Title

High Performance Liquid Chromatography Tandem Mass Spectrometry Assay for the Determination of Cobinamide in Pig Plasma

Authors

Brent A. McCracken¹ and Matthew K. Brittain¹

¹Battelle, West Jefferson, OH 43162

Corresponding author:

Brent A. McCracken

Battelle

1425 Plain City-Georgesville Rd, West Jefferson, OH 43162

Email: mccrackenb@battelle.org

Phone: 614-424-6523

Fax: 614-458-6523

Abstract

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been widely utilized for the analysis of compounds in biological matrices due to its selectivity and sensitivity. This study describes the application of an LC-MS/MS-based approach toward the analysis of cobinamide in Yorkshire pig plasma. The selectivity, accuracy, precision, recovery, linearity, range, carryover, sensitivity, matrix effect, interference, stability, reproducibility, and ruggedness of the method were investigated in pig plasma. The accuracy and precision of the method was determined to be within 10% over three different days over a range of concentrations (25-10,000 ng/mL) that spanned more than two orders of magnitude. The lower limit of quantitation (LLOQ) for dicyanocobinamide was determined to be 25 ng/mL in pig plasma. Carryover was acceptable, as the area response of the carryover blanks were $\leq 15\%$ of the area response of the nearest LLOQ standard for the analyte, while it was nonexistent for the internal standard. Specificity was ensured using six different lots of pig plasma. While the matrix effects of dicyanocobinamide in plasma were enhanced, ginsenoside Rb1 experienced signal suppression under the described conditions. The absolute recovery results for both compounds were consistent, precise, and reproducibly lower than expected at $\sim 60\%$ for dicyanocobinamide and $\sim 22\%$ for ginsenoside Rb1, confirming that a matrix standard curve was required for accurate quantitation. Cobinamide was shown to be very stable in matrix at various storage conditions including room temperature, refrigerated, and frozen at time intervals of 20 hours, 4 days, and 60 days respectively. This method was demonstrated to be sensitive, reproducible, stable, and rugged, and it should be applicable to the analysis of cobinamide in other biological matrices and species.

Key Words

Cobinamide, dicyanocobinamide, nitrocobinamide, potassium cyanide, Yorkshire pig plasma, liquid chromatography-tandem mass spectrometry

1.1 Introduction

Cyanide is a naturally occurring inhibitor of complex IV, also known as cytochrome c oxidase, a transmembrane protein found on the inner mitochondrial membrane and an integral part of the electron transport chain (ETC). The ETC is essential for aerobic respiration and the production of the cell-based energy currency, adenosine triphosphate (ATP). Cytochrome c oxidase receives an electron from each of four cytochrome c molecules and transfers them to one oxygen molecule, thus reducing the molecular oxygen to two molecules of water. During this process, cytochrome c oxidase binds four protons from the inner aqueous phase to produce water while concurrently translocating four protons across the membrane, helping to establish a transmembrane difference of proton electrochemical potential that ATP synthase uses to synthesize ATP.¹ Without the production of ATP, cellular bioenergetics fail, leading to cell death due to asphyxia. The cells requiring the greatest oxygen and energy demand, particularly those of the brain and heart, are the first cell types that are affected, which makes cyanide extremely toxic and detrimental to the neurological and cardiovascular systems.²

Cyanide poisoning is considered a terrorist threat by both military and civilian authorities. It is also an industrial hazard, as it is used in the tobacco industry, during the process of mining minerals and in the manufacture of plastics, petrochemicals, steel, aluminum, paint, pharmaceuticals, and electroplating.³ Cyanide is produced by various organisms (bacteria, fungi, algae and plants), and it can be found naturally in food substances (cassava, sweet potato, fruit pits, lima beans, corn, bamboo shoots and millet).⁴ Smoke from home fires or other fires where plastics are a predominant part of the fuel also contain high cyanide content. A darker source of cyanide poisonings is related to suicides, homicides and genocide. Cyanide has created complex problems for society, ranging from its role in normal metabolism to pollution and its rapid lethal action. Effective countermeasures are therefore necessary to deal with potential cyanide exposures as a result of its high threat potential.

The current countermeasures to cyanide lack desirable attributes, such as ease of use and toxicological tolerance. The two FDA-approved countermeasures are the Cyanide Antidote Kit (CAK, which consists of amyl nitrite, sodium nitrite and sodium thiosulfate) and hydroxocobalamin (Cyanokit®). CAK usage is a three-step process, with two of the steps requiring intravenous infusion and which must be closely monitored due to the potential for

cardiovascular collapse and death. The second approved countermeasure, hydroxocobalamin, produces minimal side effects but must also be administered through intravenous infusion, which is unrealistic in a field or mass casualty situation.

Cobinamide is a novel countermeasure for cyanide exposure that is currently under investigation. Cobinamide and cobinamide derivatives appear to possess certain advantages over current treatments, including hydroxocobalamin. Cobinamide is a water-soluble analog of hydroxocobalamin (see Figure 1); however, it possesses significantly higher affinity for cyanide. Hydroxocobalamin binds 1 mole of cyanide per mole of compound; in comparison, cobinamide has been shown to be 3 to 10 times more potent than hydroxocobalamin in both mouse and rabbit models of acute cyanide intoxication.^{5,6,7} The current use for cobinamide is strictly research only and determining the level of cobinamide in the blood is important to understand the rate of cobinamide moving from the muscle to the blood.

Current methods for the analysis of cobinamide utilize colorimetric techniques, HPLC, and UV in addition to ICP-MS approaches.^{8,9,10} However, these approaches are not suitable for a complex sample matrix. HPLC and absorbance-based techniques suffer from low sensitivity, and the ICP-MS-based approach is fairly sensitive, but lacks the method efficiency and detection limit for the sample size expected. As such, the purpose of this study was to evaluate an assay to quantify cobinamide in swine plasma samples. This method involved extraction and conversion to the dicyano form (Figure 2) using a potassium cyanide solution and acetonitrile, with subsequent analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS). Ginsenoside Rb1 was used throughout the extraction and analysis as a surrogate internal standard (Figure 2). Selectivity, accuracy, precision, recovery, linearity, range, carryover, sensitivity, matrix effect, interference, stability, reproducibility, and ruggedness were determined for this approach in pig plasma.

1.2 Material and methods

1.2.1 Materials

Dicyanocobinamide (100%) was purchased from Sigma-Aldrich (St. Louis, MO). Dinitrocobinamide (91.0%) was obtained from SRI International (Menlo Park, CA). Ginsenoside Rb1 (93.28%), purchased from Sigma-Aldrich, was used as the internal standard (IS) during the

extraction and analysis. Stock solutions of dicyanocobinamide and dinitrocobinamide were each prepared at a concentration of 2,000 µg/mL in water. Subsequent working stock solutions of dicyanocobinamide and dinitrocobinamide were prepared in water from the stock solutions for use in preparation of calibration standards and QC samples. Stock solutions of ginsenoside Rb1 were prepared at a concentration of 1,000 µg/mL in methanol. Working stock solutions of ginsenoside Rb1 were prepared in methanol from the stock solutions at 120 µg/mL. All water, methanol, and acetonitrile used for extractions and for the preparation of standards and mobile phases were Optima™ LC/MS-grade (Fisher Scientific, Hampton, NH).

Seven different sources of Yorkshire pig plasma was obtained from Bioreclamation, LLC (Westbury, NY) containing tri-potassium EDTA and was stored at $\leq -20^{\circ}\text{C}$. The plasma was thawed unassisted at room temperature and was spiked appropriately with the working stock solutions to prepare the different calibration standard levels and QC samples described below.

1.2.2 Animal Care

Swine were maintained in accordance with Battelle's guiding documents and procedures. Animals were individually housed for safety of staff with space meeting the requirements of the Guide for the Care and Use of Laboratory Animals, or as directed by a Battelle veterinarian and approved by the IACUC. The light/dark cycle was approximately 12 hours each day using fluorescent lighting. Animal room temperatures and relative humidity were maintained at $\sim 23^{\circ}\text{C}$ and $\sim 50\%$, respectively. Certified Swine Chow pellets were fed a total of approximately 2-3 percent of body weight daily. No contaminants that would affect the results of the study were known to be present in the feed. Water was supplied from the Battelle water system and was available ad libitum. Water is analyzed at a minimum of once per year. No contaminants that would affect the results of the study were known to be present in the water. The vehicle used for IV dosing was saline. The swine whole blood samples were collected in K₃EDTA tubes and processed to plasma by centrifugation at ~ 1300 rcf for 10 minutes at 4°C and stored at $\leq -70^{\circ}\text{C}$ pending analysis.

1.2.3 Sample preparation

The plasma samples were allowed to thaw unaided to room temperature if previously frozen. The plasma samples were vortexed thoroughly, and a 100 μ L aliquot of each sample was added to a 1.5 mL microcentrifuge tube, followed by 10 μ L of the 120 μ g/mL IS working stock solution and 100 μ L of 25 mM potassium cyanide in 0.1 N NaOH. The tubes were heated and mixed on a ThermoMixer C for 15 minutes at 80°C and 500 rpm. Precipitation of the plasma proteins was completed using a 600 μ L aliquot of acetonitrile added to each sample, and the samples were vortexed for 5 minutes and subsequently centrifuged at 14,000 rpm at 4°C for 15 minutes. A 600 μ L aliquot of the supernatant was transferred to the appropriate wells of a 1-mL 96 deep well plate and evaporated to dryness under a gentle stream of nitrogen. The dried residues were reconstituted using a 200 μ L aliquot of water added to the appropriate wells, the plate was sealed, and the extracts were vortexed for 1 minute and subsequently centrifuged at 3,200 rpm at 4°C for 10 minutes. The final extracts were directly analyzed using LC-MS/MS.

1.2.4 LC-MS/MS analysis and quantification

All samples were analyzed for dicyanocobinamide using LC-MS/MS. The analyses were performed on an AB Sciex Triple Quadrupole 5500 mass spectrometer (MS) that was interfaced with a Shimadzu Prominence Series HPLC system. The MS was equipped with the TurboIonSpray[®] (TIS) probe and utilized the TIS probe for positive-mode electrospray ionization (ESI+). The HPLC system consisted of a CBM-20A controller, a DGU-20A5 degasser, two LC-20ADXR pumps, an SIL-20ACXR autosampler, and a CTO-20A column oven. The injection volume was set at 10 μ L, which represented the injection of 3.7 μ L of plasma for each sample. The compounds were separated on a Phenomenex Kinetex C18 column (2.6 μ m, 2.1 x 150 mm). Mobile phase A was 0.01% formic acid in water, and mobile phase B was 0.01% formic acid in acetonitrile. The initial mobile phase composition was 10% B (90% A), which was held for 1 minute before ramping up to 80% B over 5 minutes. The composition was held at 80% B for 1 minute before bringing back to 10% B and holding for 3 minutes. The flow rate was 0.2 mL/min, the column temperature was maintained at 30°C, and the autosampler temperature was maintained at 4°C. To reduce the frequency of ion source cleaning, the mobile phase was redirected to waste during the first 4 minutes of the gradient, then to the mass spectrometer for the next 3 minutes, and then back to waste. A needle wash solution of 90:10 acetonitrile:water (v/v) was used to minimize carryover in the autosampler.

The AB Sciex software Analyst version 1.6.2 was used for data acquisition and analysis. The MS parameters for each compound were optimized to ensure the most favorable ionization and ion transfer conditions and to attain optimum signal of both the precursor and fragment ions by infusing the analytes into 80% B at 0.1 mL/min and manually tuning the parameters. The source parameters consisted of: curtain gas, 20 psi; IonSpray voltage, 5500 V; source temperature, 350°C; ion source gas 1 (nebulizer gas), 30 psi; ion source gas 2 (auxiliary gas), 30 psi; collision gas, 10 psi. The ESI probe *y*-axis was set to 5.0 mm, and the *x*-axis was positioned at 5.0 mm. The declustering potential for dicyanocobinamide and ginsenoside Rb1 was 180 V and 90 V, respectively. The entrance potentials were kept at 10 V for both analytes, the collision cell exit potentials were 21.7 V and 15 V, respectively, and the collision energy was 60 V and 40 V, respectively, for dicyanocobinamide and ginsenoside Rb1. The precursor ion of dicyanocobinamide, *m/z* 1015.5, was due to the loss of one –CN group (*i.e.*, [M–CN]⁺). The precursor ion of ginsenoside Rb1, *m/z* 1131.5, was due to the formation of a sodium adduct (*i.e.*, [M+Na]⁺).

The compounds were identified by their retention times and their specific MRM transitions (dicyanocobinamide *m/z* 1015.5→930.5; ginsenoside Rb1 *m/z* 1131.5→365.0). Quantification was performed using ginsenoside Rb1 as the internal standard. The analyte/IS peak area ratio was plotted as a function of the analyte concentration and was fitted to a linear regression ($y = mx + b$), with 1/*x* weighting.

1.3 Experimental design

1.3.1 Selectivity/sensitivity/lower limit of quantitation assessment

An analysis of six blank Yorkshire pig plasma samples from six different sources was conducted. Selectivity was ensured with no discernible peak at the retention time of the analyte or internal standard. A quantitation limit was established as the lowest calibration standard that could be quantitated to within 20% of the nominal value with an RSD ≤ 20% and have an analyte response ≥ 5 times the response compared to blank response. One replicate of plasma sample from six different sources, each spiked at 25.0 ng/mL dinitrocobinamide, were extracted and analyzed for this test.

1.3.2 Linearity and range assessment

A calibration (standard) curve processed for each analytical run was used to determine the linearity and range of the assay. Dicyanocobinamide concentrations targeting 25.0, 50.0, 100, 500, 1,000, 5,000, 8,000, and 10,000 ng/mL were analyzed in duplicate at each level for the linearity test. A weighted linear regression curve using $1/x$ as the weighting factor, with x being the concentration of dicyanocobinamide in ng/mL, and y being the ratio of the dicyanocobinamide peak area/ginsenoside Rb1 peak area was used to calculate the correlation coefficient (r).

1.3.3 Accuracy and precision assessment

Three concentrations representing the entire range of the standard curve were analyzed: one within 3 times the lower limit of quantitation (LLOQ) (low QC sample), one near the center of the standard curve (middle QC), and one near the upper boundary of the standard curve (high QC). The QC samples were prepared in Yorkshire pig plasma at 75.0 ng/mL, 750 ng/mL, and 7,500 ng/mL dinitrocobinamide on three different days and six replicates of each concentration were extracted and analyzed on each day. The intra-assay accuracy was assessed by determining the average percent relative error (RE) of the nominal concentration for each concentration level on each day. The inter-assay accuracy was assessed by determining the average RE of the nominal concentration for each concentration level over all three days. The intra-assay precision was assessed by determining the percent relative standard deviation (RSD) at each concentration level on each day. The inter-assay precision was assessed by determining the RSD at each concentration level over all three days.

1.3.4 Dilution recovery assessment

Samples above the upper limit of quantitation (ULOQ) were prepared in plasma and assessed for accuracy and precision after dilution. Six replicates of a plasma sample targeting 50,000 ng/mL was diluted in blank plasma to within the calibration standard range, extracted, and analyzed.

1.3.5 Carryover and blanks assessment

Aliquots of blank plasma were used for matrix blank preparation. Blanks lacking internal standard (matrix double blank) and blanks containing internal standard (matrix blank) were processed for each analytical run. Carryover was assessed by injecting one matrix double blank

sample immediately following each high standard for each analytical run. Carryover was ensured through the absence of any discernible peak at the retention time of the analyte or internal standard. The matrix blanks were also assessed periodically to ensure no discernible peak at the retention time of the analyte.

1.3.6 Absolute recovery assessment

Recovery experiments were performed by extracting Yorkshire pig plasma samples prepared with 75.0 ng/mL, 750 ng/mL, and 7,500 ng/mL of dicyanocobinamide with the addition of ginsenoside Rb1. The analysis results were then compared to the results with un-extracted samples prepared in blank matrix extract at concentrations equivalent to the extracted samples considering the appropriate concentration or dilution factor. The following calculation was used for recovery determination:

$$\text{Percent Recovery} = \frac{(\text{Area response of each reference QC})}{(\text{average area response of recovery QC})} \times 100$$

1.3.7 Matrix effects assessment

The matrix factor (MF) was used to determine the matrix effects in samples. MF is defined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix. Sample extracts in matrix at the LLOQ were compared to solvent standards at the same concentration as the final extract concentration of the LLOQ. The following calculation was used for MF:

$$\text{Matrix Factor} = \frac{(\text{Area response of each LLOQ in matrix})}{(\text{Average area response of LLOQ in solvent})}$$

1.3.8 Interference assessment

In order to evaluate interference caused by co-administered compounds, a set of QC samples was prepared in Yorkshire pig plasma at 75.0 ng/mL, 750 ng/mL, and 7,500 ng/mL dinitrocobinamide. This set of QC samples also contained an equivalent amount of magnesium thiosulfate. Three replicates of each concentration were extracted and analyzed to evaluate any potential interferences caused by this commonly co-administered compound.

1.3.9 Stability assessment

The stability of dinitrocobinamide in Yorkshire pig plasma samples subjected to three freeze/thaw cycles was evaluated at $\leq -70^{\circ}\text{C}$. Plasma spiked at 75.0 ng/mL and 7,500 ng/mL dinitrocobinamide was frozen at $\leq -70^{\circ}\text{C}$ for a minimum of 24 hours. The samples were removed from the freezer and allowed to thaw unassisted at room temperature for a minimum of two hours. The samples were then refrozen for a minimum of 12 hours then thawed unassisted at room temperature for a minimum of two hours. This freeze/thaw process was then repeated one last time for a total of three freeze/thaw cycles. The samples were then extracted in triplicate and analyzed along with fresh calibration curves and QC samples. The average percent relative error (RE) of the three aliquots was calculated.

The stability of dinitrocobinamide in plasma was then evaluated for short-term storage at $\leq -70^{\circ}\text{C}$ and at room temperature. Yorkshire pig plasma samples were spiked at 75.0 ng/mL and 7,500 ng/mL, and frozen at $\leq -70^{\circ}\text{C}$ for at least 24 hours. Three aliquots per concentration were then thawed unassisted at room temperature, and maintained at room temperature for approximately 20 hours prior to extraction. The samples were then extracted and analyzed along with fresh calibration curves and QC samples.

Additionally, the stability of dinitrocobinamide in plasma was evaluated for long-term storage (approximately 62 days) at $\leq -70^{\circ}\text{C}$. Yorkshire pig plasma was spiked at 75.0 ng/mL and 7,500 ng/mL, then aliquotted into micro-centrifuge tubes and frozen at $\leq -70^{\circ}\text{C}$ for 7, 14, 30, and 60 days. The samples were extracted in triplicate and analyzed along with fresh calibration curves and QC samples.

1.3.10 Reproducibility assessment

The reproducibility of the assay was evaluated by comparing the RSD of each calibration standard and QC sample between runs. For the assay to be acceptable, the grand average of the RSDs for the standards and QC samples must be $\leq 15\%$ at each concentration level except at the LLOQ, which must be $\leq 20\%$.

1.3.11 Ruggedness assessment

Two analysts were used to validate the assay for ruggedness. Each analyst independently performed their own sample preparation tasks beginning with the preparation of the calibration standards through the set-up of the instrumental system. Each analyst analyzed the data from their respective runs. The second analyst performed one accuracy and precision run which included short-term, freeze/thaw, and 7 day long-term matrix stability analyses. Two different lots of the same HPLC column from a manufacturer were also used. The data from the analytical runs were essentially equivalent, indicating that trained analysts can produce acceptable data and that the assay can utilize equivalent columns.

1.4 Results and discussion

Considerable scientific documentation exists for the swine as a predictive animal model for humans. Overall, the swine is a good model for many aspects of humans, the only issue is the size and difficulty working with them in a laboratory setting. Additionally, a good biochemical and physiological data base for the swine is available. This is an accepted non-rodent species for use in cardiovascular safety studies of drugs intended for human use. At this time, studies in laboratory animals are required to support regulatory submissions. The number of swine is considered to be the minimum number necessary to yield meaningful results. This study will provide needed safety data in a swine model. There are no known non-mammalian models, in vitro studies, structure activity relationship studies, or computer models that can replace the integrative function of the mammal.

1.4.1 Evaluation of selectivity/sensitivity/lower limit of quantitation

The analysis of six blank Yorkshire pig plasma samples from six different sources was conducted. Selectivity was ensured with no discernible peak at the retention time of the analyte or internal standard. A quantitation limit was established as the lowest calibration standard that could be quantitated to within 20% of the nominal value with an $RSD \leq 20\%$ and have an analyte response ≥ 5 times the response compared to a blank response. One replicate of plasma sample from six different sources, each spiked with 25.0 ng/mL dinitrocobinamide, was extracted and analyzed for this test. The accuracy of each of the six replicates ranged from -15% to 2.0% relative error (RE) of the nominal value, had a signal-to-noise ratio ranging from 54:1 to 100:1, and had a relative standard deviation (RSD) of 7.0% from the measured concentration. The

LLOQ for this method was determined to be 25.0 ng/mL, which was acceptable within the context of the cobinamide concentrations expected to be present in the plasma. An example chromatogram can be found in Figure 3.

1.4.2 Evaluation of linearity and range

A calibration (standard) curve processed for each analytical run was used to determine the linearity and range of the assay. Dicyanocobinamide concentrations in Yorkshire pig plasma targeting 25.0, 50.0, 100, 500, 1,000, 5,000, 8,000, and 10,000 ng/mL were analyzed in duplicate at each level for the linearity test. The correlation coefficients for the concentrations ranged from 0.9958 to 0.9993 over the course of eight different analytical runs. The average RE for standards at 25.0 ng/mL (LLOQ) was 6.8% of the nominal value, and all other standard levels ranged from -6.3% to 2.1% of their nominal values. Therefore, the assay was deemed linear and accurate over a range that spanned a factor of 400.

1.4.3 Evaluation of accuracy and precision

Three concentrations representing the entire range of the standard curve were analyzed: one within 3 times the lower limit of quantitation (LLOQ) (low QC sample), one near the center of the standard curve (middle QC), and one near the upper boundary of the standard curve (high QC). The QC samples were prepared in Yorkshire pig plasma at 75.0 ng/mL, 750 ng/mL, and 7,500 ng/mL dinitrocobinamide on three different days by two different analysts, and six replicates of each concentration were extracted and analyzed on each day. The intra-assay accuracy was assessed by determining the average RE of the nominal concentration for each concentration level on each day. The inter-assay accuracy was assessed by determining the average RE of the nominal concentration for each concentration level over all three days. The intra-assay precision was assessed by determining the percent RSD at each concentration level on each day. The inter-assay precision was assessed by determining the RSD at each concentration level over all three days. As shown in Table 1, the intra-assay RE values for the low, mid, and high levels ranged from -14% to -0.044%, -0.69% to 14%, and 7.8% to 11%, respectively. The inter-assay average RE values for the low, mid, and high levels were -6.4%, 5.1%, and 9.1%, respectively. The intra-assay RSD values for the low, mid, and high levels

ranged from 3.4% to 12%, 3.5% to 5.6%, and 3.6% to 6.3%, respectively. The inter-assay RSD values for the low, mid, and high levels was 9.7%, 7.8%, and 4.9%, respectively.

1.4.4 Evaluation of dilution recovery

Samples containing analyte at concentrations greater than the upper limit of quantitation (ULOQ) were prepared in plasma and assessed for accuracy and precision after dilution. Six replicates of a plasma sample targeting 50,000 ng/mL dinitrocobinamide was diluted in blank plasma to within the calibration standard range, extracted, and analyzed. All dilutions were determined to have appropriate accuracy and precision with the average RE of 14% and RSD of 6.5%.

1.4.5 Evaluation of carryover and blanks

Aliquots of blank plasma were used for matrix blank preparation. Duplicate blanks (matrix double blank) and blanks containing internal standard (matrix blank) were processed for each analytical run. Carryover was assessed by injecting one matrix double blank sample immediately following each high standard during each analytical run. Over the course of 8 analytical runs, the carryover of dicyanocobinamide ranged from 0 to 15% of the area response of the LLOQ, while no carryover was observed for the internal standard. The matrix blanks were also assessed to ensure that no discernible peak was at the retention time of the analyte, and that no analyte or IS peaks were observed in the matrix blanks. Results demonstrated that carryover from sample to sample was not a concern when using this assay. An example chromatogram of a matrix double blank immediately following the high-level standard can be found in Figure 4.

1.4.6 Evaluation of absolute recovery

Recovery experiments were performed by extracting Yorkshire pig plasma samples prepared with 75.0 ng/mL, 750 ng/mL, and 7,500 ng/mL of dicyanocobinamide with the addition of ginsenoside Rb1. The analysis results were then compared to the results with un-extracted samples prepared in blank matrix extract at concentrations equivalent to the extracted samples considering the appropriate concentration or dilution factor.

$$\text{Percent Recovery} = \frac{(\text{Area response of each reference QC})}{(\text{average area response of recovery QC})} \times 100$$

The average recoveries (mean $\pm$ RSD) of the low, mid, and high levels for dicyanocobinamide were 56.4% $\pm$ 4.4%, 59.6% $\pm$ 4.5%, and 62.8% $\pm$ 2.9%, respectively. The average recoveries (mean $\pm$ RSD) of the low, mid, and high levels for ginsenoside Rb1 were 22.0% $\pm$ 4.6%, 22.1% $\pm$ 3.4%, and 22.6% $\pm$ 3.9%, respectively. Based on the results, the recovery of dicyanocobinamide and ginsenoside Rb1 were consistent, precise, and reproducible. The RSD was $\leq$ 15% at each level. The lower recovery values for all levels of dicyanocobinamide confirmed that a matrix standard curve was required for accurate quantitation.

1.4.7 Evaluation of matrix effects

The matrix factor (MF) was used to determine the matrix effects in the samples. MF is defined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix. An MF equal to 1 signifies no matrix effects, an MF less than 1 suggests matrix signal suppression, and an MF greater than 1 suggests matrix signal enhancement.

$$\text{Matrix Factor} = \frac{(\text{Area response of each LLOQ in matrix})}{(\text{Average area response of LLOQ in solvent})}$$

As shown in Table 2, the results of the matrix factor indicated matrix signal enhancement for dicyanocobinamide and matrix signal suppression for ginsenoside Rb1. Ideally, analytical methods will be free from matrix effects and will result in near 100% absolute recoveries of the analytes of interest. Although recoveries were consistent, precise, and reproducible, ginsenoside Rb1 recoveries were much lower than the dicyanocobinamide recoveries. A stable isotope-labeled internal standard, if available, would recover very similar to the analyte and provide better recoveries. Signal suppression was prominent following the extraction of ginsenoside Rb1 in pig plasma. However, the agreement between analyte and internal standard with regard to the accuracy and precision values and the use of matrix-matched standards ensured that the measured values were accurate and precise regardless of signal suppression or low recoveries.

1.4.8 Evaluation of interference from magnesium thiosulfate

To evaluate potential interferences caused by the commonly co-administered magnesium thiosulfate, a set of QC samples was prepared in Yorkshire pig plasma at 75.0 ng/mL, 750 ng/mL, and 7,500 ng/mL dinitrocobinamide. This set of QC samples also contained an

equivalent amount of magnesium thiosulfate in each of the samples. Three replicates of each concentration were extracted and analyzed. The average RE for the low, mid, and high levels was 8.3%, 10%, and 13%, respectively. The RSD for the low, mid, and high levels was 9.5%, 3.7%, and 4.3%, respectively. The results of the interference evaluation indicated that magnesium thiosulfate had no effect on the dicyanocobinamide in the plasma.

1.4.9 Evaluation of stability

The average (n=3) freeze-thaw stability, short-term stability, and long-term stability of both the low- and the high-level QC samples all fell within 15% of the initial determined concentrations. Therefore, dinitrocobinamide in Yorkshire pig plasma was considered stable at $\leq -70^{\circ}\text{C}$ following three freeze/thaw cycles, up to 20 hours at room temperature after being frozen at $\leq -70^{\circ}\text{C}$, and for 60 days at $\leq -70^{\circ}\text{C}$ prior to extraction.

To demonstrate re-injection reproducibility, one entire validation set was re-capped and stored at $2-8^{\circ}\text{C}$ on the autosampler for three days. After three days, the samples were re-injected. The re-injected set had acceptable results, as the average (n=6) post-determined concentration was -14%, 5.5%, and 9.5% of nominal upon reinjection for the low, mid, and high levels, with RSD values $< 6\%$ at each level. Therefore, the dicyanocobinamide plasma extracts were considered stable when stored on the refrigerated autosampler for up to three days.

To further demonstrate the stability of the extracts, one validation set was extracted and stored at $2-8^{\circ}\text{C}$ on the autosampler for four days. After four days, the samples were analyzed along with fresh calibration curves and QC samples. The extract stability set had acceptable results. After four days, the average (n=6) determined concentration was within -0.13%, 2.4%, and 5.9% of nominal, with RSD values $< 5\%$. Therefore, the dicyanocobinamide plasma extracts were considered stable when stored on the refrigerated autosampler for up to four days.

1.4.10 Evaluation of reproducibility

The reproducibility of the assay was evaluated by comparing the RSDs of the determined concentrations of each calibration standard from 8 consecutive runs over a period of 2 months and of each QC sample from 4 consecutive runs over a period of 6 weeks. The assay was

considered reproducible because the RSDs for the standards and QC samples were $\leq 15\%$ at each concentration level.

1.4.11 Evaluation of study samples

This described method was applied to the analysis of cobinamide that was present in plasma of Yorkshire pigs after the intravenous infusion of KCN and IM injection of nitrocobinamide.¹¹ Concentration results for dicyanocobinamide in plasma ranged from 34.3 ng/mL to 28,700 ng/mL in the nitrocobinamide-treated animals. An example chromatogram can be found in Figure 5.

1.5 Conclusions

Based on the data presented here, this method was demonstrated to be highly sensitive, reproducible, stable, and rugged. This paper supports the application of the analysis of cobinamide in other biological matrices and species. Additionally, it is likely that this approach may also be applicable for other related cobinamide compounds as well.

1.6 Acknowledgments

This work was supported by the National Institutes of Health (NIH) Office of the Director through an interagency agreement (OD#: Y1-OD-0387-01) between the National Institute of Allergy and Infectious Diseases (NIAID) and Department of Defense (DoD) and prepared under the auspices of the NIH and the DoD Defense Technical Information Center (DTIC) under the Chemical, Biological, Radiological & Nuclear Defense Information Analysis Center (CBRNIAC) program, Contract No. SP0700-00-D-3180, Delivery Order Number 0687, CBRNIAC Task 832/CB-IO-OOI2.

The views expressed in this article are those of the authors and do not reflect the official policy of the NIH, Department of Health and Human Services, or the U.S. Government. No official support or endorsement of this article by the NIH or DoD is intended or should be inferred. The authors thank Patrick DeArmond and Jessica Schimmoeller (Battelle) for their critical review of the manuscript.

1.7 References

1. Biochemistry, by Jeremy M. Berg, John L. Tymoczko, and Lubert Stryer, 6th ed, Ch 17, 2007.
2. Antonini, et.al., The Interaction of Cyanide with Cytochrome Oxidase, *Eur. J. Biochem.*, 23, 396-400, 1971.
3. Chemical Warfare Agents: Chemistry, Pharmacology, Toxicology, and Therapeutics, editors, James A. Romano, Jr., Brian J. Lukey, and Harry Salem, 2nd ed, Ch 14, 2008.
4. Casarett & Doull's Toxicology: The Basic Science of Poisons 7th Edition, by Curtis D. Klaassen, Ch 27, 2008.
5. K. E. Broderick, M. Balasubramanian, A. Chan, P. Potluri, J. Feala, D. D. Belke, A. McCulloch, V. S. Sharma, R. B. Pilz, T. D. Bigby, and G. R. Boss, "The cobalamin precursor cobinamide detoxifies nitroprusside-generated cyanide," *Exp. Biol. Med.* 232(6), 789–798, 2007.
6. K. E. Broderick, P. Potluri, S. Zhuang, I. E. Scheffler, V. S. Sharma, R. B. Pilz, and G. R. Boss, "Cyanide detoxification by the cobalamin precursor cobinamide," *Exp. Biol. Med.* 231(5), 641–649, 2006.
7. A. Chan, M. Balasubramanian, W. Blackledge, O. M. Mohammad, L. Alvarez, G. R. Boss, T. D. Bigby, "Cobinamide is superior to other treatments in a mouse model of cyanide poisoning," *Clin Toxicol (Phila)*, 48:709–717, 2010.
8. J. Ma, P. K. Dasgupta, W. Blackledge, G. R. Boss, "Cobinamide-based cyanide analysis by multiwavelength spectrometry in a liquid core waveguide," *Analytical Chemistry*, 82:6244–6250, 2010.
9. S. Ford, J. Gallery, A. Nichols, M. Shambee, "High-performance liquid chromatographic analysis of the (cyanoaquo) stereoisomers of several putative vitamin B12 precursors," *J. Chromatogr*, 537, 235-247, 1991.
10. H. Chassaigne, R. Lobinski, "Determination of cobalamins and cobinamides by microbore reversed-phase HPLC with spectrophotometric, ion-spray ionization MS and inductively coupled plasma MS detection," *Anal. Chim. Acta*, 359, 227-235, 1998.

11. M. K. Brittain, F. M. Reid, M. C. Babin, D. A. Jett, G. E. Platoff, and D. T. Yeung, "Testing Cobinamide against Infused KCN in Swine Model," *unpublished work*, 2014.

Figure 1. Molecular structure of hydroxocobalamin. Structure obtained from Chemical Book (www.chemicalbook.com, accessed 9/29/15).

12.

Figure 2. Molecular structures of A) dicyanocobinamide and B) ginsenoside Rb1. Structures obtained from Sigma-Aldrich (www.sigmaaldrich.com, accessed 10/15/14).

Figure 3. LLOQ Standard (25 ng/mL) in Yorkshire Pig Plasma. Blue trace – dicyanocobinamide; red trace – ginsenoside Rb1 (IS)

14.

Figure 4. Matrix Double Blank in Yorkshire Pig Plasma. Blue trace – dicyanocobinamide; red trace – ginsenoside Rb1 (IS)

15.

Figure 5. Yorkshire Pig Plasma Sample Treated with Cobinamide. Blue trace – dicyanocobinamide; red trace – ginsenoside Rb1 (IS)

16.

Table 1. Intra-day and Inter-day Accuracy and Precision of Dicyanocobinamide Analysis in Yorkshire Pig Plasma over Three Different Days Using Two Different Analysts

Data Set	Level	Nominal Concentration (ng/mL)	Intra-assay Average RE (% , n=6)	Intra-assay RSD (% , n=6)	Inter-assay Average RE (% , n=18)	Inter-assay RSD (% , n=18)
042214	Low	75.0	-14	12	-6.4	9.7
	Mid	750	-0.69	3.5	5.1	7.8
	High	7,500	7.8	5.0	9.1	4.9
042314	Low	75.0	-0.044	3.4	-	-
	Mid	750	14	4.7	-	-
	High	7,500	8.9	6.3	-	-
042914	Low	75.0	-5.5	6.7	-	-
	Mid	750	1.8	5.6	-	-
	High	7,500	11	3.6	-	-

Table 2. Matrix Effects in Yorkshire Pig Plasma Measured at the LLOQ (25 ng/mL Dicyanocobinamide)

Level	Dicyanocobinamide Peak Area (counts)	Ginsenoside Rb1 Peak Area (counts)	Matrix Factor Dicyanocobinamide	Matrix Factor Ginsenoside Rb1
LLOQ in Matrix	13972	109306	4.37	0.115
	12395	103427	3.87	0.109
	11104	94480	3.47	0.0996
	13432	110901	4.20	0.117
	12117	80758	3.79	0.0851
	10873	86282	3.40	0.0909
LLOQ in Solvent	2947	760571	AVG = 3.85	AVG = 0.103
	3143	885991		
	3276	992163		
	3491	1006707		
	2994	1027333		
	3354	1019430		

17.