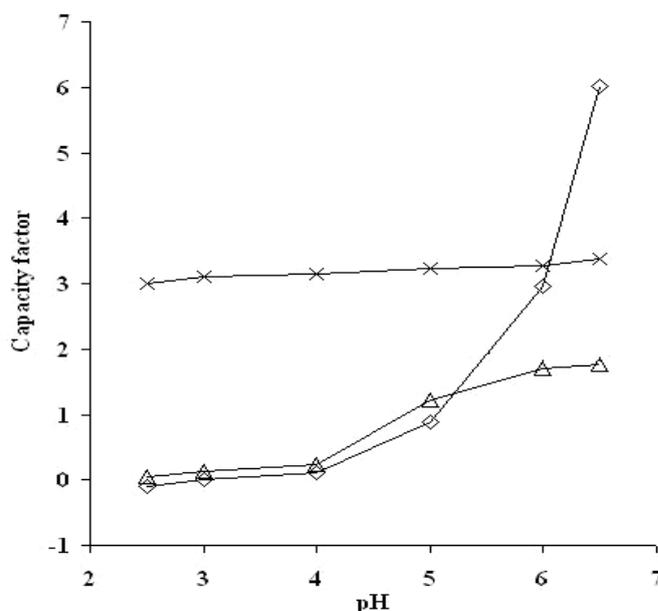
### Validation of the method

Linearity was evaluated in the concentration range 2.5–12.5 µg ml<sup>-1</sup> of vitamin B<sub>12</sub>. The data were analyzed by least-squares linear regression method. Accuracy of the developed method was studied by standard addition method. Spike sample solutions were prepared at three

different concentrations of standard added by separately added 1.0 ml of standard solution (5.0, 7.5, and 10.0 µg ml<sup>-1</sup>) into 1.0 ml of sample preparation and mixed well. Seven replications were performed at each concentration. The precision of injection was demonstrated by replicate injections of the standard solution (7.5 µg ml<sup>-1</sup>). The intra-day, and inter-day precision were investigated by injection of ten, and five replications of the same sample (different preparations). The precision of the method was expressed as the percentage of the relative standard deviation (%RSD).

## RESULTS AND DISCUSSION

The effect of eluent pH on the capacity factors of vitamins B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub> on (R)-phenylephrine-bonded silica column is shown in Figure 1. The capacity factors of the vitamins decreased markedly with decreasing pH. The decrease in retention of vitamins B<sub>1</sub>, B<sub>6</sub> and B<sub>12</sub> was 60-, 35- and 1.1-fold, respectively, when the pH changed from 6.5 to 2.5. The results showed the same behavior as organic basic compounds on this stationary phase in previous work (7). Vitamin B<sub>12</sub> was well resolved from the others between pH 2.5 and 4.0. In this study, pH 3.0 was selected for the analysis of vitamin B<sub>12</sub> in the multivitamin tablets.

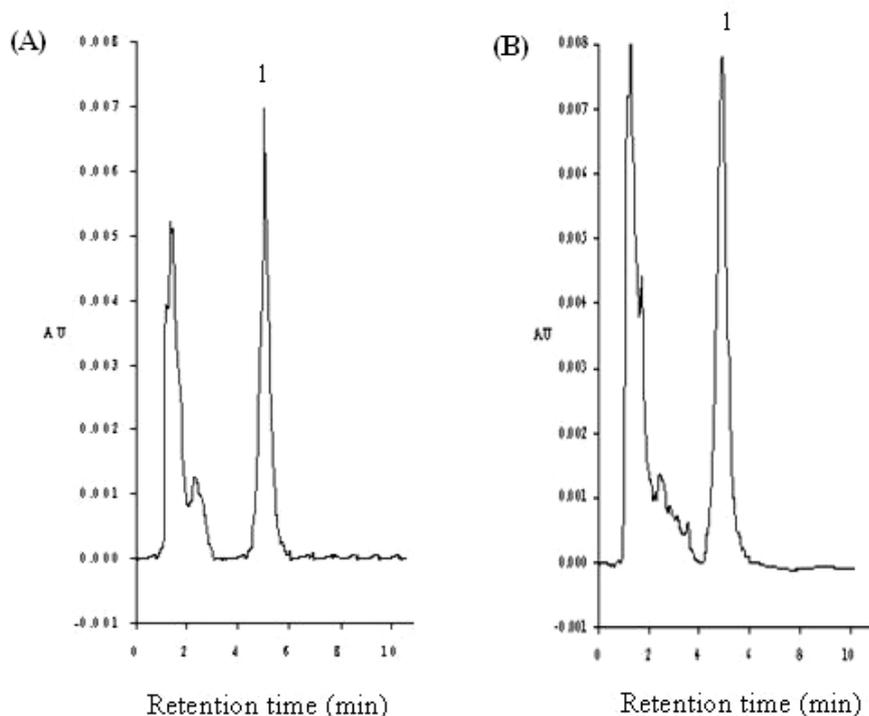


**Figure 1.** Effect of pH on capacity factors ( $k'$ ) of some basic water soluble vitamins: (◇) vitamin B<sub>1</sub>, (△) vitamin B<sub>6</sub>, and (×) vitamin B<sub>12</sub>.

Application of the method to the analysis B<sub>12</sub> in the vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets was performed by using methanol-aqueous solution as extracting solvent in order to increase the recovery of vitamin B<sub>12</sub> from the sample preparation because the solubility problem of thiamine mononitrate in water and the chromatogram is shown in Figure 2. The calibration graph was linear in the range 2.5-12.5 µg ml<sup>-1</sup> with the regression equation,  $y = -341.2 + 2304.8x$  ( $r^2=0.9999$ ), where  $x$  and  $y$  are the concentration (µg ml<sup>-1</sup>) of standard vitamin B<sub>12</sub> and peak area, respectively.

The limit of detection and quantitation were 0.25 and 1.25 (µg ml<sup>-1</sup>), respectively. The precision of injection was demonstrated by replicate injections of the standard solution (Figure 2A) and the relative standard deviation (RSD) of peak area was 0.57% (n=7). There was no interfering peak in the chromatogram of the sample (Figure 2B) and the analysis time was 6 min. The average amount of vitamin

B<sub>12</sub> in a tablet was 91.41 µg with an RSD of 1.27% (n=10) for intra-day assay. For the inter-day assay, the average amount of vitamin B<sub>12</sub> in a tablet was 90.53 µg with RSD of 1.21% (n=5). Accuracy of the method determined by the recovery study was achieved by adding three different concentrations of standard vitamin B<sub>12</sub> into the sample and the results are illustrated in Table 1. The recovery was from 98.5–101.9% with the average RSD value not more than 0.56%. The value demonstrated the high accuracy of the method and was found to be acceptable for routine drug analysis. Applications of the method to determine vitamin B<sub>12</sub> in other types of complex matrices, which contain water-soluble and fat-soluble vitamins, sugars, metal ions, oily particles or proteins will be further explored. The ability to simultaneously separate compounds of different properties renders the (R)-phenylephrine-bonded column a valuable tool in pharmaceutical analysis.



**Figure 2.** Chromatograms of (A) vitamin B<sub>12</sub> (7.5 µg ml<sup>-1</sup>), (B) Vitamin B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets containing 91.41 µg per tablet. HPLC column was (R)-phenylephrine-bonded silica (150 x 4.6 mm i.d., 5 µm) and mobile phase was 30 mM phosphoric acid, pH 3.0 with 5% (v/v) acetonitrile. The flow rate was 1 ml min<sup>-1</sup> and UV detection was 360 nm.

**Table 1.** Recovery study: final solution of sample solution containing vitamin B<sub>12</sub> at a concentration of 8.984 µg ml<sup>-1</sup>

Added (µg ml <sup>-1</sup> )	Found (µg ml <sup>-1</sup> )	Recovery (%)	RSD (%)	n
5.0	4.97	99.4	0.25	7
7.5	7.48	99.7	0.40	7
10.0	10.02	100.2	0.56	7

## CONCLUSION

Development of a method for determination of cyanocobalamin in B<sub>1</sub>-B<sub>6</sub>-B<sub>12</sub> tablets using (R)-phenylephrine-bonded silica in the isocratic mode was carried out. The application of the method to the analysis of cyanocobalamin in B-complex tablets is demonstrated and shown to be simple and rapid with a high degree of accuracy and precision.

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