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Determination of cyanocobalamin by isotope dilution LC-MS/MS

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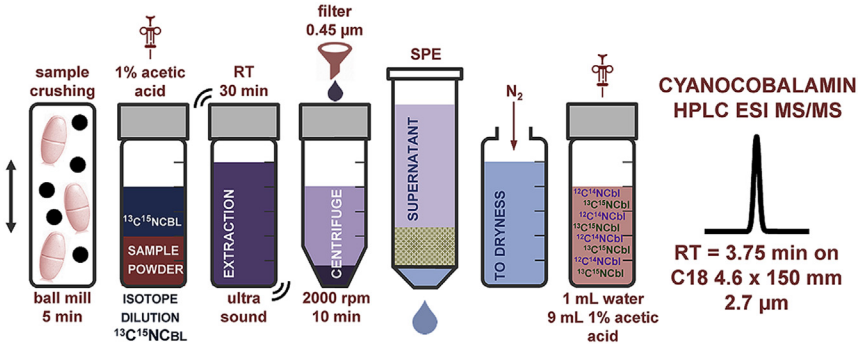
HIGHLIGHTS

- Precise quantitation of cyanocobalamin in supplements by liquid chromatography-tandem mass spectrometry.
- Quantitation at ultra-trace level.
- Use of a specific isotopically enriched internal standard produced in-house.
- Stable quadrupole isotope dilution is used.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyanocobalamin (CNCbl) is an active form of vitamin B12, commonly employed for the preparation of multivitamin supplements and fortified food. In this study, we present a novel analytical method for its determination based on stable isotope dilution liquid chromatography electrospray tandem mass spectrometry (ID LC-MS/MS). Isotopically enriched $^{13}C^{15}N$Cbl was synthesized in-house and used as internal standard. The method was validated using NIST SRM 3280 multivitamin reference material and by comparison with an independent methodology based on LC-ICPMS. The proposed method provided a detection limit of 57 pg/g and could be applied for the determination of trace level of CNCbl in multivitamin supplements with a relative standard uncertainty better than 3%. The novel ID LC-MS/MS is a primary ratio method that could become a reference for CNCbl determination in multivitamins and food supplements. The method was applied for the characterization of two NRC multivitamin tablet Certified Reference Material (CRM) candidates, VITA-1 and VITB-1 whose CNCbl levels were quantified as 2.64 ± 0.09 and 1.75 ± 0.12 µg/g, respectively.

1. Introduction

Vitamin B12 is a generic name for a family of water-soluble vitamins, commonly known as cobalamins. Cobalamins share a common structure, the so-called cobalt-containing corrinoids, and differ just for the ligand (X) on the cobalt in position (Fig. 1). Depending on the nature of the substituent, we differentiate hydroxocobalamin (OHCbl, X = OH^-), aquocobalamin (X = H_2O), methylcobalamin (X = $-CH_3$), deoxyadenosylcobalamin (X = 5′-deoxyadenosyl) [1]. Such compounds play a vital role in formation of red blood cells and in the regular physiology of nervous system

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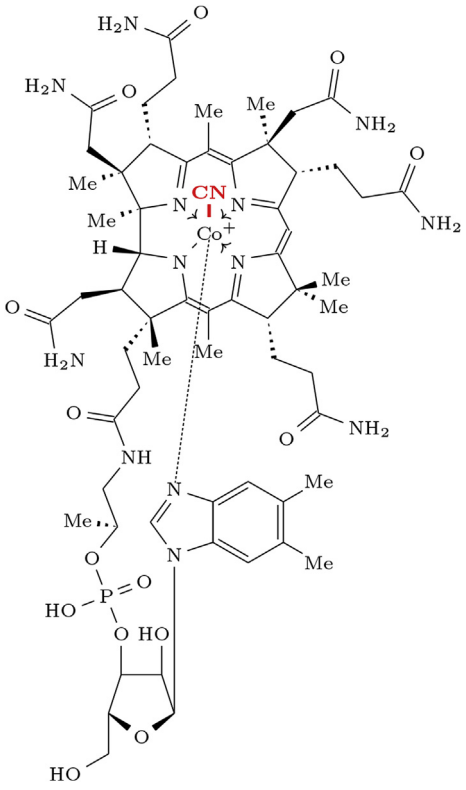

Fig. 1. Molecular structure of cyanocobalamin.

[1,2]. Vitamin B12 is mainly found in animal products such as dairy, meat and eggs [3–7], although, some recent evidences supported the presence of B12 in few vegetable species [8]. The recommended dietary intake of B12 slightly varies depending on age, gender, and physiological status (e.g. pregnancy, lactation), but an average of 2.4–2.8 μg/day is recommended for adults and of 0.9–1.2 μg/day for children [9]. B12 deficiencies can cause nervous and hematological dysfunctionalities, such as megaloblastic anemia [2,3]. These conditions can be treated with administration of vitamin B12, but neurological damages cannot be reversed. Often, B12 deficiencies can be prevented with supplements or fortified foods. Due to its stability, cyanocobalamin (CNCbl, −CN as ligand) is the molecular form commonly employed for fortification purposes [1]. CNCbl is not a natural-occurring vitamin, but it can easily be prepared from hydroxo- or deoxyadenosyl- cobalamin with an excess of cyanide [10–12]. After absorption, CNCbl is converted to methylcobalamin or adenosylcobalamin [13], which are the active coenzymatic forms. Determination of trace levels of CNCbl is required for quality control of supplements, fortified foods and pharmaceutical preparations. The official methods for its determination are microbiological assay and LC-UV [14,15]. Other methods include radioimmunoassay (RIA) [16], LC-ICPMS [17,18], AAS [16], LC-MS/MS [19,20], capillary electrophoresis with UV detection [21] and biosensors [22,23]. With the exception of LC-MS/MS, these methods lack specificity and sensitivity and, in case of RIA and biosensor, they may be too expensive for routine analysis [16,22]. Furthermore, LC-ICPMS is an indirect method with the inconvenience of lower ionization efficiency of organic Co in comparison to the inorganic one [17]. LC-MS/MS is the most suitable methodology for CNCbl determination as it combines high sensitivity and specificity for this analyte. For best analytical performance, however, the use of a specific internal standard (IS) is crucial for quantitative LC-MS/MS. Although ginsenosides and torsemide were proposed as IS [19,20], the use of an isotopically enriched form of CNCbl would provide better corrections for analyte losses in sample preparation and for fluctuation of ESI-MS response due to matrix suppression. Such an enriched CNCbl standard ($^{13}C7$) is available on the market, but its prohibitively high cost limits its use for routine quality control. For this reason, we are proposing the use of $^{13}C^{15}N$Cbl as IS for high-precision isotope dilution quantitation. Such IS was prepared in house starting from inexpensive precursors (OHCbl and $K^{13}C^{15}N$). The use of $^{13}C^{15}N$Cbl was suggested for quantitation of CNCbl in feces using single isotope dilution (ID^{1}MS) [24]. ID^{1}MS, however, links traceability of the analytical data to the chemical purity of the IS which may be challenging to establish in common analytical laboratories. To overcome this issue, in our study we implemented high-order quadruple isotope dilution quantitation, which permitted traceability of the results to a primary standard of natural isotopic composition [25]. The method was fully validated using NIST 3280 multivitamin tablet SRM and with comparison with an orthogonal methodology based on LC-ICPMS. The method was applied for the characterization of NRC VITA-1 and VITB-1 Certified Reference Material (CRM) candidates for vitamins in a pharmaceutical preparation.

2. Experimental

2.1. Chemicals

Cyanocobalamin (CNCbl, USP testing specifications) and EDTA were obtained from Sigma-Aldrich. CNCbl purity was verified by quantitative ^{1}H NMR. $^{13}C^{15}N$Cbl was prepared in-house from OHCbl (≥ 96%, Sigma-Aldrich) and $K^{13}C^{15}N$ (Sigma-Aldrich, 99% atom ^{13}C, 98% atom ^{15}N). Methanol (HPLC grade), acetone (99.5%), acetonitrile (Optima® grade) and formic acid (85%) were purchased from Fisher Scientific. Glacial acetic acid was obtained from Acros (NJ, USA). Ultrapure water (18.2 MΩ•cm) was produced in-house with a Thermo Scientific GenPure filtration system. Deuterium oxide (D_2O; D, 99.96%) was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Potassium hydrogen phthalate (KHP) SRM 84L (99.9934% ± 0.0076%) and multivitamin tablets SRM 3280 were purchased from NIST (Gaithersburg, MD, USA). VITA-1 and VITB-1 multivitamin NRC CRM candidates were produced by a nutraceutical manufacturer in the pharmaceutical form of film-coated compressed tablets (2.575 g/each). The CRM candidates contain minerals, ginsenosides, and water-soluble and fat-soluble vitamins commonly present in commercial multivitamins. The concentrations of minerals and vitamins differ in VITA-1 and VITB-1, although proportions are kept constant. For example, label values of CNCbl are 6 μg/tablet and 3 μg/tablet in VITA-1 and VITB-1, respectively. Commercial multivitamins in tablets were purchased from a local drugstore.

2.2. Synthesis of $^{13}C^{15}N$Cbl

Synthesis of $^{13}C^{15}N$Cbl was based on the known reactivity of free cyanide with hydroxyl group of OHCbl, which is converted to CNCbl [10–12]. 1.04 g of OHCbl were dissolved in 10 mL of deionized water (DIW), and 1 mL of aqueous $K^{13}C^{15}N$ (0.0493 g/mL $K^{13}C^{15}N$ in water) was slowly injected in the solution under stirring. Then 0.08 g of acetic acid were slowly dropped into the solution until a pH of 4.5 was reached. The mixture was heated to 60 °C in a water bath and 60 mL of acetone were added, under constant stirring. Then the solution was cooled down to room temperature and transferred in a fridge at 5 °C for 3 h. $^{13}C^{15}N$Cbl crystals were collected by filtration and washed first with 20 mL of water:acetone (1:9) mixture hold at 0 °C, and, finally with 20 mL of acetone. The product was recrystallized in water:acetone (1:5) solution, dried under vacuum