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ANALYSIS OF VITAMIN B_{12} (CYANOCOBALAMIN) IN MULTIVITAMIN PRODUCT USING HPLC DAN LC-MS/MS

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Abstract
Vitamin B_{12} analysis in multivitamin preparations has been developed. It is caused the analysis of vitamin B_{12} in multivitamin preparations. The influence of matrix in the preparation and the amount of vitamin B_{12} are relatively small in the preparation. Several studies have been conducted by developing methods of identification and determination of vitamin B_{12} levels in multivitamin preparations. Some studies have been done by using of HPLC and LC-MS. The application of vitamin B_{12} was performed by HPLC and LC-MS method. The validation results of specificity, linearity, precision and accuracy met the requirements.

Keywords: Vitamin B_{12}, Multivitamin, HPLC, LC-MS/MS

1. INTRODUCTION

Vitamin is an essential compound for the human body. It can be found by consuming food or taking a multivitamin supplement. (1).

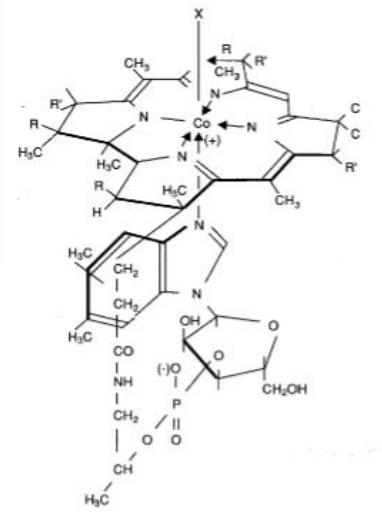

Figure 1. Vitamin B_{12} (13)

One of the vitamin B group is vitamin B_{12}. Vitamin B_{12} has an important function in the body which play an important role in the formation of red blood cells, activities in the nerve cell system and the translocation of methylene groups in DNA synthesis. The function of vitamin B_{12} is closely related to the function of folic acid. Besides, folic acid, vitamins B_{12} and B_6 and vitamin B_2 uses in the metabolism of homocysteine methionin (2, 3,4,5). The function of vitamin B_{12} for the cognitive development of children is cofactor in the central nervous system (6,7). In addition, vitamin B_{12} has a relationship with the metabolism of essential fatty acids for the maintenance of myelin (8). Deficiency of vitamin B_{12} in the body can lead to megaloblastic anemia, nervous system disorders and DNA synthesis. Vitamin B_{12} deficiency can cause megaloblastic anemia and nervous system disorders (4). The food sources containing vitamin B_{12} include meat, fish, vegetables, cereals, milk, cheese and fermented foods such as yeast (4). Vitamin supplements are available on the market to prevent and control the symptoms of avitaminosis. On the other hand, it is the purpose of strengthening the immune system and treatment in some diseases such as multivitamin tablets (9,10). From the dosage data on the market, the preparation of vitamin B_{12} in the form of multivitamin is a small degree. Some components of the matrix has the potential affect of vitamin B_{12}. Vitamin B_{12} levels may decrease in combination of trace elements (fluorine, manganese, molybdenum and copper) with ascorbic acid (11).

Analysis of Vitamin B_{12} (Cyanocobalamin) has been developed. The previous study was identification of cyanocobalamin by UV-Vis spectrophotometry. This method is based on the specific colors by cyanocobalamin and its analogue compounds. Cyanocobalamin and its analogue compounds give red, red-orange and yellow. UV-Vis electrofotometric observation was performed at 361 nm wavelengths. However, this method has a weakness if the sample is a multivitamin form. On the other hand, the method used the mirobiological test. This mecrobiological test is based on the microbiological absence within the media which has been added cyanocobalamin. However, this method has a weakness in terms of sample preparation requires, it was long incubation time (12). The present study was performed by the HPLC analysis method. It was more simple and rapid. Many researchers were optimized by the HPLC method to determine the vitamin B_{12} levels in multivitamin preparations (12). The difficulty in determining the level of vitamin B_{12} has very small concentration in the sample so that it needs to develop the method analysis using LC-MS. It can detect vitamin B_{12} in small levels (13).

II. ANALYSIS OF HPLC METHOD

Several optimizations were performed to obtain a sensitive, selective and rapid analysis method using HPLC.

A. Optimization of Wavelength Detector

Determination of the wavelength on the HPLC detector was very important. The optimum and specific uptake of vitamn B_{12} can be separated from multivitamin sample preparation. The selected wavelengths was 546 nm. Vitamin B_{12} has 3 uptake at wavelengths of 260, 360 and 546 nm. Reasons of choosing the wavelengths is not only vitamin B_{12} absorption, but also other vitamins absorb in UV area. Vitamin B_1, B_2, B_6, PP and Folic acid absorb at 360 nm wavelengths, folic acid and riboflavin absorb at 360 nm (12). This study was performed a Nucleosil C18 (250 mm x 4 mm x 8 mm) (Macherey-Nagel) column with a water motion phase: methanol: glacial acetic acid (65: 34: 1) containing 1.3 g of Sodium-1-heptan sulfonic acid . The calibration curve is made from the concentration range of 0.2 to 4.0 µg/mL. The result of research was coefficient of calibration curve variation of 1.41% (n = 10). The precision of testing multiple multivitamin samples with coefficient of variation is 0.67-1.20%.

B. Selection of Detector Type

Other development was conducted by selection of HPLC detector types. The study used fluorescence detectors to improve sensitivity and precision. Determination of vitamin B_{12} content in tablet multivitamin preparation using fluorescence detector has been conducted (14). Fluorescence detector wavelengths were 305 nm (excitation at 275 nm). The column was used µBondapack C18 (300 x 3.9 nm, 10 µm) with a methanol-water phase (30:70) isocatically. The calibration curve was prepared from 1.00 to 100.00 ng.mL with a correlation coefficient of 0.998 (n = 6), LOD 0.1 ng / mL, 94-102% recovery and precision with % RSD 1.8 - 4.1%.

C. Optimization of Sample Preparation

The matrix of samples can influence the determination of vitamin B_{12} content if it was compared with others. Therefore, the sample selection should be matched with the matrix type contained. The optimizations were conducted to solve the matrix contained by SPE Technique. The matrix contained in the multivitamin preparations was very complex, including water soluble vitamins and fat soluble (15). Solid Phase Extraction Technique or SPE was used to separate soluble and fat soluble vitamins. In this research used SPE C18 AR 3 mL catridge by eluting 500µL methanol and 500 µL deionized water. The elution solution was used to optimize based on polarity. Chloroform is the elution solution selected to release fat soluble vitamins. The results were obtained the accuracy of water soluble vitamins and fat soluble of 78-116%. The minimum analysis time is 15 minutes and 8 minutes for water soluble vitamin fraction and fat soluble vitamins respectively.

D. Optimization of Mobile Phase

There are 2 types of flow rate in HPLC method, it were isoksratik and gradient. The isocratic technique was a constant mobile phase technique from the beginning to the end of the analysis. The gradient technique was a flowing technique that various time determined based on the composition of mobile phase. The gradient technique was used to separate the matrix sample. The main impact of this matrix interference is to cause poor resolutions.

E. Application of Vitamin B12 Analysis by HPLC

The identification and determination of vitamin B_{12} in multivitamin preparations using HPLC method has been performed to determine cyanocobalamin content in multivitamin tablets containing vitamin B_1, B_6 and B_{12} (16).

i. ***Sample Preparation:*** An amount of 375 µg transfered into 50 Ml ash and added 35 ml of methanol : water (50:50), sonicated for 30 min, and homogenized. The solution was filtered by Whatman filter No. 1, the filtrate was filtered by whatman 0.45µm membrane filter

ii. ***Calibration Curves:*** The calibration curve was prepared by concentration of vitamin B12 between 2.5 - 12.5 µg/ml in a methanol : water (50:50).

iii. ***The condition of HPLC:*** Stationary phase used column (R) -Phenylephrine packed into (150x4.6 mm id), mobile phase used 30 mM phosphate buffer pH 3 and 5% (v / v) acetonitril

iv. ***Validation methods***

- Linearity was evaluated with concentration range 2.5 - 12.5µg/ml
- Precision was prepared from 1 ml standard solution (7.5 µg/ml) in 1 ml sample, it was shaked with 7 replicates of intraday, and 5 replicates of interday and obtained % RSD.
- Accuracy was evaluated from 3 standard concentration (5.0, 7.5 and 10.0 µg / ml) into sample

v. ***Analysis Results***

- Calibration curve obtained linear regression equation y = 2304.8 - 341.2, r2 = 0.9999
- Precision obtained average levels of vitamin B12 in tablets of 91.41µg, RSD = 1.27% (n = 10) and 90.53 µg tablets, RSD = 1.21% (n = 5) for intraday and interday respectively.
- Accuracy obtained % recovery 98.5 - 101.9% and RSD < 0.56%
- LOD = 0.25 µg/ml, LOQ = 1.25µg/ml

III. Vitamin B_{12} Analysis by LC-MS / MS

Analysis of vitamin B_{12} by LC-MS / MS was used to analyze vitamin B12 in relatively small levels in multivitamin preparations. The optimizations were considered to use LC-MS/MS method (column type, type of ion source and sample preparation). The research was conducted to optimize the energy cone (cone voltage) of the MS system. LC-MS/MS optimizations were performed to obtain the sensitive analysis methods (13).

A. Optimization of Mobile Phase

Selected mobile phase was optimized to obtain optimum condition in LC-MS/MS method. The researcher optimized mobile phase to determine vitamin B_{12} in food preparations and multivitamins. There were type of mobile phases, such as methanol : water, methanol : water containing 0.1% formic acid, acetonitrile : water and acetonitrile : water containing formic acid. Then it was observed an increase in response ratio of S / N (13).

B. Optimization of Column Type

The selection of columns influenced the separation result. Types of column was optimized to obtain the best results. The type's column included C18, CN and C8 columns (13).

C. Optimization of Cone Voltage and Ion Quantifiers.

Cone voltage optimization is used to obtain optimum fragmentation abundance. Different cone voltages gave differences in the abundance of fragmentation ions. The result of the cone voltage optimization was as follows (13):

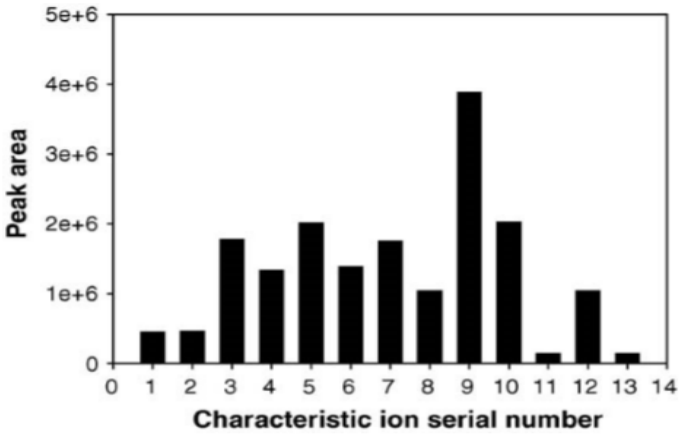

Figure 2. The effect of cone voltage on the abundance of ion fragmentation: (1) m / z 525.0 (70 V), (2) m / z 585.1 (80 V), (3) m / z 657.9 (60 V), (4) m / z 679.0 (40 V), (5) m / z 690.0 (30 V), (6) m / z 697.5 (40 V), (7) m / z 701.5 (40 V), (8) m / z 912.7 (180 V), (9) m / z 930.8 (180 V), (10) m /

z 1015.9 (130 V), (11) m / z 1356.1 (70 V), (12) m / z 1378.0 (100 V), (13) m / z 1394.1 (100 V).

D. Application of Vitamin B_{12} Analysis in LC-MS / MS

i. Preparation of standard solution and sample

- Standard solution preparation of vitamin B_{12} with concentration of 125 µg/ml was diluted in deionized water solvent
- Preparation of internal standard Ginsenoside Re with deionized water solvent obtained concentration of 40 µg/ml
- Preparation of standard curve solution was conducted by making solution with concentration range 6-150 ng/ml and added 0.1 µg/ml internal standard into concentration of standard solution.

ii. Sample preparation

An amount 1.6 g was transfered into a volumetric flask, and added 40 ml of sodium acetate buffer solution and 1 ml of 1% sodium cyanide. The solution was incubated for 30 min at 42 °C. The solution was cooled in room temperature. The solution was transferred into 50 ml volumetric flask and added 125 µl internal standard, it was diluted into deionized water,. The supernatant was filtered with a membrane filter of 0.45 µm before injected in the LC-MS/MS system.

E. Condition of LC-MS/MS

Condition LC-MS/MS was performed by Waters (Waters Corporation Milford, MA, USA) with inline degasser AF, 600 pumps, 600 controllers, 2777 C sample manager, connected to micro mass ZQ 4000 electrospray mass spectrometer (Manchester, UK). The Column was XTerra MS C18 Column (3.9 mm x 150 mm, 5 µm, Milford MA, USA), mobile phase was A (water) : B (Methanol) with gradient technique. Flow rate was 1 ml / min, Injection Volume was 50 mL, Ion Sourches was ESI, Capillary voltage was 400 V, Cone voltage was 180 V. Quantifier ion (m / z 930.8), Ginsenoside Re (m / z 969.9).

Table 1. The Composition of Mobile Phase in Gradient Technique

Time (min)	Composition of mobile phase B (methanol)
0-5	5-15%
5-10	15-30%
10-11	5%
11-15	5%

F. Validation Methods

Specificity was used to determine vitamin B_{12} with interference in the mixture. Linearity was conducted with concentration range of 6-150 ng/ml vitamin B_{12}, and internal standard 0.1 µg/ml. The result of linearity is calibration curve equation $y = (0.057 \pm 0.001)\ x + (0.0059 \pm 0.001)$, $r^2 = 0.9994$. Precision was conducted intraday and interday. Intraday conducted 5 times per day and obtained the result % RSD = 2.6%. Interday conducted in the solution for 3 days,and obtained % RSD = 3.5%. Accuracy was conducted at 3 levels of concentration and calculated % recovery. LOD obtained 2 ng/g.

Table 2. The Result of Recovery Percentage

Konsentrasi Vitamin B_{12}	% Recovery
8 ng/g	93.8 %
16 ng/g	95.6%
32 ng/g	97.5%

IV. CONCLUSION

Determination of vitamin B_{12} in multivitamin preparations has been developed. The present study used HPLC and LCMS/MS method. A sensitive and spesific of LC-MS/MS can separate the effect of matriks in analyte. The validation method met the requirement (specificity, linearity, precision and accuracy).

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