A LC/UV/Vis method for determination of cyanocobalamin (VB_{12}) in multivitamin dietary supplements with on-line sample clean-up

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A HPLC-UV/Vis method using a two-column strategy with a switching valve for on-line sample clean-up was developed for the determination of cyanocobalamin (CN–Cbl/Vitamin B_{12}) in multivitamin dietary supplement tablets. The method uses two columns: an Agilent Zorbax C8 (150 mm × 4.6 mm, 5 μm particle size) reversed-phase column and a Waters Symmetry C18 (150 mm × 4.6 mm, 5 μm particle size) reversed-phase column. Chromatographic separation was achieved using a programmed gradient mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. Because of the low levels of Vitamin B_{12} in the samples, large injection volumes, and thus much interfering material, must be used to exceed the limit of quantitation (LOQ) by UV detection. A switching valve was used to divert most of these early eluting interfering materials to waste, effecting on-line sample clean-up without excessive sample preparation steps. The recovery of CN–Cbl in the method was 99.5% and the LOQ was 10 ng per injection. The method was successfully applied to the analysis of the NIST SRM 3280 multivitamin/multimineral dietary supplement tablet. The method is specific, precise, and accurate for the intended use. Compared to off-line sample clean-up procedures, it offers the advantage of being easier, more economical, and less time-consuming.

1. Introduction

Vitamin B_{12} (cobalamin - VB_{12}) is an essential nutrient that can be found in meat and dairy products or fermented foods (derived from bacteria). VB_{12} deficiency affects the growth and repair of all body cells, namely the hematopoietic cells and the nervous system cells due to its implication in myelin generation.[1] For adults, the recommended dietary allowance of VB_{12} is 2.4 μg day^{-1}.[2] Because some older people may be unable to absorb naturally occurring VB_{12}, it has been suggested that those over 50 years of age meet their requirements with VB_{12} fortified foods or supplements.[2] An additional 0.40 μg day^{-1} is advised for children and pregnant or lactating women. The average diet generally contains adequate daily intake of VB_{12} in a non-vegetarian diet.[2]

VB_{12} is a tetrapyrrole complex which contains cobalt in the molecule and may refer to several forms of cobalamin. Cyanocobalamin (CN–Cbl) and hydroxocobalamin (OH–Cbl) forms of VB_{12} are available for medical use. Adenosylcobalamin (Ado–Cbl), methylcobalamin Me–Cbl), and cobinamide (CN_2–Cbn) are also forms of VB_{12} found in biological or food samples. In the United States, CN–Cbl is predominantly used in vitamin preparations, supplements, and medical foods because of its stability.[3,4] Generally VB_{12} is present in fortified foods or supplements at levels about 100 to 1000 times lower than other B-family vitamins such as B_1 and B_6.[5]

Today there is increased interest in accurately assessing the total dietary intake of vitamins from all sources, including foods and dietary supplements. Demand for rapid, specific, and updated methodologies for determination of vitamins is growing because of their importance for health. Several methods such as chemiluminescence,[6] atomic absorption spectrometry (AAS),[7] ultraviolet-visible (UV/Vis) spectrometry,[8] and voltammetry[9,10] have been proposed for the determination of VB_{12}. These methods do not distinguish cobalamin species and were not tested in a complex matrix, such as multivitamin or multimineral dietary supplements. Radioisotope dilution[11] and biosensor based protein-binding assays[12,13] have also been described. However, cobalamin analogues react with the binding protein used in the assays leading to lack of specificity for these methods.[14,15]

Currently, microbiological assays (MBA) from the AOAC INTERNATIONAL, which use *Lactobacillus leishmannii* as a test organism, are utilized for the routine determination of VB_{12} in foodstuffs.[16,17] These non-specific MBA are not capable of distinguishing between the cobalamin analogues because they rely on the conversion of all cobalamins to CN–Cbl using KCN. MBA may also be influenced by other food components such as deoxyribosides and deoxynucleotides.[18] Although they have high sensitivity, MBA are time-consuming and present high variability, requiring multiple determinations to get good estimates.[15]

Several studies have determined all cobalamin species together and report total cobalamin,[5,19,20] but many perform selective determination of cobalamin species. The most widely used chemical methods for VB_{12} determination are capillary electrophoresis[21,22] and high-performance liquid chromatography (HPLC) with various detection methods such as UV/VIS,[23–28] AAS,[29] fluorescence,[14,30] or mass spectrometric detectors.[20,23,31–34]

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To determine VB_{12} in a complex matrix, usually an off-line clean-up step, such as solid-phase extraction (SPE), was needed.[15,19,25]

Since the main form of cobalamin used in dietary supplements and fortified foods in the USA is CN–Cbl, this form was used in this study. The study focused on the development of an easy, economical, fast, and accurate method for VB_{12} quantitation in multivitamin dietary supplement tablets without an off-line sample clean-up procedure, thus the UV/Vis detector was selected as the detector of choice.

2. Experimental

2.1. Reagents and materials

Water, acetonitrile and methanol were Optima* grade (Fisher Scientific, Pittsburgh, PA) while formic acid is mass spectrometry grade (Sigma/Aldrich, St. Louis, MO). Cyanocobalamin (CN–Cbl) (CAS 68-19-9; MW 1,355.37) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was stored in a refrigerator (5 °C) as required to ensure stability. Stock solutions were prepared under low-light conditions and stored in the refrigerator (5 °C). Standard reference material (SRM) multivitamin/multimineral dietary supplement tablets (SRM 3280) were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). Use of a SRM provides a stable and homogeneous test material with known B_{12} values to test and validate the method. Open availability of the SRM will make it suitable for use by other laboratories to test proper implementation of this method. Five different vitamins samples from major vitamin supplement manufactures were purchased *via* internet retailers, the samples included 3 tablets, 1 chewable tablet, and 1 liquid.

2.2. Apparatus

An Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) was used, consisting of a quaternary pump with a vacuum degasser, a thermostatted column compartment, an auto-sampler, and a diode array detector (DAD).

Two analytical columns were used. Column 1 was an Agilent Zorbax C8 reversed-phase column (150 mm × 4.6 mm, 5 μm particle size, Agilent Technologies, Palo Alto, CA) and column 2 was a Waters Symmetry C18 reversed-phase column (150 mm × 4.6 mm, 5 μm particle size, Waters, Milford, MA, USA).

A mortar grinder Retsch Rm-100 (Retsch GmbH & Co. KG, Germany) and a IEC Clinical Centrifuge (Danon/IEC Division Needham H.T.S., USA) were also used.

2.3. Sample preparation

A composite of 20 tablets of solid-form vitamin supplements (including SRM 3280) was accurately weighed and ground to a uniform powder with the mortar grinder for 15 min. Approximately the weight of a single tablet of each sample (the average weight of one tablet) was weighed and transferred into a red-color 10 mL volumetric flask. 10 mL H_2O was added and the flask was sonicated in the dark for 30 min. The content was then decanted into a 15 mL centrifuge tube and was centrifuged for 15 min at 5,000 g. The supernatant was filtered through a 0.45 μm PVDF filter. Samples were prepared under low-light conditions throughout and stored in the dark at 5 °C before use. The liquid sample was diluted with H_2O to the appropriate concentration according to the label claim.

2.4. Two-column chromatography/DAD conditions

Chromatographic separation was achieved using a programmed gradient mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient is as follows: 0–12 min, linear gradient from 95 : 5 A:B (v/v) to 75 : 25 A:B (v/v); 12–15 min, linear gradient from 75 : 25 A:B (v/v) to 5 : 95 A:B (v/v); 15–17 min, isocratic at 5 : 95 A:B (v/v); 17–17.1 min, back to 97 : 5 A:B (v/v); 17.1–25 min, isocratic at 95 : 5 A:B (v/v, column equilibrating). The flow rate used was 1 mL min^{-1} and the injection volumes were 100 μL. The HPLC analysis was carried out using two columns connected in series with a switching valve sandwiched in between (Agilent Zorbax column to switching valve to Waters Symmetry column). For the first 8 min of the HPLC run, the eluent was diverted to a waste line; at 8 min, the eluent was switched from the waste to the Waters Symmetry column. UV/Vis detection was carried out at 550 nm.

3. Results and discussion

3.1. Sample preparation

Because CN–Cbl is a water-soluble vitamin and stable when protected from light, H_2O is a good extraction solvent. Water, 10 mM KCl solution, 10 mM phosphate buffers at pH 2.5 and at pH 4.3 were investigated for the extraction of CN–Cbl and no significant differences in quantitation were observed in the results.

3.2. Chromatography

Since the CN–Cbl content in a single tablet was estimated to be approximately 10 μg, it was necessary to inject the maximum possible amount of sample in order to exceed the limit of quantitation (LOQ) of the diode array detector (DAD). The amount of sample injected per analysis in this study (10 mL extraction solvent per tablet with 100 μL injection) was about 2000 times greater compared to our previous studies on the analysis of other B-vitamins in SRM 3280 (1000 mL extraction solvent per tablet with 5 μL injection).[35] For HPLC analysis of CN–Cbl in this amount of extracted material, usually a clean-up step, such as off-line solid phase extraction (SPE), is commonly used.[5,15,19,25] Off-line sample clean-up methods are usually expensive, laborious, and time consuming. One of the goals of this study was to not use any off-line sample clean-up procedure except a simple filtration. Initially, an on-line SPE method (Waters Oasis HLB on-line SPE column, 20 mm × 3.9 mm, 20 μm particle size, Waters, Milford, MA, USA) was investigated. The result was not satisfactory, due to the fact that the amount of material (other vitamins and minerals) injected exceeded the loading capacity of the column. A bigger column, such as a regular 4.6 mm HPLC column, was better suited to handle the task. Thus, a two-column strategy was investigated. The 1st column did not have to have the loading capacity to hold all of the material injected as long as it retained all of the CN–Cbl. A

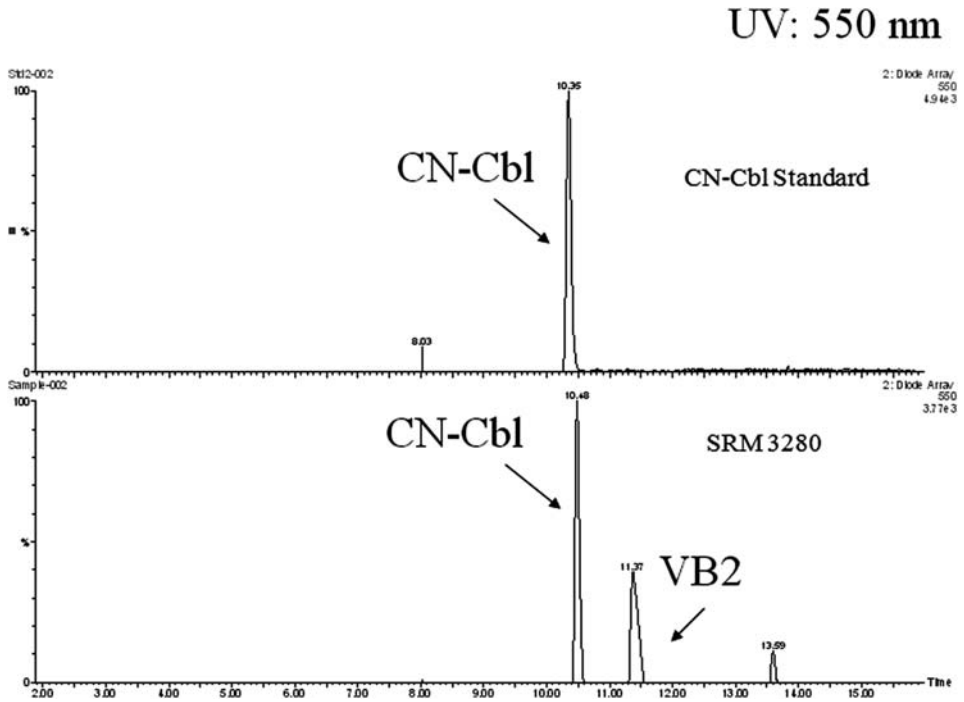

Fig. 1 HPLC-UV/Vis chromatograms of CN–Cbl standard and SRM 3280 extract (UV $\lambda = 550$ nm).

variety of reversed-phase HPLC columns from different manufacturers were tested as the 1st column. After screening a series of C18 and C8 columns, the Agilent Zorbax C8 column was found to be the best. Then, 2nd stage columns were screened and the Waters Symmetry C18 column was found to give the best peak shape and separation for CN–Cbl when combined with the Agilent Zorbax C8 column. The HPLC gradient and the time of the switching were optimized based on the combination of the two columns. The conditions selected in the developed method (see Experimental section) gave excellent separation within 25 min with a sharp and symmetric CN–Cbl peak (Fig. 1).

3.3. Sample analysis

The chromatogram at 550 nm showed that the CN–Cbl peak at 10.4 min was well separated from the vitamin B_2 (riboflavin) peak at 11.4 min in the NIST SRM 3280 multivitamin dietary supplement (Fig. 1). The identities of the CN–Cbl peaks (*m*/*z* 1356.4, 10.4 min) in both CN–Cbl standard and the NIST SRM 3280 and the B_2 peak (*m*/*z* 377.3, 11.4 min) in the NIST SRM 3280 were confirmed by mass spectrometry (Waters Quattro Micro triple-quad mass spectrometer, Waters, Milford, MA). The UV/Vis spectra from both CN–Cbl and the SRM 3280 show

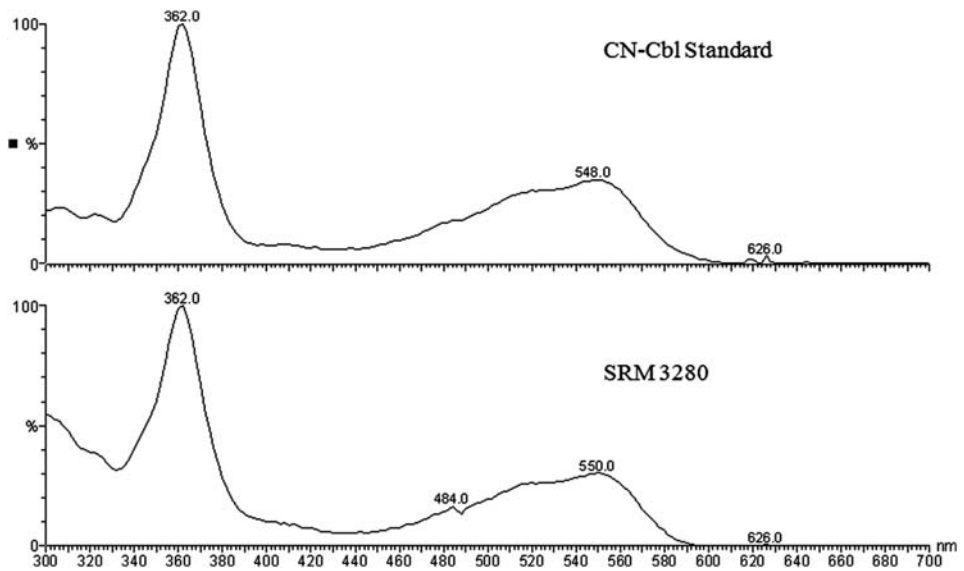

Fig. 2 UV/Vis spectrum of the CN–Cbl standard and SRM 3280 extract from the HPLC chromatogram (UV/Vis 300–700 nm).

the most distinctive peaks at 362 and 550 nm (Fig. 2). However, there is considerably more absorbance between UV 300–UV 340 nm from the matrix of NIST SRM 3280. At above 450 nm, there are no significant matrix effects on the spectrum of CN–Cbl. So 550 nm was selected as the wavelength for CN–Cbl quantitation.

The LC/UV/Vis method described was tested with respect to sensitivity [the limit of detection (LOD) and the limit of quantification (LOQ)], linearity, intra-day precision ($n = 6$) at three different concentrations, intermediate precision ($n = 3$) for five consecutive days, and accuracy.

3.3.1. LOD, LOQ, range, and linearity. The limit of detection (LOD) is 3.3 ng/inj and the limit of quantification (LOQ) is 10.0 ng/inj. The LOD and LOQ were calculated based on signal to noise ratios of 3 and 10 (S/N = 3 and 10), respectively. Compared with previously reported HPLC methods using UV/Vis detection, the obtained LOD with the present method (3.3 ng mL^{-1}) was more sensitive than methods optimized by Moreno *et al.*[19] (4 ng/inj), Klejdus *et al.*[25] (9.7 ng/inj) and Wongyai *et al.*[5] (100 ng/inj), and similar to the one reported by Heudi *et al.*[15] (3 ng/inj). The working range of 10–1000 ng mL^{-1} is estimated from the LOQ (10 ng mL^{-1}) up to 1000% of the estimated concentration of the CN–Cbl level existing in the extract. Excellent linearity was observed for the calibration plot of peak area *versus* concentration ($y = 0.3622x - 2.3987$, $R^2 = 0.9998$).

3.3.2. Intra-day and inter-day precisions. The intra-day precision of the chromatographic system was evaluated by injection of CN–Cbl standards at three different concentration levels ($n = 6$, Table 1). The intermediate precision of the method performance was tested for 5 days ($n = 3$ except the 1st day, $n = 6$, Table 2). The overall relative standard deviation (RSD) for the 5 days' results was 0.84%.

3.3.3. Accuracy. Averaging of all 5 days' results from the intermediate precision study (Table 2), the amount of the CN–Cbl contained in SRM 3280 is 6.02 ± 0.05 μg g^{-1} (mass fraction). Our values are accurate within the certified value and uncertainties in the Certificate of Analysis for NIST SRM 3280 of 4.9 ± 1.9 μg g^{-1} (mass fraction).[36]

Table 1 Intra-day precision of the LC/UV method[a,b]

Concentration/μg g^{-1}	SD ($n = 6$)	RSD%
3.02	0.04	1.42
6.00	0.02	0.30
10.56	0.12	1.17

[a] Within-day precision, UV 550 nm. Expressed as mass fraction (μg g^{-1}) of the NIST SRM 3280. [b] In order to better judge the intra-day precision of the method, one diluted, one normal, and one spiked SRM 3280 sample were used in the experiment.

Table 2 Intermediate precision of the LC/UV method[a,b]

Day 1	Day 2	Day 3	Day 4	Day 5	Overall RSD (%)
6.04 ± 0.04	6.07 ± 0.06	6.01 ± 0.02	5.99 ± 0.01	6.00 ± 0.02	0.84%

[a] Inter-day precision, UV 550 nm. Expressed as mass fraction (μg g^{-1}) of the NIST SRM 3280. [b] $n = 6$ (Day 1) and $n = 3$ (Day 2–5).

Table 3 Analysis of commercial multi-vitamin dietary supplements [a,b]

Samples	Sample 1	Sample 2	Sample 3 [b]	Sample 4	Sample 5
Measured amount	74.48 ± 0.16	144.10 ± 0.62	0.27 ± 0.01	3.30 ± 0.03	4.40 ± 0.07
Labeled amount	57.10	122.67	0.30	2.68	3.34

[a] Expressed as mass fraction (μg g^{-1}) for all samples except for sample 3 (liquid form, μg mL^{-1}). [b] $n = 3$.

3.3.4. Recovery and matrix effects. For recovery studies, known amounts of CN–Cbl (equivalent to 50%, 100%, and 200% of expected vitamin concentration of the analyzed sample) were added to the respective aliquots of the tablet powder prior to sample preparation. The recovery of added CN–Cbl calculated was 99.5 ± 2.8%, the recovery from the certificate analysis was 122.8% ± 31.6%.

Since it was impossible to obtain the blank matrix for the SRM 3280 used, the standard addition method was used to evaluate matrix interference[37] in the detection system. Different amounts of CN–Cbl standards were added to a sample extract at approximately 50%, 100%, 150%, and 200% of the estimated CN–Cbl level. Expressed as relative error, the magnitude of matrix effects can be calculated by the following equation:

$$\text{Relative Error (\%)} = (100 \times |X - A|)/A$$

Where X = mean value obtained through the standard curve and A = mean value obtained through the standard addition curve.

The relative error due to matrix effects calculated for the LC/UV/Vis method is 0.35%, showing matrix effects to be non-significant.

3.3.5 Analysis of commercial samples. The results of the amount of CN–Cbl contained in each of the commercial samples are listed in Table 3. Generally the measured amounts of the CN–Cbl are higher than the label claims. This is not surprising since it is well-known that manufacturers often deliberately add more vitamins than the label claims in order to have extended shelf-life. The lone exception among the commercial samples is the liquid form vitamin supplements, the measured amount is about 10% lower compared to the label claim. The reason might be that CN–Cbl in liquid is not as stable as in a tablet and degradation might have occurred.

4. Conclusion

This study provided an improved approach for the analysis of CN–Cbl, which normally exists at low concentration in multi-vitamin dietary supplements. The method does not use any off-line sample clean-up/concentration procedures that are expensive and laborious. The proposed 2-column HPLC-UV/Vis method is

sensitive and reproducible with good precision and accuracy. This method is easy, economical, and efficient. Consequently, this analytical method can be considered as a valid alternative to those that require an off-line sample clean-up procedure or require a mass spectrometer for analysis of VB_{12} in dietary supplements.

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