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Abstract

Cyanocobalamin (CNCbl) is an active form of vitamin B12, commonly used in multivitamin supplements and fortified food. Although several analytical methods are available for the determination of CNCbl, most of them lack a proper standardization and/or are too tedius or pricey for routine analysis. In this paper, we present a new analytical method for the determination of CNCbl in multivitamin tablets based on stable isotope dilution liquid chromatography-electrospray-tandem mass spectrometry (ID LC-ESI-MS/MS). A labeled CNCbl ($^{13}C^{15}N$Cbl) was synthesized in-house and used as internal standard. Quadrupole isotope dilution (ID^4) was selected as calibration strategy for the quantitation of CNCbl. The method was validated with NIST standard reference material 3280 and by an comparison with LC-ICPMS. Our results showed that CNCbl values were in agreement with both NIST certification and the LC-ICPMS data. The new method provided a limit of detection of 57 ng/g, with good linearity

in the range of calibration, and was further applied for determining CNCbl in VITA-1 candidate certified reference materials from National Research Council Canada and commercial multivitamin supplements. Thanks to its accuracy and precision (3%), ID LC-ESI-MS/MS could be proposed as a primary analytical method for the analysis of CNCbl in supplements.

Introduction

Vitamin B12 is a generic name for a large 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 on the β-position of the cobalt (Figure 1). Depending on the substituent on the cobalt, we differentiate hydroxocobalamin (OH^- as ligand), aquocobalamin (H_2O as ligand), methylcobalamin (-CH_3 as ligand), deoxyadenosylcobalamin (5'-deoxyadenosyl as ligand).[1] Cobalamins play a vital role in the formation of red blood cells and in the regular functioning of the nervous system.[1,2] Vitamin B12 is mainly found in animal products such as dairy, meat and eggs,[3,4] although, some recent evidences reported the presence of B12 in a few plants.[5] The recommended dietary intake of B12 slightly varies depending on age, sex and physiological status (e.g. pregnancy, lactation), but it is usually 2.4-2.8 ug/day for adults and 0.9-1.2/day for children.[6] Deficiencies of B12 might arise from insufficient dietary intake or malabsorption, and cause nervous and hematological dysfunctionalities, such as megaloblastic anemia.[2,3] These conditions can be treated by administrating vitamin B12, although, the resulting neurological damages cannot be reversed. Often, deficiency can simply be prevented by assuming supplements or fortified foods containing B12. Cyanoconalamin (CNCbl, -CN as ligand) is commonly used for fortification purpose thanks to its higher stability compared to other cobalamins.[1] CNCbl is not a natural-occurring vitamin, but can be easily synthesized from any cobalamin in an excess of cyanide.[7–9] After absorption, CNCbl is then converted to methylcobalamin or adenosylcobalamin,[10] which are the coenzymatic forms of the vitamin in the human body. Determination of cobalamins is thus crucial for quality control of supplements, fortified foods and pharmaceutical preparations. For simplicity and stability purposes, cobalamins are often converted to CNCbl by treatment with NaCN or KCN prior to instrumental analysis.[11,12] The total cobalamins are then determined as CNCbl and reported in the final assay as "vitamin B12" value. Currently, there is no "gold-standard" method available for the analysis of CNCob. The official methods for determination of CNCbl are microbiological test and LC-UV.[12,13]

Other methods include radioimmunoassay (RIA),[14] LC-ICPMS,[15] AAS,[14] LC-MS/MS,[16,17] capillary electrophoresis-UV[18] and biosensors.[19] With the exception of LC-MS/MS, these methods lack specificity and/or sensitivity or, in the case of RIA and biosensor approaches, they may be too expensive for routine analysis.[14,19] Even LC-ICPMS, which is however an indirect method, provides limited sensitivity due to the lower ionization efficiency of the Co in the cobalamins, compared to inorganic Co.[15] LC-MS/MS has been so far the most promising method since it combines both sensitivity and specificity. The only limitation lies in the lack of an appropriate internal standard (IS), which limits the calibration modes to external and standard addition. However, given the instability of the ESI-MS signal, the use of an appropriate IS would be crucial to obtain more accurate and precise results. Although ginsenosides and torsemide were proposed as IS with decent results,[16,17] their non-specific nature poses still doubts on the possibility to correct the signal variation and any kind of analyte loss that might occur during sample preparation. The ideal IS candidate would be a stable isotope labelled compound, behaving exactly as the natural analyte. A labeled IS would allow isotope dilution (ID) calibration which is a primary ratio method, providing high accuracy. Such a standard (^{13}C7) is available on the market, but the prohibitively high price (over 2000 USD per μg) strongly limits its use in routine analysis. In this work, we present a new method for the determination of CNCbl with LC-ID-MS/MS in supplements. Quadrupole ID (ID^4) was accomplished thanks to the labelled standard ^{13}C^{15}NCbl, which was synthesized in-house from hydroxocobalamin (OHCbl) and isotope labelled cyanide (K^{13}C^{15}N). Although ^{13}C^{15}NCbl was already used for fundamental studies and determination of cobalamins in feces, appropriate analytical method validation and standardization have never been carried out.[20,21] The method was tested on various commercial multivitamin formulations, including VITA-1 certified reference material (CRM) candidate and on NIST standard reference material (SRM) 3280, and was able to accurately quantify CNCbl in those supplements. As a further validation, results from LC-ID^4-MS/MS were also quantitatively compared with those obtained with LC-ICPMS for VITA-1 CRM candidate.

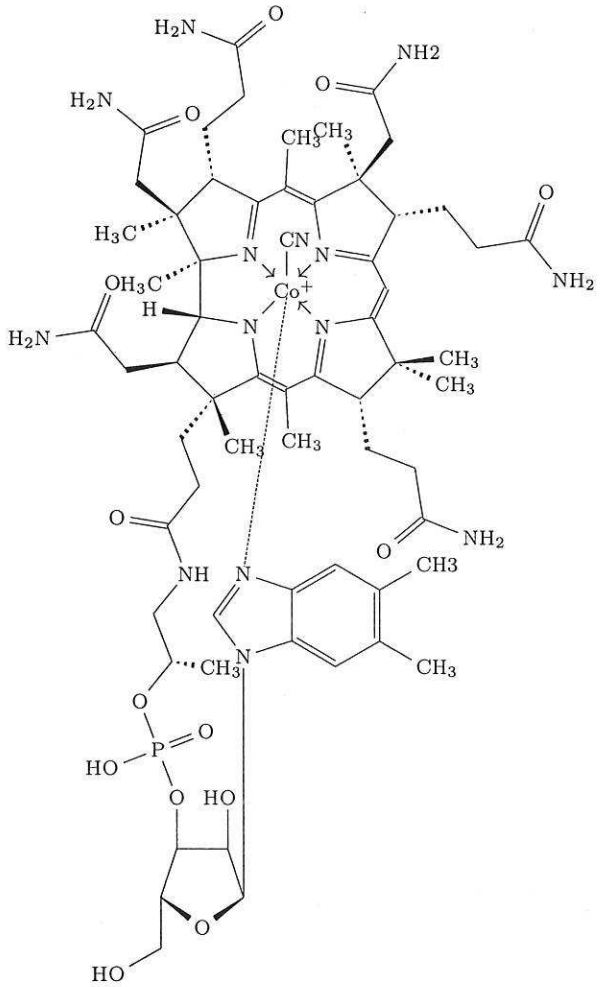

Figure 1: Molecular structure of CNCbl.

Experimental

Chemicals

Cyanocobalamin (CNCbl, meets USP testing specifications) and EDTA were from Sigma-Aldrich. $^{13}C^{15}N$Cbl was semi-sithesized in house from hydroxocobalamin (OHCbl) ($\geq$ 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 from Fisher Chemicals. Glacial acetic acid was from Acros (NJ, USA). Ultrapure water was

produced in-house with a Genpure filtration system (Thermo Scientific, Canada). Deuterium oxide (D_2O; D, 99.96%) was from Cambridge Isotopes Laboratories (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 multivitamin tablets CRM candidate was produced by Suntrition Inc. (Tecumseh, ON, Canada) according to NRC specifications. Commercial multivitamin tablets A and B were purchased from a local drugstore.

Synthesis and characterization of $^{13}C^{15}N$Cbl

Synthesis of $^{13}C^{15}N$Cbl was based on the well-known reactivity of free cyanide with the hydroxy group of OHCbl, which is converted to CNCbl.[7–9] 1.04 g of OHCbl was dissolved in 10 mL deionized water (DIW), while 1 mL of $K^{13}C^{15}N$ (0.0493 g/mL in DIW) was slowly injected in the solution under stirring. 0.08 g of acetic acid was slowly dropped into the solution until pH 4.5 was reached. The solution was heated to 60°C in a hot water bath and 60 mL acetone was added, under constant stirring. Then the solution was let to cool down until room temperature was reached and, finally, transferred in a fridge where it was left at 5°C for 3 hours. The formed crystals were collected by filtration and washed with 20 mL cold (0°C) water : acetone (1 : 9) first, and, finally with 20 mL pure acetone. The solid was recrystallised in water : acetone (1 : 5) and dried under vacuum. Characterisation of $^{13}C^{15N}$Cbl was performed with an ESI-high resolution mass spectrometer (Orbitrap Fusion Lumos, Thermo Scientific). $^{13}C^{15}N$Cbl was dissolved in acetonitrile:water (1 : 1) containing 0.1% formic acid to obtain a final concentration of 1 μg/g. The solution was then infused in the mass spectrometer with a 0.25 glass syringe (flow rate: 5 μL/min). Resolution was set at 30000 and scans were collected in the m/z range 600-1450. Fragmentation was induced with collision-induced dissociation (CID) with normalised collision energy set at 35%.

Sample preparation

As many of the vitamins, CNCbl is light-sensitive and degrades to OHCbl by losing the -CN functionality from the Co.[22,23] Working with subdued lighting and amber vials is therefore necessary to prevent degradation of standards and samples.

Preparation of solutions

Stock solutions of CNCbl and $^{13}C^{15}N$Cbl were prepared in ultrapure water. Solutions, which were stored at +4°C and protected from light, were stable for a month. 1% (v/v) acetic acid was prepared by diluting glacial acetic acid in ultrapure water.

Preparation of calibration blends and multivitamin tablets for LC-ID^4-MS/MS analysis

Qualdrupole ID (ID^4) requires the preparation of three calibration blends,[24,25] which hereafter will be identified as blend1, blend2 and blend3. For the successful accomplishment of ID^4, a few conditions must be met:

1. Each blend differs in the ratio of $^{13}C^{15}N$Cbl/CNCbl, which is 2, 1 and 0.5 for blend1, blend2 and blend3, respectively. The amount of labelled standard is kept constant, while the one of the natural spike varies.

2. The concentration of CNCbl in the blends should bracket the one in real samples.

3. The amount of $^{13}C^{15}N$Cbl spiked in the real sample must match that of CNCbl (exact matching). Therefore, CNCbl level should be known or estimated beforehand.

4. Equilibration between labelled and natural standard must be achieved prior to analysis.

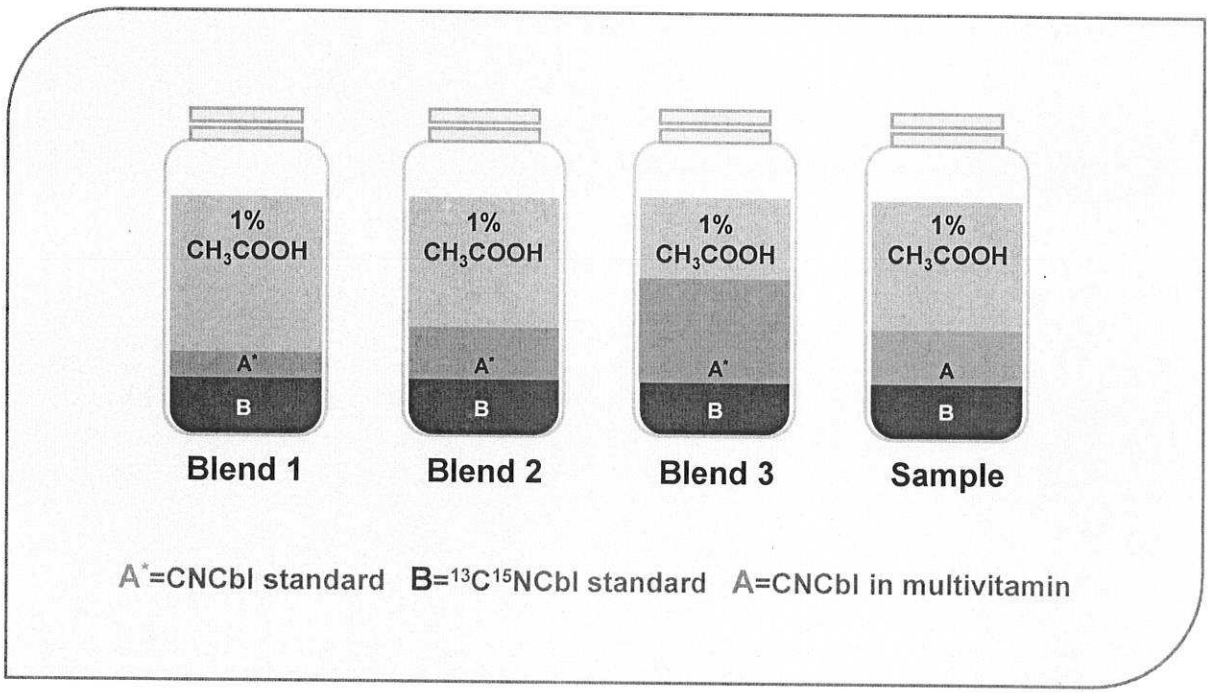

Figure 2: Preparation of calibration blends and sample for ID[4].

In our case, the blends were prepared by adding an appropriate amount of $^{13}C^{15}N$Cbl and CNCbl working stock solution in a 60 mL amber vial, equipped with screw caps, and by bringing to 60 mL volume with 1% acetic acid. For each sample, 15 tablets were finely milled for about 10 min, using automatic agate mortar and balls (Chemplex Industries, Inc., Palm City, FLA, USA). This process generates a fine powder with a good degree of homogeneity, which cannot otherwise be obtained by simple manual grinding. The powder was stored in amber vials tightly closed with screw caps and found to be stable for 2 weeks at -20°C. Depending on the level of CNCbl in the supplement, 0.6-2.5 g of powder was sampled into 60 mL amber vial. A proper amount of $^{13}C^{15}N$Cbl working stock solution and 1%

acetic acid were then added to the sample, so that the $^{13}C^{15}N$Cbl + 1% acetic acid volume equals 60 mL. The samples, including the blends, were sonicated at room temperature for 30 minutes and, then, centrifuged at 2000 RPM for 10 minutes (rotor 804). The supernatant was filtered through 0.45μ nylon filter and concentrated by solid phase extraction (SPE). Prior to adding the multivitamin extracts and the blends, the SPE tubes (Strata^{TM}-X 33 μm Polymeric Reversed Phase, 500 mg/12 mL Phenomenex®) were conditioned with 10 mL methanol, followed by 10 mL ultrapure water. 60 mL multivitamin extract was then loaded in multiple steps to be absorbed on the SPE solid phase. The sample was then washed with 10 mL 5% MeOH in ultrapure water to remove the minerals. CNCbl was finally elute with 10 mL MeOH. The organic solvent was evaporated under nitrogen and the solid residue reconstituted with 1 mL ultrapure water. The samples were diluted 1:10 in 1% acetic acid and transferred into 1 mL amber vials prior to LC-MS/MS analysis.

Preparation of multivitamin tablets for LC-ICPMS analysis with standard addition

Sample preparation of tablets for LC-ICPMS was similar to the one reported above, with the exception of the SPE step, which was not required in this case. 15 tablets of VITA-1 were finely ground with an automatic mill, equipped with an agate mortar and balls, for about 10 min. About 1 g of powder was sampled into 60 mL amber vial, equipped with screw caps, and 60 mL of 1% acetic acid was then added. The samples were sonicated at room temperature for 30 minutes and, then, centrifuged at 2000 RPM for 10 minutes (rotor 804). The supernatant was finally filtered through 0.45μ nylon filter. For calibration purposes, about 9 mL extract was sampled into three 20 mL amber vials and about 0.5 and 1 mL CNCbl stock solution (2.0242 μg/g in water) spiked in two of the three vials. The three samples were then brought to 10 mL volume with water.

LC-ID^4-MS/MS analysis

Analyses were peformed with a HPLC (Infinity 1290, Agilent Technologies) coupled with an electrospray (ESI)-triple quadrupole mass spectrometer (Quantiva TSQ, Thermo Scientific). 5 uL sample was injected in a reversed phase column (Ascentis® Express C18, 4.6x150 mm, 2.7μ, Supelco). CNCbl and $^{13}C^{15}N$Cbl were eluted isocratically with 15% acetonitrile+0.1% formic acid and 85% water+0.1% formic acid (flow rate: 0.5 mL/min). The column was held at 25°C. Detection was carried out in SRM mode, with m/z transitions 678→912 and 679→359 observed for CNCbl and $^{13}C^{15}N$Cbl. Conditions for ESI source and mass spectrometer are reported in Table 1.

Table 1: ESI-MS/MS conditions

ESI Source		**MS/MS**	
Spray voltage	+4 kV	CID	1 mTorr
Sheath gas	40 AU	Fragmentation source	0 V
Aux gas	11 AU	Dwell time	100 ms
Sweep gas	3 AU	RF lenses CNCbl	113 V
Ion transfer temperature	325°C	RF lenses $^{13}C^{15}N$Cbl	109 V
Vaporizing temperature	300°C		

LC-ICPMS analysis

Analyses were carried as reported by Raju *et al.*[15] A HPLC (1200 series, Agilent Technologies) coupled with an ICP-triple quadrupole mass spectrometer (model 8800, Agilent Technologies) was used through all experiments. 5 uL sample was injected in a reversed phase column (Luna C5, 2.0x100 mm, 5μ, Phenomenex), and CNCbl was eluted isocratically and separated from inorganic Co with 70% 20 mM EDTA in 25% methanol and 30% ultrapure water (flow rate: 0.2 mL/min). The column was held at 25 °C. CNCbl was detected as ^{59}Co. Operating conditions for the mass spectrometer are reported in Table 2.

Table 2: Plasma conditions for LC-ICPMS analysis

Plasma gas flow	15 L/min
RF power	1550 W
RF matching	1.80 V
Sample depth	8.0 mm
Carrier gas	1.01 L/min
Nebulizer pump	0.10 rps
S/C Temp	2 °C

Determination of CNCbl purity with quantitative NMR (qNMR)

For qNMR, 5 mg of CNCbl were dissolved in 1 mL deuterium oxide, along with 2.5 mg of KHP (SRM 84L), which was selected as internal standard. The solution was transferred in a NMR tube (Wilmad LabGlass, USA) and submitted to NMR analysis. NMR analyses were performed using a Bruker AV-III 400 NMR spectrometer equipped with a 5 mm BBFO probe. All experiments were performed at 25°C. Prior to qNMR measurements, the longitudinal relaxation times (T1) were measured for a sample containing CNCbl and the internal standard in deuterium oxide. From this experiment, the longest relevant T1 was calculated to be 3.8 s. 1H-qNMR experiments were performed using a 90° pulse, a sweep width of 30 ppm (12000 Hz), 32 scans preceded by 2 dummy scans, and a relaxation delay of 40 s. Spectra were processed using the Bruker Topspin software (version 3.2).

Results and discussion

Synthesis and characterization of $^{13}C^{15}N$Cbl

Synthesis of $^{13}C^{15}N$Cbl was based on the ligand exchange on the Co atom.[26] OHCbl is a good starting material for the synthesis of CNCbl, although other cobalamins (e.g. methylcobalamin, adenosylcobalamin) could be used as well. Because of the high affinity, $^{13}C^{15}N^-$ straightforwardly replaces the OH^- on the Co to obtain the more stable $^{13}C^{15}N$Cbl. An equimolar amount of OHCbl:$K^{13}C^{15}N$ was used to avoid the formation of dicyanocobal-

amin.[27] Synthetic $^{13}C^{15}N$Cbl was then characterized with an Orbitrap mass spectrometer. As shown on Figure 3, characteristic masses of $^{13}C^{15}N$Cbl were observed at 1357.5795 (M^+), 1379.5607 ($[M+Na]^+$), 679.2916 (M^{2+}), 690.2829 ($[M+Na]^{2+}$), 701.2733 ($[M+2Na]^{2+}$), where M is the molecular ion. No trace of OHCbl (molecular weight 1346.37 g/mol) was found in the scans, indicating completeness of the reaction.

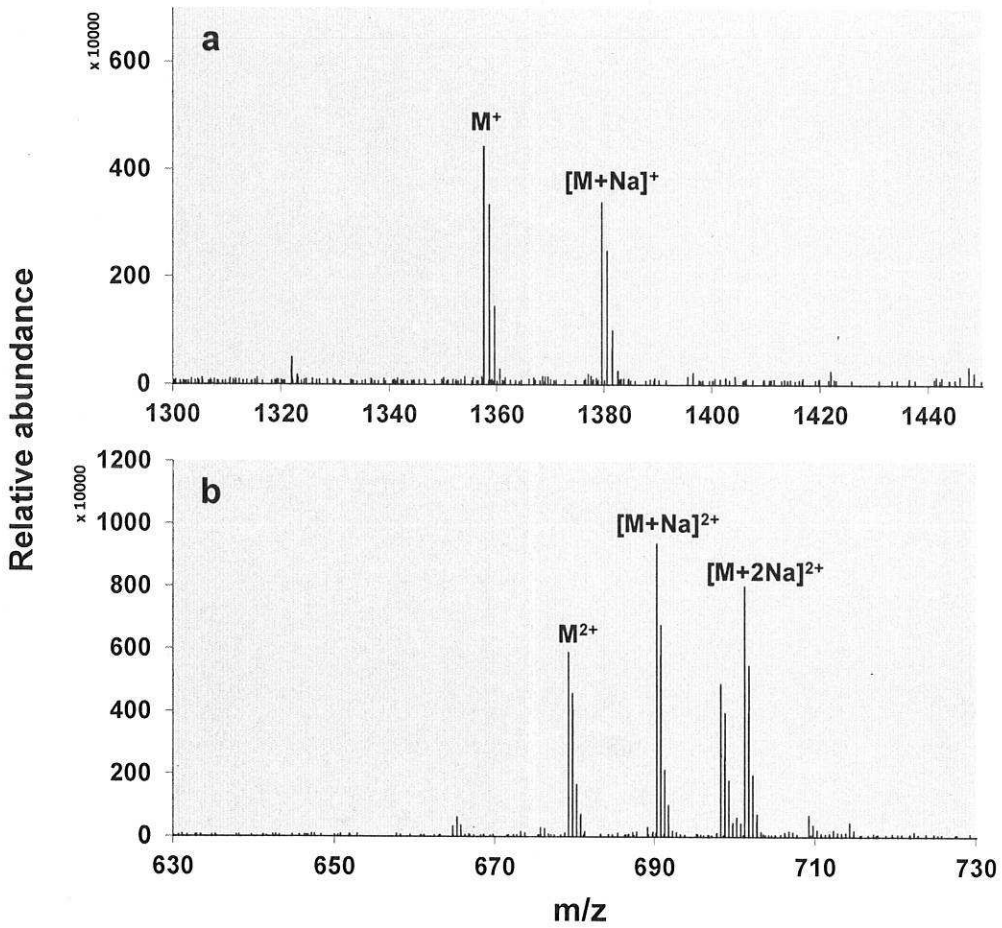

Figure 3: Orbitrap scan of $^{13}C^{15}N$Cbl. Part (a) shows M^+ and relatived adducts, while part (b) M^{2+} and associated adducts.

ID^4 for quantitation of CNCbl

An detailed explanation of ID^4, as well of the mathematical aspects involved, has been given elsewhere *et al.*[24,25] In the current work, the selection of ID^4 over single ID was dictated by the

complexity of CNCbl molecule. In single ID model, information about isotopic composition of both natural and labelled standards, and purity of labelled standard are required. On the other hand, ID^4 model does not require knowledge on the isotopic composition of the labelled standard, making it particularly useful for the analysis of large molecules for which this information might be difficult to asses.

Purity determination with qNMR

Quantification with ID^4 requires the knowledge of the concentration of natural spike (CNCbl in our case), while the one of labelled spike ($^{13}C^{15}N$Cbl) is not needed. The concentration must then be corrected by the purity of the natural standard. When the available sample quantity is low, qNMR is often selected for purity assessment. qNMR requires the presence of internal standard (IS) and at least one resolved peak for both the analyte and IS. In our case, KHP was chosen as IS. Commonly, degradation of CNCbl involves the loss of CN^- on the Co, which is usually replaced by OH^-.[22,23] Although a Co-NMR would be feasible, the low sensitivity, in terms of S/N, reported in former papers discouraged us to go in that way.[28,29] Given the complexity of CNCbl molecule, a 1H-NMR might appear tricky to interpret. However, Brash *et al*[30] accurately determined impurities in CNCbl performing a 1H-NMR and using the aromatic part of the molecule. As showed in Figure 4, proton peaks in the aromatic regions are well resolved from each others and from those of the IS.

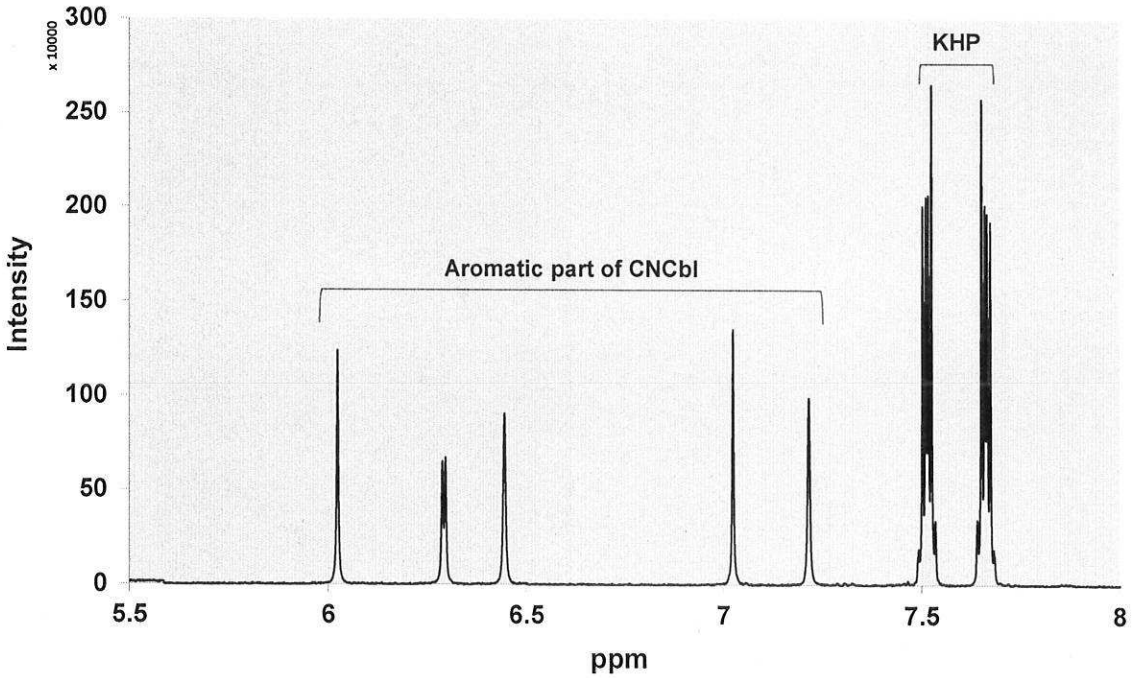

Figure 4: HNMR of the aromatic region of CNCbl. KHP is used as internal standard.

CNCbl signal with 7.2 ppm chemical shift was selected, along with the 4 protons of KHP, for quantitative scopes. Purity of CNCbl was calculated with the following equation:

$$W_{\mathrm{an}} = \frac{\mathrm{I_{an}}}{\mathrm{I_{cal}}} \frac{\rho_{\mathrm{cal}}}{\rho_{\mathrm{an}}} \frac{\mathrm{M_{an}}}{\mathrm{M_{cal}}} \frac{\mathrm{m_{cal}}}{\mathrm{m_{an}}} W_{\mathrm{cal}} \tag{1}$$

Where $\mathrm{I_{an}}$ and $\mathrm{I_{cal}}$ are the intensities (area times number of protons assigned) of the protons for the analyte and the calibrant, respectively; ρ_{an} and ρ_{cal} are the densities of analyte and calibrant solutions, respectively; $\mathrm{M_{an}}$ and $\mathrm{M_{cal}}$ are the molecular weights of analyte and calibrant, respectively; $\mathrm{m_{an}}$ and $\mathrm{m_{cal}}$ are the weights of analyte and calibrant, respectively; $\mathrm{W_{cal}}$ is the purity of the calibrant. CNCbl purity was assigned as 85.79% (n=3).

Comparison between LC-ICPMS and LC-ID^4-MS/MS for quantitation of CNCbl

LC-ICPMS was successfully applied for the determination of CNCbl in supplements and fortified breakfast cereals.[15] For intercomparison purpose, 20 tablets of VITA-1 CRM candidate were finely ground and independently analysed with LC-ICPMS and LC-ID^4-MS/MS, as reported in the Experimental section. Figures 5 and 6 show the chromatogram of CNCbl in LC-ICPMS and LC-ID^4-MS/MS, respectively. As can be observed, the chromatographic runs are much shorter in LC-ID^4-MS/MS, which allows complete elution of CNCbl within 5 minutes, against the 10 minutes required in LC-ICPMS. Shorter retention times could be achieved also in ICP-based analysis by a steeper solvent gradient. However, an increased carbon load could be of an issue for the ICPMS instrument and, therefore, it was avoided. As shown on Figure 5, inorganic Co elutes first and its ionization efficiency is much higher than CNCbl, as observed by Raju *et al.*[15]

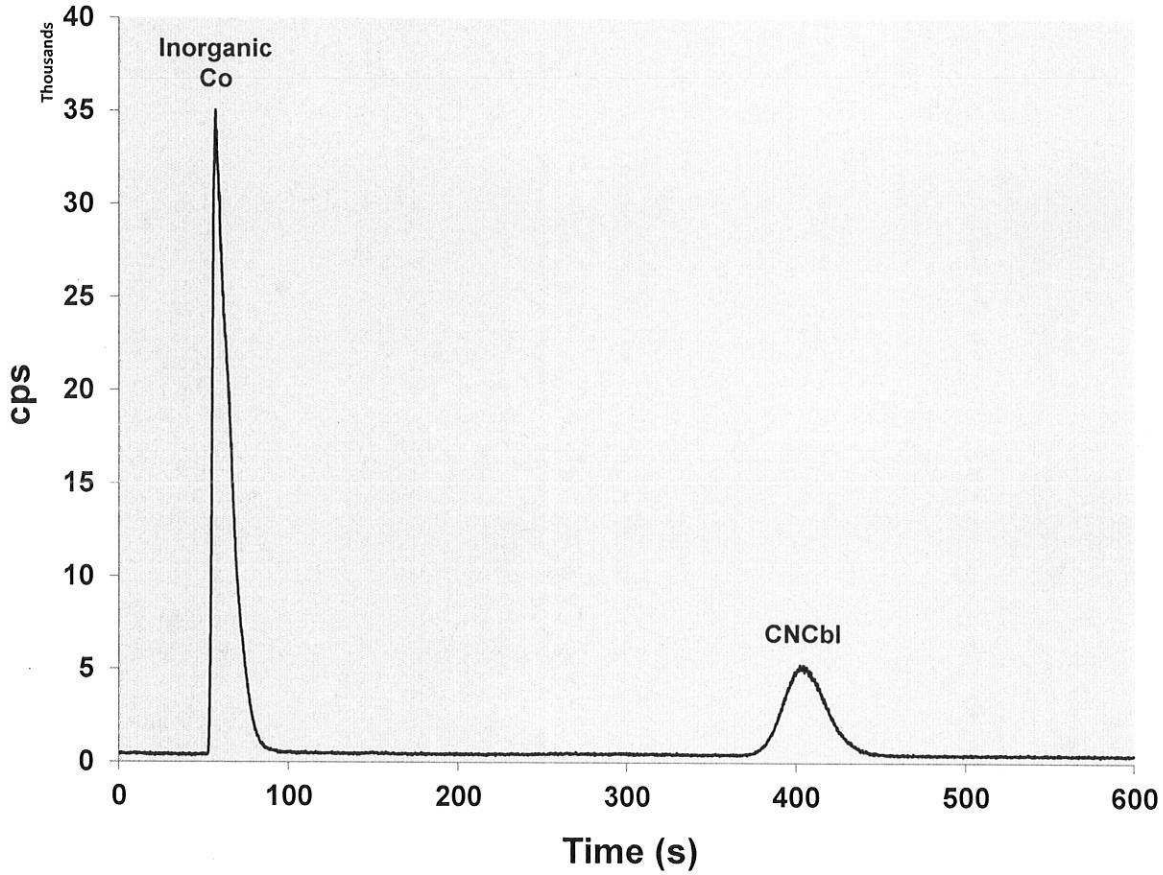

Figure 5: LC-ICPMS chromatogram of CNCbl in VITA-1 CRM candidate. Injection volume: 5 uL. Column: Luna C5, 2.0x100 mm, 5μm. Elution: isocratic in 70% 20 mM EDTA in 25% methanol and 30% ultrapure water. Flow rate: 0.2 mL/min. Temperature: 25 °C. CNCbl detected as ^{59}Co.

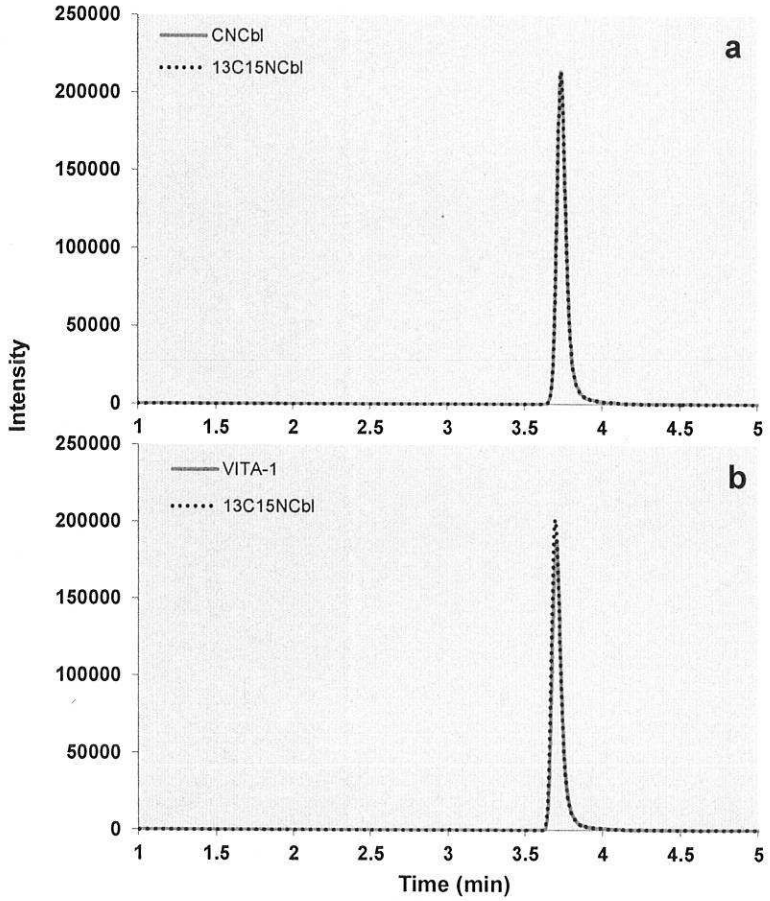

Figure 6: LC-ID^4-MS/MS chromatogram of CNCbl and $^{13}C^{15}N$Cbl in blend2 (a) and VITA-1 multivitamin tablets (b). Injection volume: 5 uL. Column: Ascentis Express C18, 4.7x150 mm, 2.7μm. Elution: isocratic in 85% ultrapure water+0.1% formic acid and 15% acetonitrile+0.1% formic acid. Flow rate: 0.5 mL/min. Temperature: 25°C. CNCbl and $^{13}C^{15}N$Cbl detected in SRM mode (transitions: 678→912 and 679→359, respectively)

Table 3 shows the results obtained for the quantitation of CNCbl in VITA-1 CRM candidate in four independent samples. Although ID^4 data are slightly higher than those obtained with ICPMS, values can be considered in good agreement with each others.

Table 3: Comparison between LC-ICPMS and LC-ID^4-MS/MS methods for the determination of CNCbl in VITA-1 CRM candidate.

	LC-ICPMS (μg/g)	LC-ID^4-MS/MS (μg/g)
1	2.52	2.68
2	2.33	2.74
3	2.45	2.52
4	2.41	2.61
Mean	2.43	2.64
RSD (%)	3.32	3.68

Determination of CNCbl in multiple supplements with LC-ID^4-MS/MS: figures of merit

LC-ID^4-MS/MS method was applied to the determination of CNCbl in commercial multivitamin tablets (hereafter defined as A and B) and NIST 3280 SRM. As showed in Table 4, CNCbl value was in agreement with that reported for NIST 3280 SRM (4.8$\pm$1 μg/g), which demonstrated the accuracy of the method. For A and B commercial tablets, declared label values for CNCbl are 14 and 2.6 μg/tablet, respectively. Considering that each tablet weighs about 1.2 g, our results might look as an overestimation of the declared value. However, we have to remember that pharmaceutical manufacturers use to add an amount of vitamins higher than what declared on the label. These "overages" are meant to compensate possible losses occurring during manufacturing and storage.[31] In solid multivitamin formulation, USP allows and overages of 30-65%, depending on the documented stability of the vitamin.[32] In light of that, assay value is usually higher than label one. According to NIST certificate, CNCbl value in SRM 3280 was obtained by combining results from microbiological and LC-ICPMS analysis. Unfortunately, microbiological assessment is notorious for its biases and poor accuracy, due to the many interferences.[19] In the first release of SRM 3280, CNCbl amount was just reported as reference value.[33] This number was obtained with microbiological test only and showed, indeed, an uncertainty of about 38%.[33] The following development of a LC-ICPMS method has allowed decreasing the uncertainty to 20%, as reported in the

NIST certificate of SRM 3280. By looking at data in Table 3 and NIST value obtained with LC-ID^4-MS/MS, it appears that LC-ICPMS might slightly underestimates the amount of CNCbl in the supplements. Another interesting aspect is that uncertainty of the measurement differs depending on the type of tablet tested. This could be related to the amount of CNCbl in the supplement, as a lower variation is expected in tablets with higher level of the vitamin. Indeed, measurements performed on multivitamin A show much lower uncertainty than those on B. However, this could be also due to the non-uniform dispersion of vitamins during the manufacturing. CNCbl is indeed present in the tablets in much lower amount than other vitamins, as e.g. pantothenic acid that can reach 5000 μg/tablets. This makes the manufacturing process more challenging as a small quantity is harder to homogenise than a high one.

Table 4: Amount of CNCbl in various multivitamin tablets, determined with LC-ID^4-MS/MS. Results are reported in μg/g. Certified value of NIST 3280 and labelled values of VITA-1, multivitamin A and B are reported in parenthesis

	NIST 3280 (μg/g)	**VITA-1 (μg/g)**	**Multivitamin A (μg/g)**	**Multivitamin B (μg/g**
1	5.48	2.68	15.70	4.37
2	5.33	2.74	15.83	3.99
3	5.58	2.52	15.67	4.44
4	5.21	2.61	15.78	3.89
Mean	5.40 (4.8±1)	2.64 (2.33)	15.75 (14)	4.17 (2.6)
SD	0.16	0.09	0.07	0.27

LC-ID^4-MS/MS showed an excellent linearity in the concentration range of interest (R^2=0.9996) (Figure 7). Limit of detection (LOD) and quantitation (LOQ), estimated as 3 and 10 times S/N ratio, respectively,[34] for CNCbl in multivitamin tablets were 57 and 155 ng/g, respectively. CNCbl spike recoveries were assessed on NIST 3280 SRM with standard addition on two different concentrations (2.4 and 14 μg/g). Recoveries were 109.7% (n=3) and 112.2% (n=3) for the low and high-level concentrations, respectively.

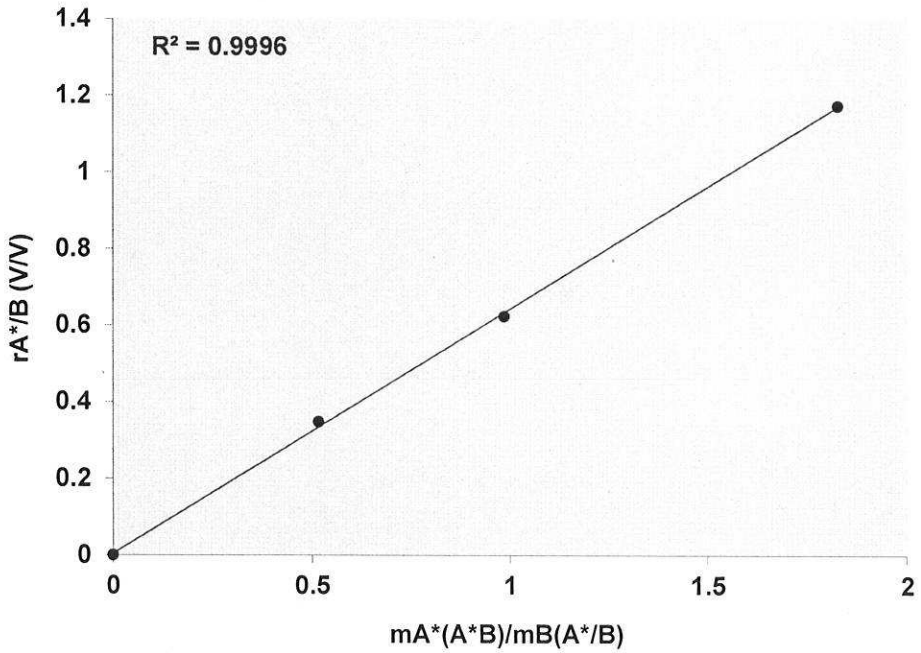

Figure 7: ID^4 calibration curve for CNCbl. A and A* are the CNCbl amount in the sample and in the blends, respectively. B is the $^{13}C^{15}N$Cbl amount.

Conclusions

In this paper, LC-ID^4-MS/MS is proposed as new method for the determination of CNCbl in multivitamin tablets. The technique was successfully validated with NIST SRM 3280 multivitamin tablets and by an intercomparison with LC-ICPMS. Thanks to the presence of a labelled IS ($^{13}C^{15}N$Cbl), the method showed excellent accuracy and linearity. It is anticipated that LC-ID^{arg}-MS/MS can be adopted as primary method for certification of CNCbl in supplements.

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