INSTRUMENTAL PHARMACEUTICAL ANALYSIS OF VITAMIN B12 IN PHARMACEUTICAL PRODUCTS AND DIETARY SUPPLEMENTS: A REVIEW

Gjylai ALIJA[1*], Dorentina BEXHETI, Merita DAUTI, Drita Y.- HAVZIU, Nexhibe NUHII, Qail IBRAIMI, Sihana A.- LIKA, Edita A.-IDRIZI, Arlinda H.-ZAJMI, Lulzime BALLAZHI, Arijeta SHABANI, Taulant IDRIZI

[1*]*Department of Pharmacy, Faculty of Medical Sciences, State University of Tetova, Tetova R. N. M.*
[*]*Corresponding Author: e-mail: gjylai.alija@unite.edu.mk*

Abstract

Vitamin B12, or cobalamin, is a water-soluble vitamin that is vital for the proper functioning of the human body. It plays important roles in the formation of red blood cells, DNA synthesis, and the functioning of the nervous system. A deficiency in this vitamin can result in megaloblastic anemia, fatigue, weakness, neurological issues, and memory problems. Consequently, Vitamin B12 supplements are commonly utilized, particularly among older adults, vegetarians, vegans, and individuals with gastrointestinal absorption disorders.
The growing use of dietary supplements has made the analysis of Vitamin B12 essential in pharmaceutical and food quality control. The primary goal of this analysis is to assess the actual quantity of vitamin present in the product and confirm whether it aligns with the value stated on the label. LC-MS/MS is an advanced analytical technique used for determining Vitamin B12 in dietary supplements and pharmaceutical preparations. Furthermore, the analysis is used to examine the stability of the vitamin during storage, detect potential degradation, and ensure the safety and effectiveness of the supplement.

Keywords: vitamin B12, public health, dietary supplements, analysis

Introduction

Vitamins are a category of organic compounds that are necessary in minimal quantities for the proper functioning of the human body. They have diverse chemical and physiological roles and are commonly found in a variety of natural food sources. Vitamins are not produced by the body, so they must be obtained from food. They are categorized into water-soluble and fat-soluble types. Fat-soluble vitamins D, A, E, and K are easily processed by the kidneys and eliminated from the body. Water-soluble vitamins, including folate, riboflavin, vitamin C, vitamin B12, vitamin B6, niacin, and thiamine, are stored in the body's cells (Alija *et al.* 2024).
Vitamin B12 is a complex water-soluble vitamin, is a term that refers to four cobamides, which share a common lower ligand known as 5,6-dimethylbenzimidazole (DMBI) at the α-position and one of four upper ligands—adenosyl, methyl, hydroxo, or cyano—at the β-position. These forms are identified as adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxocobalamin (OHCbl), and cyanocobalamin (CNCbl). A central cobalt (Co) atom is coordinated with four pyrrole rings, with the lower ligand connected through a phosphate–sugar bond and the upper ligand linked via a metal–carbon bond.
AdoCbl and MeCbl are the coenzyme forms necessary for the function of the enzymes methionine synthase and methylmalonyl-CoA, is shown in Figure 1. (Chamlagain, 2016).

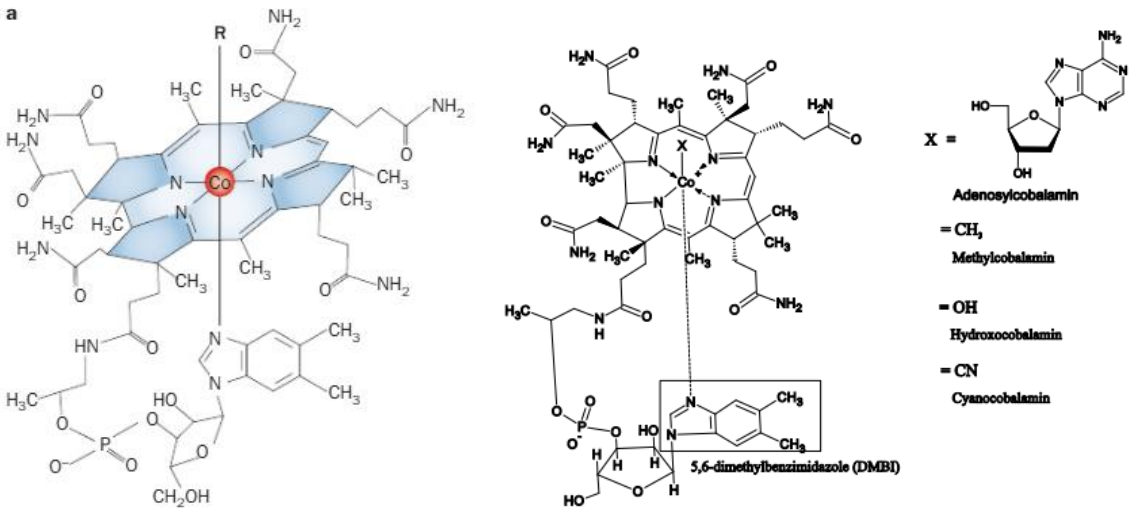

Figure 1. Structure of vitamin B12 ((Nielsen et al. 2012, Chamlagain 2016)

Vitamin B12 is vitamin essential for hematological and neurological functions. It serves as a cofactor in the processes of DNA synthesis, fatty acid metabolism, erythrocyte formation, aminoacid metabolism, nervous system function and cellular energy production. It is crucial for DNA synthesis, which directly influences cell division and growth. Without it, the maturation of blood cells can be hindered, resulting in megaloblastic anemia. Furthermore, vitamin B12 is vital for the upkeep of myelin, an important element for the proper functioning of the nervous system. (Green, 2011; Herrmann & Obeid, 2008).

Vitamin B12 is essential for two enzymatic processes: the conversion of homocysteine to methionine through methionine synthase, and the conversion of methylmalonyl-CoA to succinyl-CoA via methylmalonyl-CoA mutase (Nielsen *et al.* 2012).

Humans cannot synthesize vitamin B12 and therefore depend on dietary sources as well as a complex intracellular process for the vitamin's processing and delivery to its intended locations. (O'Leary & Samman, 2010).

Its absorption requires several steps: first, it is released from food proteins in the stomach, then it binds to intrinsic factor, followed by absorption in the terminal ileum, and finally, it is transported in the blood via trans cobalamin. Any disruption in these steps may result in a deficiency. Deficiency in vitamin B12 caused by malabsorption and insufficient intake is a global public health concern, is shown in Figure 2 (O'Leary & Samman, 2010; Hannibal *et al*., 2016; Allen, 2008).

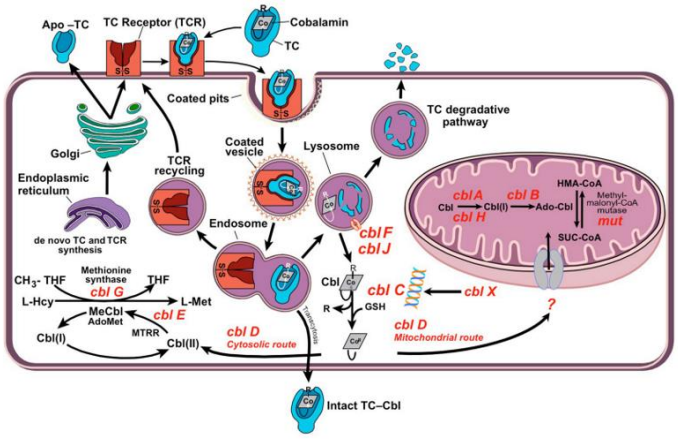

Figure 2. Cellular processing and trafficking of dietary vitamin B12 (Hannibal et al., 2016)

Vitamin B12 deficiency in humans is characterized by anemia and neuropathy. The anemia is clinically similar to that caused by folate deficiency and results from the inactivity of a vitamin B-dependent enzyme involved in folate metabolism. When megaloblastic anemia occurs due to a vitamin B12 deficiency, it is referred to as pernicious or Addisonian anemia. The neurological changes are not related to folate deficiency and arise from the inability to produce the lipid component of myelin, leading to a general demyelination of nerve tissue. Neuropathy initially affects the peripheral nerves, starting with the feet and fingers, and then progresses to the spinal cord and brain. If there is a deficiency in vitamin B12, taking folic acid supplements without including vitamin B12 may lead to irreversible neurological damage. While folate treatment may alleviate the apparent symptoms of anemia, it can also allow nerve degeneration to go unnoticed. Therefore, preventative vitamin supplementation should always include vitamin B12 along with folic acid (Stabler, 2013; Ball, 2005).

Proper evaluation of vitamin B12 levels in the body is important for the early detection and prevention of permanent complications.

A more typical presentation involves low or marginal levels of cobalamin ranging from 200 to 300 pg/mL (148–221 pmol/L) without accompanying symptoms. The groups most affected include older adults, individuals with pernicious anemia, pregnant and lactating women, and those with gastrointestinal disorders. People following a vegan diet may want to consider supplementation, in addition to the elderly population. High doses of cobalamin are generally considered safe and unlikely to result in toxicity (Hanna *et al*. 2022)

As a result, laboratory analysis of Vitamin B12 is essential to ensure the quality, efficacy, and safety of dietary supplements.

Vitamin B12 is available in multiple chemical forms, including cyanocobalamin, methylcobalamin, hydroxocobalamin, and adenosylcobalamin.

1. Cyanocobalamin is a more stable form and is most commonly found in supplements.
2. Methylcobalamin is the biologically active form and is frequently used for neurological disorders.
3. Hydroxocobalamin is administered through injections and has a longer duration of action.
4. Adenosylcobalamin is the metabolically active form found in mitochondria.

The most widely used form in supplements is cyanocobalamin due to its stability. The structure of Vitamin B12 is complex and features a cobalt atom at the center of the molecule.

The molecular formula of cyanocobalamin is $C_{63}H_{88}CoN_{14}O_{14}P$ (Ball, 2005)

The main purpose of the analysis is to assess the actual quantity of vitamin in the product and to confirm if it corresponds with the value stated on the label. Furthermore, the analysis is conducted to examine the stability of the vitamin during storage, detect any potential degradation, and ensure the supplement's safety and effectiveness.

Methods for assessing vitamin B12 include polarographic, spectrophotometric, and electrophoretic techniques, as well as various chromatographic procedures such as paper, thin-layer, open-column, gas chromatography (GC), liquid chromatography (LC), and liquid chromatography combined with mass spectrometry (LC-MS). Other methods include microbiologic and radio-ligand binding techniques (Eitenmiller, 2008)

One of the frequently utilized techniques for Vitamin B12 analysis is ultraviolet-visible spectroscopy. This technique relies on Vitamin B12's capacity to absorb electromagnetic radiation in the ultraviolet and visible regions of the spectrum. The concentration of the vitamin is calculated using the Beer–Lambert law: $A=\varepsilon \cdot b \cdot c$

which states that absorbance (A) is related to the molar absorptivity coefficient (ε), the path length of the cuvette (b), and the concentration of the analyte (c). This method is straightforward, quick, and economical, although it may be influenced by interference from other substances present in the supplement (Mendham, 2000).

Spectrophotometric methods are used for the analysis of high concentration pharmaceutical products. Özgür and Koyuncu (2002) employed second derivative spectrophotometry to analyze vitamin B6, thiamin, and vitamin B12 in pharmaceuticals. The calibration graphs were linear for thiamin at 228.9 nm, vitamin B6 at 309.6 nm, and vitamin B12 at 361.7 nm, up to a concentration of 20 μg mL^{-1}. An analysis of tablets with 1 mg of vitamin B12 yielded results that were close to the labeled amount, with a %RSD of 3.58 (Özgür & Koyuncu, 2002).

Nepote et al. developed a simultaneous analysis method for vitamin B6, dexamethasone, and vitamin B12 using UV-vis spectral measurements in conjunction with partial least squares (PLS-1) multivariate calibration. When applied to injectable products containing vitamin B12, the results were comparable to those obtained through capillary electrophoresis (Nepote *et al.* 2006).

Several methods utilizing chemiluminescence for the assay of vitamin B12 have been published. Most of these methods are suitable for flow injection analysis (FIA), which allows for rapid throughput procedures. For instance, Song and Hou (2003) presented a method that enhances the chemiluminescence reaction between luminol and dissolved oxygen in a flow injection system through the addition of cobalt (II). This method demonstrated high sensitivity, with a detection limit of 5×10^{-11} g L^{-1}. It is applicable to pharmaceuticals, serum, and foods. The evaluation by the authors highlighted the speed and simplicity of the procedure, positioning it as a useful routine analytical method (Song & Hou, 2003).

The analysis of Vitamin B12 in dietary supplements is a significant component of pharmaceutical and food quality control. Research has shown that modern analytical techniques allow for more precise and selective measurement of Vitamin B12 in multivitamin products and dietary supplements.

The analysis of Vitamin B12 using liquid chromatography coupled with mass spectrometry (LC-MS/MS) is regarded as a highly advanced and reliable analytical technique in pharmaceutical and food analysis. This method integrates the separation capability of high-performance liquid chromatography with the sensitivity and selectivity of mass spectrometry, facilitating accurate identification and quantification of Vitamin B12, even at trace concentrations.

Liquid chromatography can be effectively used to analyze vitamin B12 in high-concentration supplements and pharmaceutical formulations. It can successfully separate natural cobalamins. However, due to the low concentrations of the analytes, early studies often employed isotope dilution and radio-ligand binding assays to quantify eluted cobalamins. Currently, the integration of mass spectrometry with liquid chromatography has been limited (Li *et al*.2000; Wongyai,2000).

The analytical procedure typically starts with sample preparation. Solid dosage forms like tablets and capsules are crushed and homogenized, while liquid formulations are thoroughly mixed. The vitamin is extracted using solvents such as water, methanol, or acetonitrile, sometimes combined with buffer solutions to enhance extraction efficiency and stability. The resulting solution is then filtered and diluted before being injected into the chromatographic system.

Chromatographic separation is carried out using reverse-phase columns, commonly C18 columns, where compounds are separated based on polarity and interaction with the stationary phase. The mobile phase usually consists of water and organic solvents like methanol or acetonitrile, often containing formic acid to improve ionization efficiency during mass spectrometric detection.

Following chromatographic separation, the analyte enters the mass spectrometer, where ionization occurs, typically through electrospray ionization (ESI) in positive ion mode. During this process, Vitamin B12 molecules become protonated and form charged ions that are detected based on their mass-to-charge ratio (m/z) (Wongyai,2000; Lindemans *et al*. 2000).

Nonetheless, Lindemans (2000) indicates that soft ionization techniques suitable for nonvolatile and thermolabile compounds will be relevant for LC-MS methods for vitamin B12. Improved LC-MS instrumentation and LC interfaced with inductively coupled plasma emission mass spectrometry (LC-ICP-MS) have been identified as yielding sensitive and specific methods for the analysis of cobalamins.

A notable study conducted in North Macedonia was published by Stefova *et al.* which focused on developing a high-performance liquid chromatography (HPLC) method for determining Vitamin B12 in multivitamin tablets. The authors found that the HPLC method offered good sensitivity and precision for the routine analysis of supplements. This study was significant as it illustrated the applicability of modern chromatographic techniques in pharmaceutical laboratories in Macedonia and contributed to the enhancement of quality control for multivitamin preparations (Stefova *et al.1997).* HPLC provides greater selectivity than conventional spectrophotometric methods and minimizes interference from other components in the supplement matrix. The advancement of analytical techniques, particularly Mass Spectrometry coupled with Liquid Chromatography (LC-MS/MS), has significantly enhanced Vitamin B12 analysis. Research from various international studies indicates that LC-MS/MS allows for highly precise identification and quantification of various cobalamin forms, such as cyanocobalamin, methylcobalamin, and hydroxocobalamin, even at low concentrations.

The research conducted by Chamlagain *et al.* showed that LC-MS/MS offers high sensitivity and specificity for analyzing Vitamin B12 in food and dietary supplements. The authors noted that this method is particularly useful for detecting degradation products and for examining complex samples that may contain interfering compounds. These results suggest that LC-MS/MS is a current standard for vitamin analysis in both the pharmaceutical and food industries (Chamlagain *et al.* 2015).

Yanes and Miller-Ihli demonstrated that CNCbl, OHCbl, AdoCbl, and MeCbl were effectively resolved by LC-ICP-MS, resulting in clear chromatographs due to the element specificity of ICP-MS. The method proved to be robust and suitable for the analysis of foods and supplements, with minimized matrix interference (Yanes & Miller-Ihli, 2004).

There are limited methods that utilize LC-MS technology.

Chassaigne and Łobiński compared various detection modes, including spectrophotometric, ICP-MS, and electrospray ionization mass spectrometry (MS-ESI) paired with microbore reversed-phase LC on C8. When using standards such as CNCbl, AdoCbl, OHCbl, MeCbl, and cobinamide dicyanide (CN)2Cbn, ICP-MS demonstrated greater sensitivity compared to other online detection systems; however, total ion current chromatograms facilitated clear species identification (Chassaigne & Łobiński, 1998).

The authors Jiang *et al*. describe a straightforward LC-MS quantification method for Vitamin B12. Using the Waters Atlantis C18 Column, Vitamin B12 exhibited good retention and peak shape. A simple binary gradient was employed without the addition of a buffer.

The quantification linear range spanned from 5 ppb to 4000 ppb, with a limit of detection of 2 ppb for a 10 μL injection (Jiang *et al*. 2003)

Conclusion

Vitamin B12 is important for cellular metabolism and neurological health. A deficiency in vitamin B12 is a significant public health concern worldwide, especially among older adults, vegetarians, and individuals with malabsorption disorders. Proper evaluation of vitamin B12 levels is essential for early diagnosis and the prevention of complications. As a result, vitamin B12 supplements are commonly used as prophylactic therapy. The increasing use of dietary supplements has made vitamin B12 analysis essential in pharmaceutical and food quality control.

LC-MS/MS is an advanced analytical technique used for determining Vitamin B12 in dietary supplements and pharmaceutical preparations. This method provides enhanced sensitivity, selectivity, and precision when compared to traditional analytical techniques. Its capability to accurately identify and quantify various forms of Vitamin B12 is important for pharmaceutical quality control, food analysis, and research applications.

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