World Journal of Biology Pharmacy and Health Sciences
eISSN: 2582-5542
Cross Ref DOI: 10.30574/wjbphs
Journal homepage: https://wjbphs.com/

(REVIEW ARTICLE)

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A review: On HPLC method for estimation of methylcobalamin

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World Journal of Biology Pharmacy and Health Sciences, 2025, 21(01), 282-289

Publication history: Received on 30 November 2024; revised on 08 January 2025; accepted on 10 January 2025

Article DOI: https://doi.org/10.30574/wjbphs.2025.21.1.0018

Abstract

Methylcobalamin, a vital vitamin B_{12} analog, is susceptible to degradation under thermal stress, leading to changes in PH. Trigeminal neuralgia, diabetic neuropathy, facial paralysis and megaloblastic anemia can be treated by the use of Methylcobalamin injection. A new simple, precise, economically viable and efficient High-Performance Liquid Chromatographic (HPLC) method for the estimation of Methylcobalamin (MeB_{12}) in bulk drugs and pharmaceutical dosage formulations was developed and validated. The method was validated for linearity, accuracy, precision, LOD, LOQ and robustness as per ICH guidelines. The method was tested with commercially available MeB12 drugs and the concentration methylcobalamine was found to be accurate.

Keywords: Methyl cobalamin; HPLC; Method validation; Vitamin

1. Introduction

Chemically Methylcobalamine (Me B_{12} or Me Cbl, MF: $C_{63}H_{91}CoN_{13}O_{14}P$) belongs to cobalamin group compounds [1]. Methylcobalamin had pronounced susceptibility to hydrolysis under acidic, alkaline, and photolytic conditions. Methylcobalamin followed pseudo-first-order kinetics upon exposure to acidic and alkaline hydrolysis with highest stability at pH 5 and least stability at pH 2 [2]. Methylcobalamin is used for the treatment of megaloblastic anaemia. Methylcobalamine (MC; carbanide; cobalt; [5-(5,6-dimethylbenzimidazol-1 propanoylamino] propan-2-yl hydrogen phosphate) is derivative form of vitamin B_{12}. It is used for the treatment of peripheral neuropathy, diabetic neuropathy [3]. Methylcobalamin is essential for cell growth and replication. In the present investigation an attempt was made to develop an HPLC method. Methylcobalamin is the metabolically active form prerequisite for cobalamin-dependent enzyme.

1.1. Methylcobalamin

Methylcobalamin Literature review revealed that vitamin B_{12} is determined traditionally by microbiological assay using Lactobacillus; although the assay is sensitive, it is not specific as inactive cobalamin can interfere with microorganism. The most scientific and environmental major significancant role of MeB_{12} is biomethylation of certain heavy metals It is used in peripheral neuropathy, diabetic neuropathy and for preliminary treatment of amyotrophic lateral sclerosis. Structurally, cobalamins have centrally coordinated cobalt atom whose fifth position is covalently occupied by the groups such as CH_3. For the different commercial drugs of methylcobalamin namely Neurokind, Neurostar, Keeneuron and Merck sample were used to test the accuracy of the developed methods. Methylcobalamine is official in USP, EP and BP. Methylcobalamine is a routine estimation in liver, plasma, milk, intestinal fluids and faeces has been reported. Methylcobalamine is essential for cell growth and replication.

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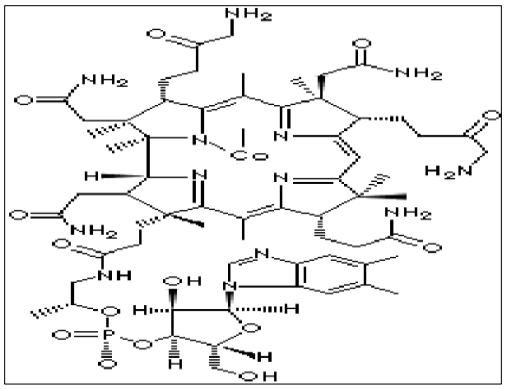

Figure 1 Chemical Structure of Methyl cobalamin

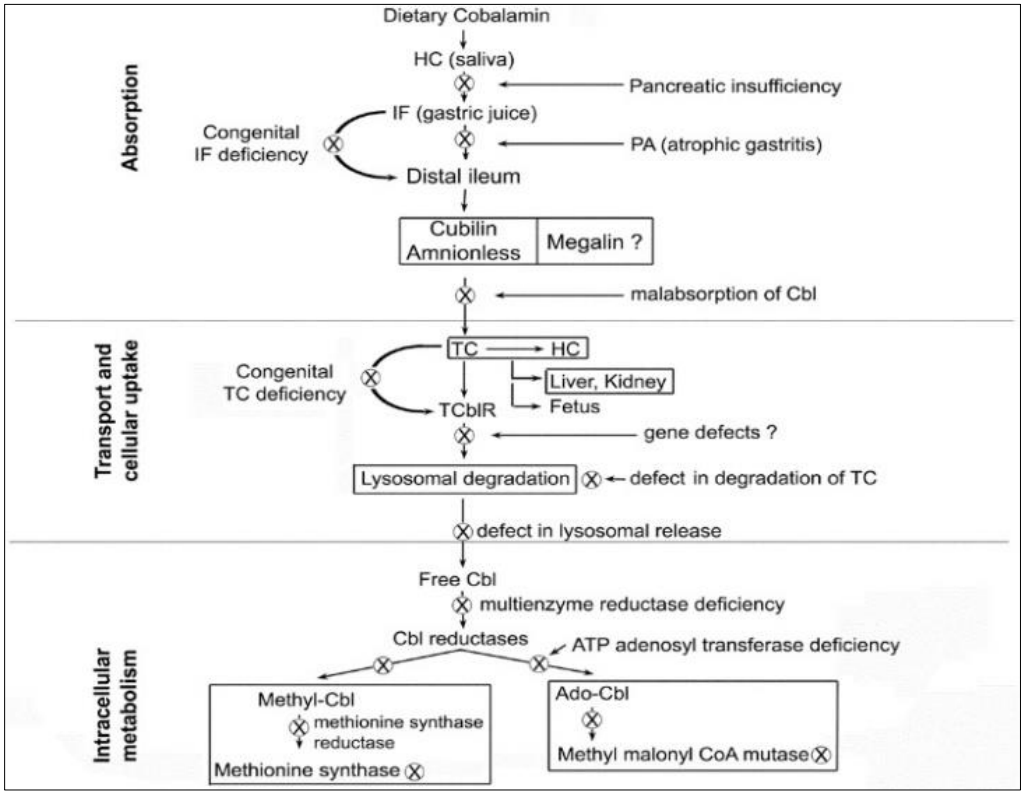

Figure 2 Mechanism of action of methylcobalin

Methylcobalamine is essential for cell growth and replication. Methylcobalamine are natural forms of vitamin B_{12} commercially available in different types of pharmaceutical dosage forms. Methylcobalamine is the metabolically active form prerequisite for cobalamin- dependent enzyme function of the drug. If extensive degradation occurs, limit exposure should be considered [6].

2. High performance liquid chromatography

High-performance liquid chromatography (HPLC) stands impurities, synthesis intermediates, and degradants. As a as a powerful analytical tool in modern chemistry. HPLC is an analytical technique in which solutes are resolved by differential rates of elution as they pass through a chromatographic column [6]. Now a day reversed-phase chromatography is the most commonly used separation technique in HPLC due to its broad application range. It is estimated that over 65% (possibly up to 90%) of all HPLC separations are carried out in the reversed phase mode [7].

HPLC is quantitative and qualitative drug product analysis, playing prized for its precision in both quantitative and a pivotal role in determining drug product stability [8].

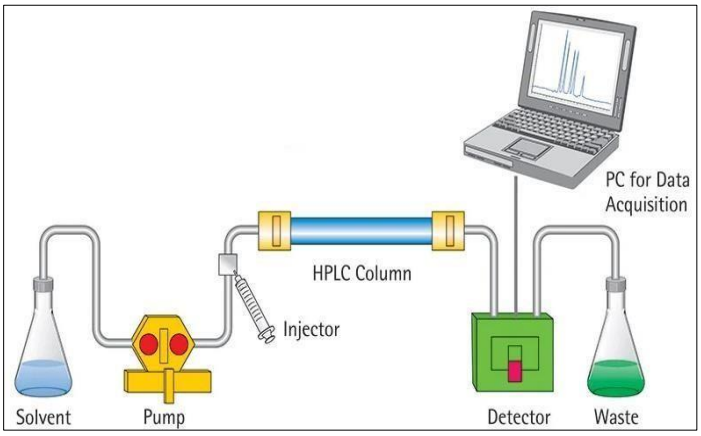

Figure 3 Instrumentation of HPLC

3. Types of HPLC

Depending upon substrate used i.e. stationary phase used, the HPLC was divided into following types:

3.1. Reverse Phase HPLC

Reversed phase chromatography has found both analytical and preparative applications in the area of biochemical separation and purification. Uses water-organic as mobile phase, columns may be C_{18} (ODS), C_8, phenyl, Trimethyl Silane (TMS), cyano as a stationary phase [8].

3.2. Normal Phase HPLC

It is first choice for mixtures of isomers and for preparative scale HPLC and second choice for lipophilic samples that cannot dissolve well in water-organic mixtures [8].

3.2.1. Bio-affinity chromatography

Based on specific interactions, making affinity chromatography a valuable technique in biochemistry and protein. most samples especially neutral or non-ionized compounds, that dissolve in water organic mixtures [9].

3.3. RP-HPLC (Reversed-Phase HPLC)

RP-HPLC is widely used for its ability to separate compounds based on hydrophobicity, providing effective analysis in various fields, including pharmaceuticals, biochemistry [9].

3.4. Instrumentation

The HPLC instrumentation involves the following parts:

3.4.1. Data collecting device (computer):

The computer not only controls all the modules of the HPLC instrument but it takes the signal from the detector and uses it to determine the time of elution (retention time) of the sample components (qualitative analysis) and the amount of sample (quantitative analysis) [10].

3.4.2. Sample Injection:

The injector can be a single injection or an automated injection system. Referring to load / inject valve in step (2e) verify valve handle is in the load position. And flow rate is 3ml/min. If valve handle is not in load position the plug will be forced out upon release by 2000 PSI. This should start the data acquisition. The display should change from "Waiting for Injection" to Running" [10].

3.4.3. Clean Syringe:

Rinse syringe thoroughly with DI water until clean [11].

3.4.4. Pumps:

It is necessary to pump the eluent at a constant flow rate and pressure. Conventional, analytical HPLC pumps are the most common type, but semi-micro and a preparative A pump can be compared to the human heart which continuously pumps blood throughout the body but through the human heart can withstand changes in blood pressure within a specified limit due to stress and strain the HPLC pump is required to deliver a low of mobile phase at constant pressure and low rate. e pumps are also used depending on the range of the eluent flow rate required [11].

3.4.5. Stationary Phase:

The stationary phase in HPLC refers to the solid support contained within the column over which the mobile phase continuously flows. The chemical interactions of the stationary phase and the sample with the mobile phase, determines the degree of migration and separation of the components contained in the sample. Columns containing various types of stationary phases are commercially available.

3.4.6. Columns

Columns are usually made of polished stainless steel, are between 50 and 300 mm long, and have an internal diameter of between 2 and 5 mm. The column is one of the most important components of the HPLC chromatograph because the separation of the sample components is achieved when those components pass through the column.

Column temperature control: For some applications, close control of column temperature is not necessary and columns are operated at room temperature.

Table 1 Columns Normal vs Reversed Phase

	Normal	**Reverse**
Packing polarity	High	Low
Solvent polarity	Low	High
Elution order	Non-polar first, then polar	Polar first, then non-polar
Effect of increasing solvent polarity	Decreases retention time	Increases retention time

3.4.7. Detector

The components eluted from the column are detected, and the detection data are converted into an electrical signal. The detector is selected to suit the sample. The detector provides an output to a recorder or computer that results in the liquid chromatogram

Table 2 Major types of detectors

UV detector	The light source is a D_2 lamp. This detector is used mainly to detect components having an absorption wavelength of 400 nm or less in the ultraviolet region.
UV-VIS detector	A D_2 lamp and a W lamp are used as the light source. This detector is effective in the detection of coloring components such as dyes and stains because of coverage of the visible light region
Diode array detector (DAD)	Data on the spectrum from the ultraviolet to visible light range is also collected
Fluorescence (FL) detector	Fluorescent substances can be detected specifically with high sensitivity.

Mass spectroscopy detector: Universal detector The MS detector can sometimes identify the compound directly since its mass spectrum is like a fingerprint and is unique to that compound.

3.4.8. Chromatograph Data Acquisition

save chromatogram, close chromatogram - go to Acquisition – Quick Start – Select Method – Be sure to select the method previously used, make any notes concerning injection volume etc. If you get an "In use" error close program and reboot computer.

3.4.9. Size Exclusion Chromatography (SEC)

This technique is widely used for the molecular weight determination of polysaccharides. In SEC, there is no interaction between the sample compounds and the column packing material. It is also useful for determining the tertiary structure and quaternary structure of purified proteins [11].

3.5. Method development of HPLC

The goal of the HPLC-method is to try & separate, quantify the main active drug, any reaction impurities, all available synthetic inter-mediates and degradation.

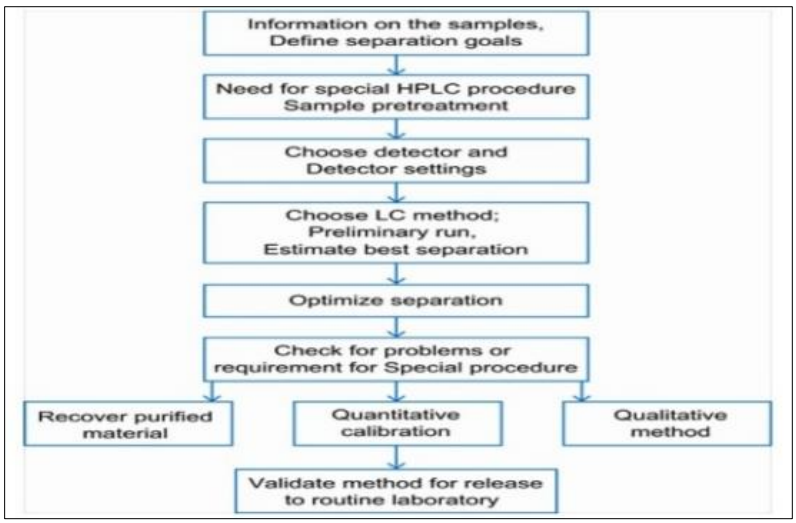

Figure 4 Flow chart of method development of HPLC

Chromatographic information. The PC coordinates the reaction of the indicator to every part and places it into a Chromatograph that is anything but difficult to interpret.

3.6. Components of method validation

- Accuracy
- Precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity Range
- Robustness
- Ruggedness

3.6.1. Accuracy

To study of the reliability suitability and accuracy of the method recovery experiments are carried out for MeB_{12} in two stages and the average recovery of drugs was calculated. The accuracy of the proposed method was assessed by triplicate analysis of three different concentrations (40, 80, and 120 µg/ml).

$$Accuracy = \frac{Correct\ prediction}{Total\ cases} * 100\%$$

$$Accuracy = \frac{(TP + TN)}{(TP + TN + FP + FN)} * 100\%$$

the assay was performed as per the test method. Then from these results the values calculated were, percentage recovery and quantity present (mcg).

3.6.2. Precision

Precision was evaluated at the repeatability and intermediate precision levels. For repeatability analysis, six independent portions of a sample solution of Methylcobalamin. The intra- and interday precision was carried out by analyzing three concentrations using in the same day or three successive days. %RSD of the results obtained. The values of %RSD within a day, day to day variation (<1%) proves that the method is precise.

$$\text{Precision} = \frac{True\ Positive}{True\ Positive + False\ Positive}$$

$$= \frac{True\ Positive}{Total\ Predicted\ Positive}$$

3.6.3. Linearity

The linearity of measurement was evaluated by analyzing different concentrations of the standard solutions MeB_{12}. A calibration curve was obtained for MeCbl by triplicate injection of MeCbl standard in the range of 2–160 µg/ml. Mean peak area of each concentration was plotted against the corresponding concentration linearity of standard Methylcobalamine powder was determined with the help of UV spectrophotometer at 353 nm and recorded their absorbance.

3.6.4. Detection and quantitation limits

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the following equations: LOD = 3.3* σ/S, LOQ = 10* σ/S, respectively, where S is the value of the calculated slope and σ is the residual standard deviation of regression line, and LOD and LOQ were found to be 0.6556 and 1.9867 µg/ml, respectively.

$$LOD = 3.3 \times S\ /SD \text{ and } LOQ = 10 \times S\ /SD$$

3.6.5. Specificity

Specificity was determined by quantifying MeCbl in the presence of probable excipients in different types of dosage forms (tablet, ampoule and oral dissolvable film) also in presence of all potential degradation products produced under stress degradation conditions. The forced degradation study for the triple combination drug revealed that drug was degraded under the influence of acid, alkali, thermal, water, hydrogen peroxide and photolytic conditions.

3.6.6. Ruggedness

Inter-day variations were performed by using six replicate injections of standard and sample solutions of concentrations which were prepared and analyzed by different analyst on three different days over a period of one week. Ruggedness also expressed in terms of percentage relative standard deviation [11].

3.6.7. Robustness

The analytical method robustness was tested by evaluating the influence of minor modifications in HPLC conditions on system suitability parameters of the proposed method such as the composition of the mobile phase, temperature and flow rate. that the percent of recovery was within acceptable limits and the %RSD was within limit of not more than 2.0%. The tailing factors and number of theoretical plates were found within acceptable limits as well.

3.6.8. Range

The range of method is interval between the upper lower levels of an analyte that have been determined

Table 3 Literature review of Methyl cobalamin

S.NO	COLUMN	MOBILE PHASE	RESULT ss	REFEERENCE
1	Kromasil C18	Methanol: water (ratio 20:80 v/v)	Retention time: 6 min Linearity: 0.999 Accuracy: 99.53% Precision:0.632-0.764% LOD: 0.15 µg/ml LOQ: 0.5 µg/ml	[12]
2	USP C18	Methanol: ACN (55:35:10, v/v)	Retention time: 6 min Linearity: 2–160 µg/ml Accuracy: 96.17% Precision: 96.17 % LOD: 0.6556 µg/ml LOQ: 1.9867 µg/ml	[13]
3	Cosmosil C18	Methylcobalamin: distilled water (50µg/ml)	Retention time: 6 min Linearity: 10-50 µg/ml Accuracy: 99.29 -100.5 % LOD: 0.00342 µg/ml RSD: 1.33	[14]

4. Conclusion

A stability indicating assay method for MeCbl determination was developed and validated. The proposed method is simple, accurate and applicable to the analysis of MeB_{12} in bulk drugs and tablet formulations. Percentage recovery was proved to be in par with ICH guidelines and the proposed method was accurate and simple.

This method was validation for linearity, accuracy, precision, repeatability, LOD and LOQ of Methylcobalamin.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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