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ORIGINAL

A Stability-Indicating HPLC Method for the Determination of Nitrosylcobalamin (NO-Cbl), a Novel Vitamin B_{12} Analog

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Abstract Nitrosylcobalamin (NO-Cbl), a novel vitamin B_{12} analog and anti-tumor agent, functions as a biologic 'Trojan horse', utilizing the vitamin B_{12} transcobalamin II transport protein and cell surface receptor to specifically target cancer cells. A stability-indicating HPLC method was developed for the detection of NO-Cbl during forced degradation studies. This method utilized an Ascentis® RP-Amide (150 mm × 4.6 mm, 5 µm) column at 35 °C with a mobile phase (1.0 mL min^{-1}) combining a gradient of methanol and an acetate buffer at pH 6.0. Detection wavelengths of 450 and 254 nm were used to detect corrin and non-corrin-based products, respectively. NO-Cbl, synthesized from hydroxocobalamin and pure nitric oxide gas, was subjected to degradative stress conditions including oxidation, hydrolysis and thermal and radiant energy challenge. The method was validated by assessing linearity, accuracy, precision, detection and quantitation limits and robustness. The method was applied successfully for purity assessment of synthesized NO-Cbl and for the determination of NO-Cbl during kinetic studies in aqueous solution and in solid-state degradation assessments. This HPLC method is suitable for the separation of cobalamins in aqueous and methanolic solutions, for routine detection of NO-Cbl and for purity assessment of synthesized NO-Cbl. Additionally, this method has potential application in identification and monitoring of diseases involving altered nitric oxide homeostasis where vitamin B_{12} therapy is utilized to scavenge excess nitric oxide, subsequently resulting in the in vivo production of NO-Cbl.

Keywords Column liquid chromatography · Stability-indicating method · Nitrosylcobalamin · Vitamin B_{12} · Nitric oxide

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Introduction

Vitamin B_{12}, a natural cyano-cobalt organic complex, was first isolated in 1948 [1]. Since that time, vitamin B_{12} has been verified as an absolute requirement for the differentiation, proliferation and metabolic maintenance of all cells [2–4]. Vitamin B_{12} is also an effective scavenger of nitric oxide (NO) [5, 6], an inflammatory mediator that, in excess concentrations, may be adversely involved in various diseases including cancer [7, 8], asthma [9, 10] and autism [11, 12].

As early as 1951, researchers began to synthesize analogs of vitamin B_{12} by substituting the cyano group with hydroxo-, chloro-, bromo-, sulfato-, nitro- and cyanto groups [13]. Optimal sites of conjugation have been identified that are capable of maintaining the biologic activity of these analogs [14–16]. Nitrosylcobalamin (NO-Cbl), the most recent vitamin B_{12} analog to be described, comprises nitric oxide (NO) bound to the upper axial ligand position of vitamin B_{12} [17]. The chemical structure of NO-Cbl is shown in Fig. 1. Developed as a potential anti-tumor agent, NO-Cbl functions as a biologic 'Trojan horse', utilizing the vitamin B_{12} transcobalamin II transport protein (TCII) and cell surface receptor (TCII-R) to specifically target NO-Cbl to cancer cells [18]. Once NO-Cbl is internalized within the cancer cells through TCII-R-mediated endocytosis, NO

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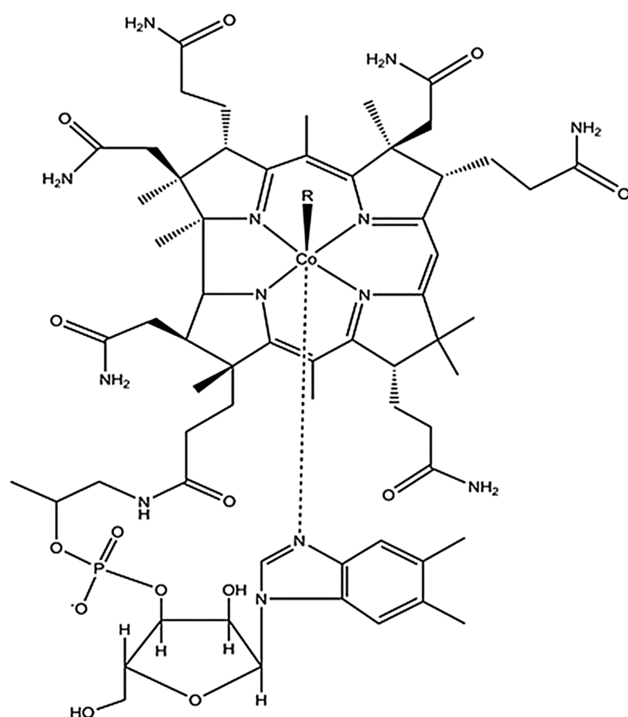


Fig. 1 Lewis structure of the cobalamin corrin ring and coordinated mononucleotide. The ligand *R* can be represented by a variety of functional groups including methyl (Me), hydroxo- (OH), cyano- (CN), adenosyl- (Ado), nitrogen dioxide (NO_2) and more recently nitric oxide (NO) groups

is liberated from vitamin B_{12}, resulting in decreased cellular metabolism, activation of apoptotic mechanisms and inhibition of survival pathways [18–20]. The anti-tumor efficacy of NO-Cbl has been demonstrated in human cancer cell lines [18–20], in murine xenografts [18–20] and in spontaneous tumors in dogs [21].

Development of vitamin B_{12} analogs has been accompanied by the requirement for new methods to detect them. Vitamin B_{12} and its analogs, with the exception of NO-Cbl, have been detected and quantified by a variety of assays, including high-pressure liquid chromatography (HPLC), microbiologic assay, reverse isotope dilution assay (RIDA), radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) [22–25]. The majority of HPLC methods used to identify vitamin B_{12} have been reverse phase and have utilized a variety of detection methods such as ultraviolet (UV) fluorescence, mass spectrometry and electrochemical approaches [26, 27]. In preparation for HPLC analysis, immunoaffinity columns utilizing vitamin B_{12} binding proteins such as intrinsic factor and haptocorrin have often been used to isolate and/or purify cobalamins prior to analysis [25, 28].

To date, assessment of vitamin B_{12} analogs by traditional reverse-phase HPLC methods has been challenging, and published HPLC methods have not addressed NO-Cbl analysis. Attempts to adapt published conditions to the separation and identification of NO-Cbl have been unsuccessful due to a variety of compound-specific factors. For instance, many vitamin B_{12} analogs are unstable under HPLC analysis conditions and require an indirect assay for purity determination [29]. Additionally, coordination of the NO free radical to cobalt presents significant challenges for NO-Cbl analysis using traditional aqueous mobile phases at low pH, because NO-Cbl characteristically releases NO below pH 5.0. Furthermore, most NO donors spontaneously release NO in aqueous solution, often resulting in erratic peak symmetry and retention times with traditional HPLC methods [20]. The aim of this work was to develop and validate an HPLC method that can be used for the identification, determination and stability assessment of NO-Cbl.

The method presented here describes an HPLC procedure suitable for the separation of NO-Cbl from: (i) hydroxocobalamin (OH-Cbl), the synthetic starting material for NO-Cbl; (ii) cyanocobalamin (CN-Cbl), the commercially available form of vitamin B_{12}; (iii) nitrocobalamin (NO_2-Cbl), a potential synthetic by-product; and (iv) dicyanocobinamide (DCC) and 5,6-dimethylbenzimidazole (DMBI), which are cobalamin precursors. Parameters including linearity, accuracy, precision, detection and quantitation limits and robustness have been evaluated to validate the method. The validated HPLC method has been applied to the assessment of synthetic NO-Cbl purity, and to the stability of NO-Cbl following chemical, thermal and radiant energy stress.

Experimental

Standards and Reagents

Glacial acetic acid (BDH Aristar® Plus) was purchased from VWR (Radnor, PA, USA). Sodium hydroxide (Emsure®), HPLC-grade water, methanol, acetonitrile and biosynthesis-grade dichloromethane (OmniSolv®) were obtained from EMD Millipore Chemicals (Billerica, MA, USA). Hydroxocobalamin acetate (vitamin B_{12a}, OH-Cbl) was purchased from Hebei Huarong Pharmaceutical Co. (Hebei Province, China). Cyanocobalamin (CN-Cbl), dicyanocobinamide (DCC) and 5,6-dimethylbenzimidazole (DMBI) reference standards were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Nitrocobalamin (NO_2-Cbl) was synthesized as described previously in the literature [13]. Nitric oxide gas (99.5 %) was purchased from Praxair (Bethlehem, PA, USA).

Synthesis of NO-Cbl

NO-Cbl was synthesized as previously described [17]: OH-Cbl was dissolved in dichloromethane and exposed to pure

NO gas. The reaction was performed at room temperature in a closed system within a high-pressure 8 L aluminum reactor (Luxfer Gas Cylinders, Riverside, CA, USA) and stirred continuously using a standard laboratory magnetic stirring plate. The reaction system was purged daily and evacuated prior to fresh NO exposure. After 5 days, the solid NO-Cbl product was collected following rotary evaporation of the solvent and stored in a desiccator at −20 °C, protected from light.

Stock and Working Cobalamin Standards

Stock standards of all three cobalamins (OH-Cbl, CN-Cbl and NO-Cbl) at 2.0 mg mL^{-1} were prepared by adding 20.0 mg of the pure solid standards separately to clean methanol-rinsed 10.0 mL volumetric flasks, adding 4 mL of HPLC-grade methanol to dissolve the solid and then *quantum satis* (QS) to 10.0 mL. Working standards of NO-Cbl (20–200 µg mL^{-1}) and all cobalamins analyzed were prepared by appropriate dilutions of stock standards. The diluting solvent was either HPLC-grade methanol or mobile phase A (see below). 15 µL of working standards or samples was injected for all analyses described. All samples were either analyzed the same day or stored for no more than 1 week at −20 °C, protected from light.

Instrumentation

The HPLC system consisted of a Shimadzu model LC-20AD liquid chromatograph with column oven (model CTO-20AC), pump (model DGU-20A5), degasser (model CBM-20A) and UV–VIS detector (model SPD-20AV) (all Shimadzu Corp. products, Kyoto, Japan). HPLC data were processed using LC Solutions software, version 1.2 (Shimadzu Corp., Kyoto, Japan). Separations were performed using an Ascentis® RP-Amide column (150 mm length × 4.6 mm internal diameter, 5 µm particle size) (Supelco Inc./Sigma-Aldrich, Bellfonte, PA, USA). All samples were injected manually using a 25 µL glass syringe (model 1705 N, Hamilton Co., Reno, NV, USA). During analysis, the column temperature was maintained at 35 °C. Mobile phase flow rate was 1 mL min^{-1}. Mobile phase A consisted of glacial acetic acid (440 mM, pH 6.0), adjusted using 10 M sodium hydroxide in HPLC-grade water. Prior to use, this acetate buffer was filtered through a Whatman™ 0.2 µm filter (GE Healthcare, Piscataway, NJ, USA). Mobile phase B was HPLC-grade methanol. The system was equilibrated for 30 min with mobile phase A at 1 mL min^{-1} prior to injection of any standard or sample. A stepwise gradient with a total run time (including recycle time) of 25 min was performed. The final gradient program used for this study was 100 % A for 1.5 min; to 65 % A at 12.0 min; to 100 % B at 12.5 min, all linear gradients. The mobile phase was then switched back to 100 % A at 17.0 min and re-equilibrated for 15 min between injections. The relatively sharp ramp to 100 % methanol was necessary to drive the cobalamins off the column and create good peak symmetry. An unusually high salt concentration was also needed to enable good chromatographic separation. Spectrophotometric detection of cobalamins was performed at 254 nm (ultraviolet) for detection of potential non-corrin ring organic degradation products and at 450 nm (visible) for detection of corrin ring-based structures. Prior to use, the RP-amide analytical column was pre-conditioned as recommended by the manufacturer. Between analytic studies, the column was left at room temperature with mobile phase A trickling at 0.2 mL min^{-1} for 24 h daily. These procedures extended the usable life of the column and helped to maintain a more stable baseline and to improve chromatographic behavior.

Procedures for Forced Degradation Studies of NO-Cbl

Oxidative Degradation with Aqueous Hydrochloric Acid

Since NO-Cbl is only sparingly soluble in water, solid-form NO-Cbl was first dissolved in a minimal amount of methanol, and the solution was then diluted with water, enabling NO-Cbl to remain in solution. Degradation of NO-Cbl in aqueous solution was studied at 90 °C (363 K) in 0.3 mol L^{-1} hydrochloric acid (HCl). The ionic strength of all solutions was adjusted to 0.5 mol L^{-1} with a solution of 4 mol L^{-1} sodium chloride. A stock solution of NO-Cbl in methanol was prepared by dissolving 50 mg of NO-Cbl into 10 mL of solution (5 mg mL^{-1}). 200 µL of this solution (1,000 µg) was then transferred into a total volume of 10 mL of the HCl/NaCl solution in a 25 mL sealed glass vial. The solution was placed into a water bath at 90 °C and 15 µL samples were removed at 5 h, 10 h and 24 h and analyzed by HPLC for NO-Cbl and degradation products.

Oxidative Degradation with Aqueous Hydrogen Peroxide

NO-Cbl solutions were prepared as described above. Degradation of NO-Cbl in 2 mL of 30 % hydrogen peroxide (H_2O_2) solution was evaluated. Solutions were placed in a water bath at 90 °C. 15 µL samples were removed at 5 h, 10 h and 24 h and analyzed by HPLC for NO-Cbl and degradation products.

Oxidative Degradation with Sodium Hypochlorite (Bleach)

NO-Cbl solutions were prepared as described above. Degradation of NO-Cbl in 2 mL of 10 % sodium hypochlorite (NaClO) solution was evaluated. Solutions were placed in a

water bath at 90 °C. 15 μL samples were removed at 5, 10 and 24 h and analyzed by HPLC for NO-Cbl and degradation products.

Oxidative Degradation with Acidified Hydrogen Peroxide

NO-Cbl solutions were prepared as described above. 2 mL of 30 % H_2O_2 was added to 0.3 mol L^{-1} HCl. The solutions were placed in a water bath at 90 °C. 15 μL samples were removed at 5, 10 and 24 h and analyzed by HPLC for NO-Cbl and degradation products.

Oxidative Degradation with Acidified Sodium Hypochlorite (Bleach)

NO-Cbl solutions were prepared as described above. 2 mL of 10 % NaClO was added to 0.3 mol L^{-1} HCl. The solutions were placed into a water bath at 90 °C. 15 μL samples were removed at 5, 10 and 24 h and analyzed by HPLC for NO-Cbl and degradation products.

Thermal Degradation of Solid NO-Cbl

Samples of solid NO-Cbl (5 mg) were weighed and placed in clean, dry 25 mL glass scintillation vials and sealed. Samples were then placed in an oven at 100 °C for 28 days. At 24, 48 and 72 h, the samples were removed and cooled to room temperature. The solid contents were dissolved in 50 mL of methanol (100 μg mL^{-1}) and analyzed by HPLC for NO-Cbl and degradation products.

Degradation of Solid NO-Cbl with UV Radiation

Solid samples of NO-Cbl (5 mg) were weighed and spread out on clean, dry watch glasses to maximize surface exposure. The samples were placed in the dark at room temperature and then exposed to long (360 nm) and short (260 nm) wave UV radiation. At 24, 48 and 72 h, the samples were removed, dissolved in 50 mL of methanol (100 μg mL^{-1}) and analyzed by HPLC for NO-Cbl and degradation products.

Results and Discussion

Optimization of Chromatographic Conditions and Separation of Cobalamins

Over the course of development, both normal phase and reverse phase systems were evaluated for their ability to produce a suitable HPLC method for NO-Cbl analysis. System-limiting factors included NO-Cbl's minimal solubility in all of the low-carbon water-soluble organic solvents (with the exception of methanol), and a predictably labile cobalt–nitrogen coordination bond below pH 5.0. As a result, phosphate and acetate buffers with a pH >5.0 were first utilized, and methanol was employed as the sole organic phase component. Initial gradient work with an Ascentis® C_{18} column (Supelco Inc./Sigma-Aldrich, Bellfonte, PA, USA) and methanol and phosphate buffers ranging in pH from 5.0 to 7.5 yielded broad and somewhat tailed cobalamin peaks; however, separation of OH-Cbl, CN-Cbl and NO-Cbl was observed. Based on an NO-Cbl isoelectric pH ~3.0, experiments were conducted using a lower pH to decrease NO-Cbl polarity and to allow for potentially better partitioning on the Ascentis® C_{18} column. Although use of a lower pH could potentially risk loss of NO from the cobalt atom over the course of several hours, it was reasoned that a fast analysis time might decrease this risk of loss. A modified XTerra® C_{18} column (Waters Corp., Milford, MA, USA) with espoused higher thermal stability and lower pH was evaluated. This column was selected for its unique hybrid particle technology, in which silanols are replaced with methyl groups to produce a rugged hybrid (inorganic/organic) particle able to function both at high temperatures and pH extremes. Using the modified C_{18} column, OH-Cbl, CN-Cbl and NO-Cbl eluted in under 8 min, with OH-Cbl and CN-Cbl presenting as very sharp symmetrical peaks and with NO-Cbl eluting as a broader but still symmetrical peak. Although the XTerra® column functioned well, it exhibited poor performance characteristics at pH 3.0 after only 100 injections. Consequently, testing reverted back to use of the Ascentis® C_{18} column, with sodium heptanesulfonate used as a paired ion chromatography (PIC) reagent in an acetate buffer (25 mM, pH 6.0) with a buffer:methanol gradient. This combination produced very sharp cobalamin peaks that eluted in under 20 min. However, the Ascentis® C_{18} column demonstrated signs of degradation after a few hundred injections. After evaluating several different mobile phase compositions and column phases, optimum results were obtained with the Ascentis® RP-Amide column at 35 °C using an acetate buffer:methanol gradient. No PIC reagent was required and all three cobalamins produced sharp and symmetrical peaks in under 15 min. The Ascentis® RP-Amide column remained stable and consistent for many hundreds of injections.

During method development using either C_{18} or RP-Amide stationary phases, it was noted that NO-Cbl in particular required a minimum of 10–12 min of residence time on the stationary phase with a buffer of pH 6.0 to enable good peak symmetry when eluting with methanol. Improved peak symmetry was observed with the RP-Amide column and with relatively high buffer molarity. The pH of the mobile phase buffer was tested at pHs ranging from 5.0 to 7.0 to minimize any loss of the NO ligand from the corrin ring cobalt atom. The buffer at pH 6.0 produced optimal peak symmetry

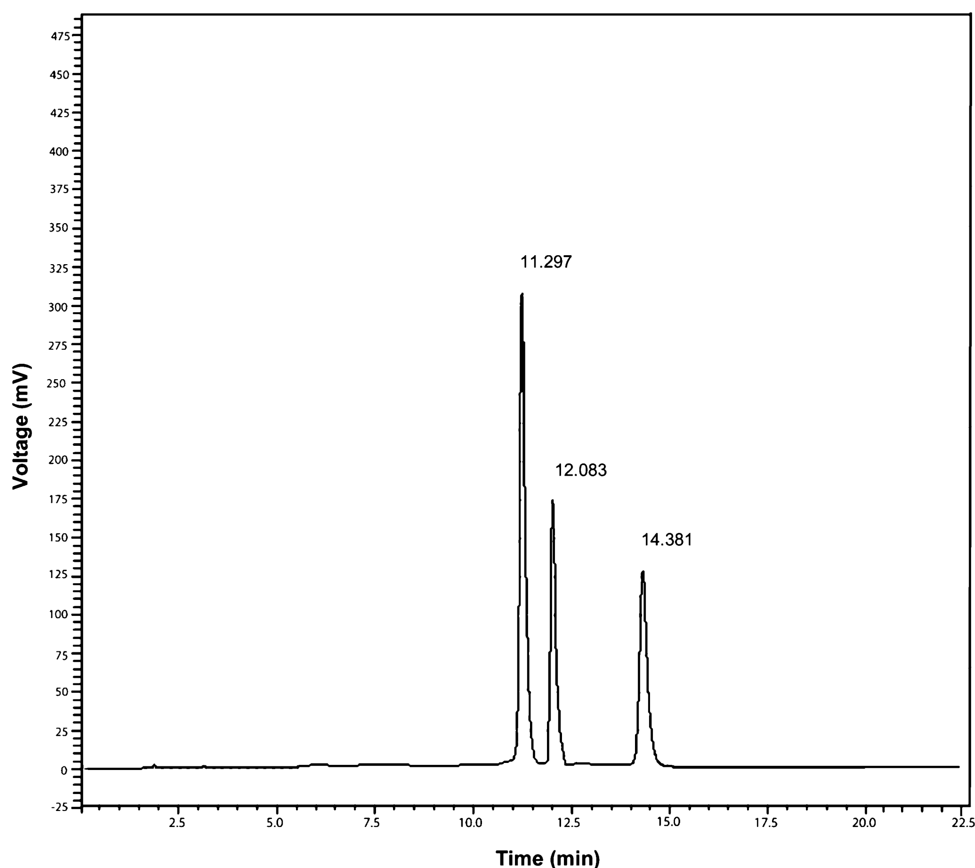


Fig. 2 HPLC chromatogram of OH-Cbl (11.3 min elution), CN-Cbl (12.1 min elution) and NO-Cbl (14.4 min elution) on the RP-Amide reverse phase column, with detection at 450 nm

for NO-Cbl. A chromatogram showing the separation of OH-Cbl, CN-Cbl and NO-Cbl on the RP-Amide column is provided in Fig. 2. Respective elution times, rounded to 0.1 min, were OH-Cbl 11.3 min; CN-Cbl 12.1 min; and NO-Cbl 14.4 min. The elution order indicated that NO-Cbl was more highly retained on the non-polar RP-Amide phase at pH 6.0 than the other cobalamins. NO-Cbl resolved completely from the other cobalamins, especially OH-Cbl, the synthetic starting material, by at least 1 min. Nitrocobalamin (NO_2-Cbl), a potential synthetic impurity, eluted at 13.1 min (data not shown). The cobalamin precursors dicyanocobinamide (DCC) and 5,6-dimethylbenzimidazole (DMBI) eluted at 11.9 and 13.1 min respectively (data not shown). DMBI was detected at 254 nm based on its lack of a corrin ring.

Method Validation

The HPLC method reported here was validated in accordance with the International Conference on Harmonization Guidelines (ICH Q2B, Validation of Analytical Procedures: Methodology). Specific parameters evaluated included linearity, accuracy, precision, detection limit, quantitation limit and robustness. Regarding range, since no therapeutic range currently exists for NO-Cbl, a range of linearity from the detection limit of 10–200 μg mL^{-1} proved to be suitable for this method application.

Linearity

The linearity of the method was evaluated in the concentration range of 10–220 μg mL^{-1}, which represents 10–120 % of the nominal concentration of NO-Cbl (100 μg mL^{-1}) that was used during forced degradation studies and which produced a solid reproducible signal. Samples of each of six solutions were injected three times for each series; both methanolic and aqueous standards were evaluated. Calibration curves were constructed by plotting peak areas against corresponding concentrations and by performing linear regression analysis according to the formula: $y = mx + b$ (y = peak area; m = slope; x = concentration of NO-Cbl; b = 0 intercept). Methanolic and aqueous standards demonstrated identical results. Analysis confirmed that the method was linear in the concentration range of 10–220 μg mL^{-1} ($n = 6$, $r = 0.9992$).

Accuracy

The accuracy of the method was determined by recovering NO-Cbl from spiked buffered standards. The recovery

Table 1 Intra-day and inter-day precision ($n = 10$) and recovery ($n = 3$) studies

Spiked concentration (μg mL^{-1})	Mean concentration ± SD (μg mL^{-1})	RSD (%)
Recovery		
80.0	81.11 ± 0.73	101.2
100.0	100.56 ± 1.25	100.6
120.0	119.28 ± 1.69	99.4
Intra-day precision		
80.0	81.03 ± 0.64	0.80
100.0	100.88 ± 1.23	1.23
120.0	119.47 ± 1.17	1.00
Inter-day precision		
100.0	102.04 ± 1.88	1.09

SD standard deviation, *RSD* relative standard deviation

test was performed using 80, 100 and 120 % of the nominal concentration of NO-Cbl (100 μg mL^{-1}) that was used during forced degradation studies. Three samples were prepared for each recovery level. The solutions were analyzed and percent recovery was calculated; data are reported in Table 1. Calculated %RSD was 101.2, 100.6 and 99.4 % for 80, 100 and 120 μg mL^{-1} NO-Cbl-spiked samples, respectively, indicating that the method was accurate. Mean recovery of 99.4 % for the 120 μg mL^{-1} NO-Cbl-spiked sample may have been associated with irreversible adsorption of NO-Cbl to the surfaces of the system.

Table 2 Results of robustness studies (NO-Cbl at 100 μg mL^{-1})

Parameter	t_R	R_S	% Area difference[▲]	Peak symmetry
Optimal[a]	14.46	3.75	–	1.95
Methanol = 30–100 %	14.84	3.69	0.29	1.78
Methanol = 35–95 %	14.15	3.80	0.02	1.82
Acetate = 400 mM	14.42	3.72	0.09	1.93
Acetate = 480 mM	14.45	3.76	0.57	1.96
pH 5.7	14.44	3.79	0.02	1.90
pH 6.3	14.43	3.71	2.61	1.93
$f = 0.8$ mL min^{-1}	14.51	3.73	0.56	1.89
$f = 1.2$ mL min^{-1}	14.26	3.71	3.06	1.92
$\lambda = 445$ nm	14.42	3.75	0.04	1.94
$\lambda = 455$ nm	14.43	3.76	0.02	1.95
$T = 33$ °C	14.79	3.80	0.56	1.90
$T = 38$ °C	14.28	3.74	0.05	1.95

[a] Acetate = 440 mM, pH 6.0, $f = 1.0$ mL min^{-1}, $\lambda = 450$ nm, $T = 35$ °C

f flow rate, λ wavelength, *T* temperature, t_R retention time, R_S resolution

[▲] % Area difference $= \frac{\text{Optimal area} - \text{parameter area}}{(\text{Optimal area} + \text{parameter area})/2} \times 100$

Table 3 Results of forced degradation studies

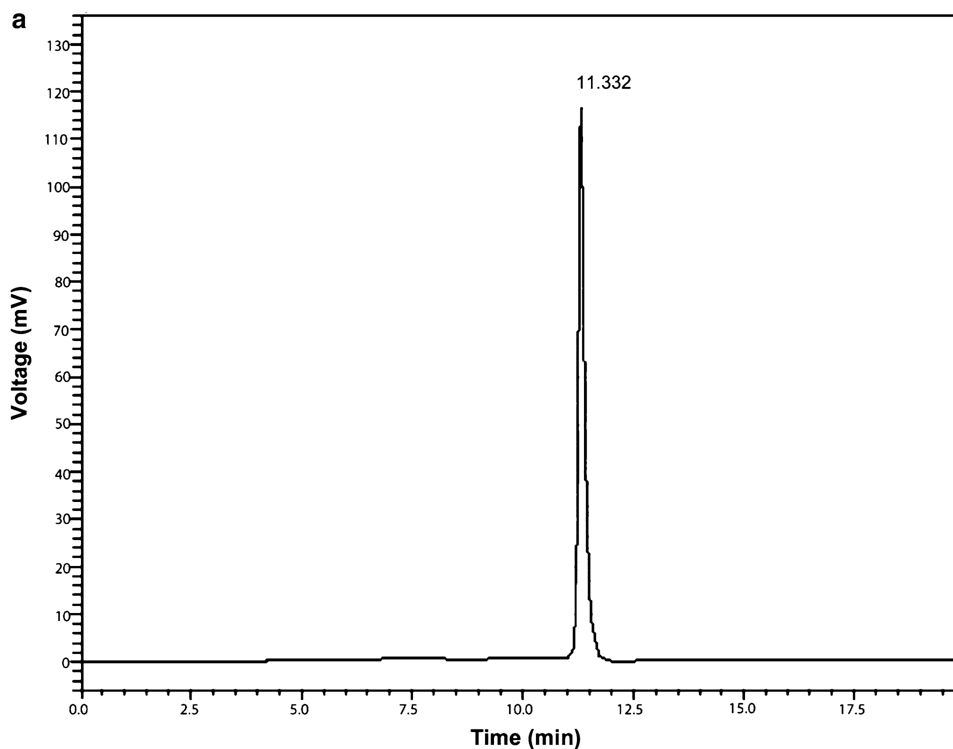
Stress condition	Degradation (%)
0.3 M HCl solution	
5 h	No change
10 h	No change
24 h	No change
10 % H_2O_2 solution	
5 h	No change
10 h	No change
24	No change
10 % NaClO solution	
5 h	No change
10 h	No change
24 h	No change
10 % H_2O_2 + 0.3 M HCl solution	
5 h	40.0
10 h	48.0
24 h	52.0
10 % NaClO + 0.3 M HCl solution	
5 h	6.9
10 h	11.4
24 h	21.6
Thermal (100 °C)[a]	
24 h	No change
48 h	2.4
72 h	3.3
UV radiation (360 nm)[a]	
24 h	No change
48 h	No change
72 h	No change
UV radiation (260 nm)[a]	
24 h	No change
48 h	1.4
72 h	1.5

HCl hydrogen chloride, *H_2O_2* hydrogen peroxide, *NaClO* sodium hypochlorite (bleach), *UV* ultraviolet

[a] Degradation study performed on solid NO-Cbl

Precision

For precision studies, both repeatability and intermediate precision were evaluated. The repeatability (intra-day) study was conducted using six injections of each of three different concentrations of NO-Cbl, analyzed in a single day. Intermediate precision (inter-day) was assessed by comparing assays performed on three different days. Results for repeatability and intermediate precision studies of NO-Cbl are reported in Table 1. Calculated relative standard deviation (RSD) values were 0.8, 1.23, 1 %



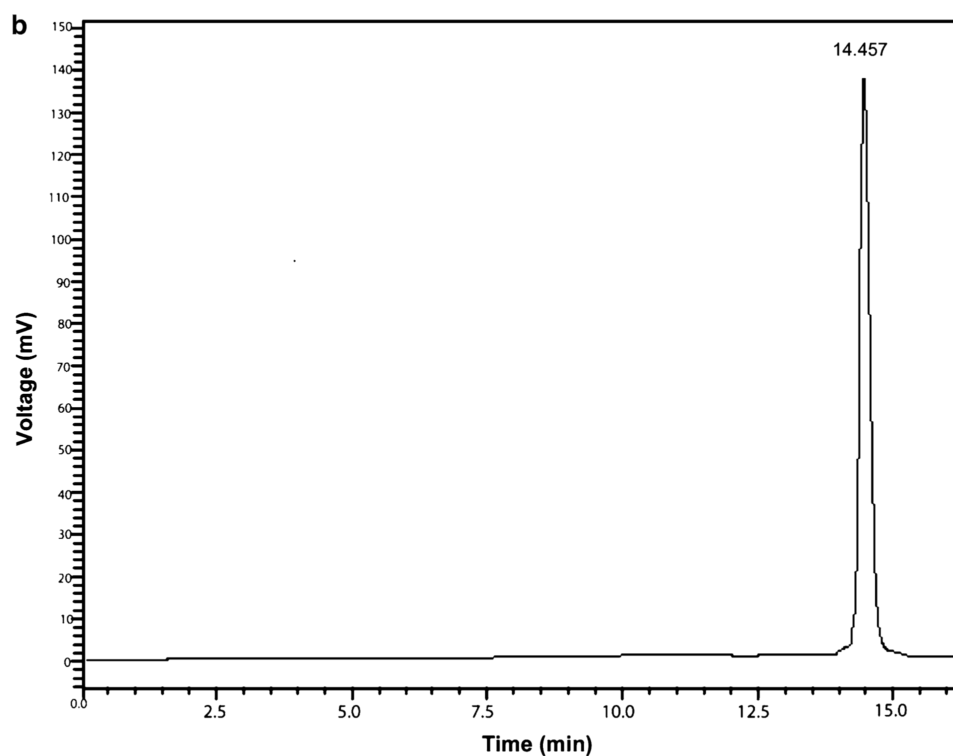


Fig. 3 **a** HPLC chromatogram of OH-Cbl starting material dissolved in dichloromethane and following evaporation of dichloromethane with nitrogen gas and reconstitution of OH-Cbl in methanol, with detection at 450 nm. **b** HPLC chromatogram of synthesized NO-Cbl after 5 days of exposure to nitric oxide gas in dichoromethane. The sample was removed from the reaction mixture, dichloromethane was removed by evaporation with nitrogen gas and the NO-Cbl solid was reconstituted in methanol, with detection at 450 nm

(repeatability) and 1.09 % (intermediate precision). The method was determined to be precise as the %RSD values for both repeatability and precision studies were <2 % as recommended by ICH guidelines.

Detection and Quantitation Limits

Signal-to-noise ratio (S/N) was used to evaluate both detection and quantitation limits by comparing measured signals

from samples with known low NO-Cbl concentrations with those of blank samples. Samples between 1.0 and 50 μg mL^{-1} were injected, and the S/N was measured as the mean and standard deviation for six injections. Under applied chromatographic conditions, the detection limit of NO-Cbl was 10 μg mL^{-1}; an S/N of 3:1 indicated that the method was acceptable for establishing this detection limit according to ICH guidelines. The quantitation limit of NO-Cbl was determined to be 50 μg mL^{-1}; an S/N of 10:1 was considered acceptable for estimating the quantitation level with suitable accuracy and precision.

Robustness

The HPLC procedure was evaluated for robustness by assessing the effect of varying: mobile phase methanol gradient (altered beginning and ending methanol percentage from 30 to 95 %), acetate buffer (400, 440 and 480 mM), mobile phase pH (5.7, 6.0 and 6.3), mobile phase flow rate (0.8, 1.0 and 1.2 mL min^{-1}), detector wavelength (445, 450 and 455 nm) and column temperature (33, 35 and 38 °C). The influence of each parameter change on retention time (t_R), resolution (R_S, relative to hydroxocobalamin), area (A) and peak symmetry was evaluated. The results are outlined in Table 2. No significant changes in retention time, resolution, area or peak symmetry were observed, indicating that the method was reliable under normal usage conditions.

Results of Forced Degradation Studies

Degradation of drug substances between 5 and 20 % has been accepted as reasonable for validation of stability-indicating chromatographic assays [30]. When treated separately with 0.3 M HCl, 10 % H_2O_2 or 10 % NaClO solutions at 90 °C for up to 24 h, HPLC analysis of NO-Cbl did not provide evidence of any measureable degradation products; NO-Cbl peak area of 15-μL injections was 100 ± 0.2 %. When treated with acidified H_2O_2 and hypochlorite solutions, NO-Cbl peak area decomposed by 52 and 21.6 %, respectively, in 24 h, indicating suitable NO-Cbl degradation. When solid NO-Cbl was exposed to 100 °C for up to 72 h, HPLC analysis indicated that NO-Cbl had less than a 4 % loss of peak area. Exposure of solid NO-Cbl to long wave (360 nm) ultraviolet (UV) radiation for up to 72 h produced no measureable loss of NO-Cbl. Exposure to short wave (260 nm) UV radiation produced less than 2 % degradation after 72 h of constant exposure.

In all cases, no specific degradation product peaks appeared under the chromatographic conditions used. The absence of degradation signals at 450 nm suggested that the extended pi-system of the corrin ring was destroyed, causing lack of absorption at 450 nm. Lack of signals at 254 nm may have been associated with loss of fragmented degradation products within the void volume. Forced degradation study results are summarized in Table 3.

Analysis of NO-Cbl Synthetic Purity

A chromatogram of OH-Cbl starting material prior to addition of NO gas is shown in Fig. 3a. After 5 days of synthesis reaction, a 500 μL sample was removed from the reaction mixture, dichloromethane was evaporated under a stream of nitrogen gas, and the residue was dissolved in 20 μL of methanol and in sufficient mobile phase A to produce a minimum 100 mV peak height. During synthesis, a progressive loss of OH-Cbl peak was observed with a simultaneous increase in NO-Cbl peak, with an ultimate calculated NO-Cbl peak area of 98.9 % (relative to all other areas detected) after 5 days exposure to NO gas. A chromatogram of NO-Cbl is shown in Fig. 3b. These results provide evidence for the synthesis of high-purity NO-Cbl relative to the starting material.

Conclusion

The development of a first-of-kind method for the separation of NO-Cbl, a novel cobalamin analog, from other common cobalamins is significant. The gradient HPLC procedure described in this report is suitable for the separation of various cobalamins in aqueous and methanolic solutions, for routine separation of NO-Cbl from other common cobalamins, and for assessment of synthetic NO-Cbl purity and stability. This HPLC method may also be potentially useful in the identification and monitoring of diseases involving altered NO homeostasis where vitamin B_{12} therapy is employed to scavenge excess NO, resulting in the in vivo production of NO-Cbl. The results of validation studies, including linearity, accuracy, precision, detection and quantitation limits and robustness, indicate that the HPLC method is reliable and can differentiate between cobalamin precursors as well as various cobalamins, including OH-Cbl, CN-Cbl, NO_2-Cbl and NO-Cbl. Currently, a solid-phase extraction system for quantitative analysis of NO-Cbl in biological samples is being developed for use in pharmacokinetic applications, as well as a fluorescence-based detection approach that will help to improve sensitivity.

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